

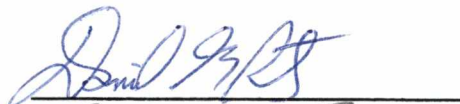
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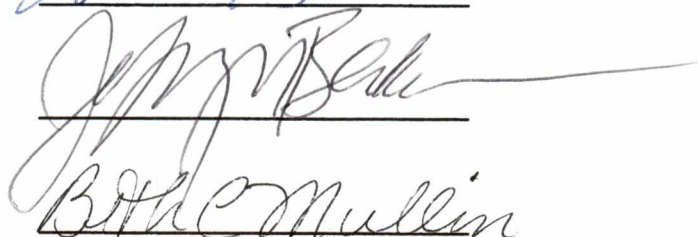
I am submitting herewith a dissertation written by Wei Song entitled "Isolation and Characterization of two Peptide Transporter cDNAs from *Arabidopsis thaliana*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major of Microbiology.

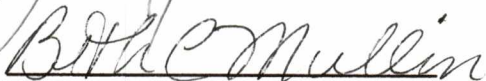

Gary Stacey, Major Professor

We have read this dissertation
and recommend its acceptance:









Accepted for the Council:


Associate Vice Chancellor and
Dean of The Graduate School

**Isolation and Characterization of two Peptide Transporter cDNAs
from *Arabidopsis thaliana***

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Wei Song

December, 1996

DEDICATION

This work is dedicated to my parents and my husband, whose love, support and encouragement made it possible for me to pursue higher education.

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Finally, I would like to thank my husband Dr. Yuxin Zhang for all his guidance, encouragement and love.

ABSTRACT

The transport of small peptides of 2 to 5 residues across the plasma membrane, a phenomenon shown to occur in a range of prokaryotes and eukaryotes, is mediated by specific membrane proteins in an energy-dependent manner. Peptide transport has been found to be involved in a number of biological processes such as nutrition, antibiotic and toxin absorption. Peptide transport systems have been well studied in a wide variety of organisms including bacteria, fungi, and animals, but there are only a handful of reports on their occurrence in plants. However, several lines of evidence have demonstrated that peptide transport not only exists in plants, but may also participate in important physiological processes in plants such as nutrition. The major goal of my project was to isolate and characterize the genes required for peptide transport in plants, and further elucidate the physiological role of these identified peptide transporters with regard to plant growth and development.

This document describes the isolation of two peptide transporter cDNAs, *AtPTR2-A* and *AtPTR2-B*, from an *Arabidopsis thaliana* cDNA library by functional complementation of a yeast peptide transport mutant deficient in *ptr2*, a yeast peptide transporter gene. Transfer of *AtPTR2-A* or *AtPTR2-B* to the yeast *ptr2* mutant restored the ability to grow on di- and tripeptides but not peptides of four residues or longer. Transformants expressing *AtPTR2-A* or *AtPTR2-B* were also sensitive to toxic di- and tripeptides. Kinetic studies indicated that *AtPTR2-A* and *AtPTR2-B* have K_m values of 47 μM and 14 μM , respectively,

with $V_{\max}$ values of 0.061 and 0.013 nmoles/mg cell dry weight (sec)⁻¹, respectively, when dileucine was used as a substrate. DNA sequence analysis showed that *AtPTR2-A* encodes a putative 68 kD protein (611 aa) and *AtPTR2-B* encodes a putative 64 kD protein (585 aa). Hydropathy analysis indicated that both proteins are highly hydrophobic with twelve putative transmembrane domains. A search of the protein sequence data base reveals a high degree of similarity of *AtPTR2-A* and *AtPTR2-B* to the yeast peptide transporter Ptr2p, the *A. thaliana* nitrate transporter AtCHL1, the rabbit intestinal peptide transporter PepT1, the *Candida albicans* peptide transporter CaPtr2p, and *Lactococcus lactis* peptide transporter DtpT. Comparison of these protein sequences to a number of other transport proteins suggested that these transporters comprise a new family of membrane transport proteins, called the PTR (Peptide TRansport) family. Northern blot analysis showed that *AtPTR2-B* was highly expressed in roots, leaves, germinating seeds, stems, flowers, and siliques. *AtPTR2-A* mRNA could not be detected by Northern blot analysis, but was detectable in roots after reverse transcription and PCR amplification.

Transgenic *Arabidopsis* plants were constructed expressing antisense or sense *AtPTR2-B* or *AtPTR2-A*. Southern blot analysis indicated that four independent antisense and three independent sense *AtPTR2-B* transgenic lines were regenerated. Southern blot analysis showed that these lines had 1 or 2 T-DNA copies integrated into their genome. This was supported by the segregation data following kanamycin resistance encoded on the T-DNA. Northern blot data showed that the endogenous *AtPTR2-B* mRNA levels was significantly reduced in transgenic leaves and flowers. Consistent with this reduction, all antisense lines

and one sense transgenic line exhibited significant phenotypic changes including late flowering, arrested seed development, and increased lateral root and root hair formation. These phenotypic changes may reflect a nutritional deficiency due to reduced peptide transport mediated by *AtPTR2-B*, suggesting that *AtPTR2-B* may play a general role in plant nutrition.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Peptide transport can be defined as a cellular process by which small peptides of 2 to 5 amino acid residues are transported across the cellular membrane in an energy-dependent manner (Payne et al., 1994). Once transported, the peptides are rapidly hydrolyzed into amino acids by intracellular peptidases and serve as a source of amino acids, carbon, or nitrogen. The peptide transport process has been well documented physiologically in a wide variety of organisms including bacteria, fungi, and animals (Becker and Naider, 1980, 1994; Ganapathy et al., 1991; Payne et al., 1994), but there are only a handful of reports in plants (Higgins et al., 1980). Information gathered over the past two decades reveals that, from unicellular to multicellular organisms, peptide transport systems have similar structural and functional features. This apparent evolutionary conservation underscores the importance of peptide transport. In this review, I will describe our current understanding of peptide transport focusing on such aspects as the components involved in the transport systems of different organisms, regulation, energetics, and derived functions, as well as the nutritional role of peptide transport.

Potential Benefits of Peptide Transport to a cell

Before starting a detailed review, I would like to discuss the possible advantages of

transporting small peptides in light of the fact that every organism possesses a well defined amino acid transport system. One important advantage may lie in the conservation of metabolic energy. Active transport of nutrients expends considerable amounts of energy and thus any mechanism that will reduce this consumption will be advantageous to cells. It is now known that ATP or a proton motive force drives amino acid or peptide uptake (Payne and Smith, 1994). Although the stoichiometry of ATP-coupling or proton-coupling and amino acid or peptide transport has yet to be clarified, a transporter that has the potential ability to handle the oligomer of an amino acid such as Ala₄ or Leu₄ is certainly an energetically superior system. For example, if the histidine permease has a stoichiometry of one (or two) ATP molecules per molecule of histidine, an oligopeptide permease that operates with the same stoichiometry for transport of His₅ will be definitely a more efficient and economic system. In the case of the *Enterococcus faecalis*, a stoichiometry of two protons for each peptide was derived for uptake (Payne, 1980). Transported peptides are also a nutritionally superior source of amino acids because peptides improve the variety of amino acids presented to cells. Peptides are absorbed faster than amino acids in *E. coli*, as well as by animal cells (Mathews, 1991). Moreover, peptide transport systems allow neutral, positively- or negatively-charged amino acids to be handled within the same peptide and avoid the competition for uptake occurring between free amino acids. In addition, peptide transport systems could circumvent defects of amino acid transport. Examples can be found in microorganisms as well as in human beings. In the case of Hartnup's disease (an inability to transport Ala, Phe and Leu) and cystinuria (an inability to transport Cys, Lys and Arg), cells can obtain these amino acids via peptide transport (Alagramam, 1994). The peptide

transport system can also transport a variety of peptide derivatives, in which case non-transportable and physiologically active compounds can be transported by cells. For example, phaseolotoxin, N-phosphosulfamyl-ornithylalanylhomarginine, a phytotoxin, is a tripeptide analogue. Its structure mimics triornithione. The peptide backbone of phaseolotoxin facilitates transmembrane passage via the oligopeptide permease (Opp) system in *E. coli* and *S. typhimurium* (Staskawicz and Panopoulos, 1980). The intracellularly released toxic moiety, N-phosphosulfamyl, serves as an inhibitor of ornithine carbamoyltransferase (OCTase). Another example is the uptake of a peptide-based herbicide, bialaphos. Bialaphos is a phosphinothricin-dialanine peptide, and upon uptake it releases the toxic moiety phosphinotricinyl (PPT) as an inhibitor of glutamine synthetase (Damm, 1993).

General Characteristics of Peptide Transport Systems

Peptide transport is an active process independent of amino acid transport with high stereoselectivity but low specificity with regard to amino acid side-chains. All systems prefer α -peptide bonds and the *L*-forms of amino acid residues. Loss of a peptide transport system due to mutation is not lethal to the organism. The transport of peptides in mammalian, plant, and yeast is H^+ -dependent, Na^+ -independent, while peptide transport systems in bacteria require phosphate bond energy for transport (Steiner et al., 1995). The transporters that utilize ATP to drive the transport process are grouped to a unique transporter family, termed ABC (ATP-binding cassette) family. The one exception in bacteria is the *Lactococcus lactis* di- and tripeptide transporter, DtpT, which utilizes a proton motive force to drive peptide

uptake (Hagting et al., 1994). So far, no eukaryotic ABC-type peptide transport systems mediating uptake of peptides from the extracellular milieu into the cytoplasm has been identified. Bacterial systems have the capability of transporting peptides up to five residues long while in those eukaryotic organisms examined, with the exception of *Candida albicans* (Basrai et al., 1995), only peptides of three residues or less are transported (Payne and Smith, 1994; Ganapathy et al., 1991; Becker and Naider, 1980). Eukaryotic cells appear to have a single peptide transport system, while in bacteria, multiple systems may be present. *Candida albicans* possesses multiple peptide transport systems, a di- and tripeptide transport system (Basrai et al., 1995) and a recently identified oligopeptide transport system allowing transport of tetra- and pentapeptides (Lubkowitz et al., submitted).

The difference in energy source divides peptide transporters into two groups, ABC-type and proton motive force-driven transporters. This difference in energy-coupling suggests that these two groups of peptide transporters arose through different evolutionary pathways. It is likely that the variation in energy coupling reflects unique structural features that differ between the two groups of transporters. Indeed, a number of superfamilies of transport proteins have been characterized by sequence comparison (Saier, 1994). These comparisons show that correlations exist between function and the degree of sequence similarity over the either the entire protein, or discrete regions of the protein.

The primary function of peptide transport is utilization of peptides as amino acids and nitrogen sources (Payne, 1980; Becker and Naider, 1980; Higgins and Payne, 1982;

Mathews, 1991; Wolfenbarger, 1980). In bacteria, peptide transport has been shown to be involved in other cellular processes (described below). In yeast, the expression of a peptide transporter, PTR2, is highly regulated, being induced by certain amino acids and repressed by inorganic nitrogen sources such as ammonium sulfate (Island et al., 1987). Such regulation suggests that the peptide transport system may serve some sort of developmental or adaptive function in these cells. During seed germination in plants, a large part of the nitrogen supplied to the growing embryo is in the form of peptides transported from the endosperm (Higgins and Payne, 1980). Peptide transport is particularly important in humans where it has been observed that protein hydrolysates are nutritionally superior to equivalent amino acid mixtures and that peptides are absorbed at a much faster rate than amino acids (Mathews, 1991).

Peptide Transport in Prokaryotes

Peptide transport can play an important nutritional role in prokaryotes (Payne et al., 1994). In addition, a few reports have recently shown involvement of this system in the recycling of bacterial cell wall components (Goodell et al., 1987), bacterial chemotaxis towards peptides (Manson et al., 1986), bacterial sporulation (Perego et al., 1991; Mathiopoulos et al., 1991; Koide et al., 1994), and bacterial transformation (Pearce et al., 1994).

In bacteria, peptide transport is mediated by three genetically distinct systems with

overlapping substrate specificity. The dipeptide permease (Dpp) system preferentially transports dipeptides (Abouhanmed et al., 1991; Olson et al., 1991). The tripeptide permease (Tpp) system is specific for the transport of di- and tripeptides (Gibson et al., 1984). The oligopeptide permease (Opp) system is capable of transporting peptides up to five amino acid residues in length (Tynkkynen et al., 1993; Andrews et al., 1986; Kunji et al., 1993). Commonly, the three peptide transport systems lack a high degree of amino acid side chain specificity and are composed of multiple membrane associated components (Payne et al., 1994). One component of each system is a periplasmic peptide -binding protein which recognizes and binds the peptide substrate and brings the substrate to the membrane; The second component is an integral membrane protein which is responsible for the translocation of the peptide across the plasma membrane. The third component is a member of the ATP-binding cassette (ABC) family of transporters (Higgins et al., 1992). Hydrolysis of ATP provides energy to drive peptide transport.

The Dpp system has been identified in *E. coli*, *Salmonella typhimurium*, *Lactococcus lactis*, and *Listeria monocytogenes* (Verheul et al., 1995). The Dpp system preferentially transports dipeptides, but has lower affinity for tripeptides. The Dpp system not only transports nutrient dipeptides, but also peptide analogues, such as S-nitrosoglutathione (GSNO). In *Salmonella typhimurium*, glutathione (a tripeptide) acts as a carrier of nitric oxide (NO) by forming S-nitrosoglutathione and is involved in the transport and regulation of NO cytotoxicity (De-Groote et al., 1995). The Dpp system has also been shown to be involved in chemotaxis by recognizing peptide attractants (Manson et al., 1986). The

periplasmic dipeptide-binding protein (DppA) in *E. coli* can act as a periplasmic chemoreceptor for a peptide substrate (Dunten et al., 1995). Mutations at the *dpp* locus have been mapped to a single site on the *S. typhimurium* chromosome that differs from the known *opp* and *tpg* loci (Higgins and Gibson, 1986).

The Opp system has been reported in a variety of bacteria, such as *E. coli*, *Salmonella typhimurium*, *Lactococcus lactis*, *Bacillus subtilis*, and *Streptococcus pneumoniae*. The Opp system of *E. coli* and *Salmonella typhimurium* is encoded by five genes, *oppA*, *oppB*, *oppC*, *oppD* and *oppF* which map to a single genetic locus, comprising an operon (*oppABCDF*) (Hile et al., 1987, Hogarth, 1983). OppA is the periplasmic binding protein. OppB and OppC comprise the two integral membrane proteins. OppD and OppF are two ATP-binding components.

The Opp system is capable of transporting peptides up to five amino acids in length without specificity as to amino acid side chain composition. In *E. coli* and *Salmonella typhimurium*, the recycling of cell wall peptides was found to be mediated by the Opp system (Goodell et al., 1987). During bacterial growth, cell wall peptides, such as L-Ala-D-Gly- γ -meso-diaminopimelic acid (L-Ala-D-Gly- γ -m-A₂pm) and L-Ala-D-Gly- γ -m-A₂pm-D-Ala, two major cell wall peptides, are released from the peptidoglycan and reused for the synthesis of new cell wall materials. These peptides are transported intact into the cytoplasm prior to reincorporation into the cell wall. Studies of the uptake of radiolabeled cell wall peptides and mutants defective in peptide transport indicated that these cell wall peptides were transported

into cells solely by the oligopeptide permease (Opp). Neither the dipeptide permease nor the tripeptide permease played a significant role in this process. Furthermore, the periplasmic oligopeptide-binding protein has more than one substrate-binding site, each with different but overlapping specificities. Opp also plays a role in the initiation of sporulation in *Bacillus subtilis* (Perego et al., 1991; Mathiopoulos et al., 1991; Koide et al., 1994). A *Bacillus subtilis* SpoOK⁻ mutation defective at the first step in sporulation was localized within the oligopeptide transport operon (*opp*). The OppA peptide-binding protein has a signal sequence characteristic of lipo-proteins with an amino-terminal lipo-amino acid anchor (Perego et al., 1991). During exponential growth, OppA is associated with the cell, but is released into the medium in stationary phase. Since a major role of the Opp system in Gram-negative bacteria is the recycling of cell wall peptides released from the growing peptidoglycan, it was postulated that the accumulation of such peptides may play a signalling role in the initiation of sporulation. More recently, an *opp* homolog, *app*, has been identified from *Bacillus subtilis*. The AppF protein shares 54% identity with OppF, while AppA has 22% identity with OppA (Koide et al., 1994). In the wildtype cell, the *app* operon has a frameshift mutation in *appA*. The App system can substitute completely for the Opp system in both sporulation and competence for genetic transformation (see below). Oligopeptide permeases have been reported to modulate transformation in *Streptococcus pneumoniae* (Pearce et al., 1994) and *Bacillus subtilis* (Koide et al., 1994). Analysis of mutants of *Streptococcus pneumoniae* exhibiting decreased transformation efficiency led to the identification of a locus, *plpA* (permease-like protein). The *plpA* gene encodes a putative substrate-binding protein sharing 80% similarity to AmiA, a peptide-binding protein from

Pneumococcus and 50% similarity to SpoOK. The use of a *plpA-PhoA* fusion revealed that *plpA* is found in the periplasm and plasma membrane (Pearce et al., 1994). Therefore, it was proposed that the *plpA* permease may bind a specific ligand in the periplasm and then the permease-ligand interaction modulates the process of transformation.

The Tpp system can transport, with varying ability, a wide variety of tripeptides and less effectively dipeptides. This system has an apparent affinity for hydrophobic peptides, particularly with N-terminal Val or Met residues (Payne and Smith, 1994). Mutational analysis led to identification of two loci, *tppA* and *tppB*, which map at different sites on the *S. typhimurium* chromosome (Gibson et al., 1984). The *tppB* locus has been cloned and, although the nucleotide sequence was not determined, it was considered from the size of the cloned DNA to consist of more than one gene. Transcription of *tppB* is induced 10-fold by exogenous leucine, alanine, or anaerobic growth (Gibson et al., 1984; Jamieson et al., 1986). The product of the *tppA* gene was reported to act as a positive regulator. Although transport by the Tpp system is generally considered to play a minor role in nutrition, it could become more important for certain bacteria growing in the anaerobic environment of the mammalian gut (Payne and Smith, 1994).

In addition to the ATP-dependent peptide transport systems, a proton motive force (PMF)-driven peptide transporter (i.e., DtpT) has been recently identified in *Lactococcus lactis* by complementation of a dipeptide transport-deficient and proline auxotrophic *Escherichia coli* strain (Hagting et al., 1994). The DtpT transporter mediates the uptake of

di- and tripeptides. Amino acid sequence comparisons showed no significant homology with other bacterial peptide transport systems, but showed high homology to eukaryotic H⁺-coupled peptide transporters (discussed below). DtpT was found to be the only transport protein in *L. lactis* for hydrophilic di- and tripeptides. The identification of the proton-driven peptide transporter, DtpT, in bacteria provides valuable information for understanding the evolutionary connection between prokaryotic and eukaryotic peptide transport systems. The proton-mediated peptide transport system has been extensively described in lower and higher eukaryotes.

Peptide Transport in Eukaryotes

Peptide transport systems have been well studied in eukaryotes (Becker and Naider, 1980; Adibi and Kim, 1981; Ganapathy and Leibach, 1991). Peptide transport systems in eukaryotes display common functional features to peptide transport systems in prokaryotes. For example, eukaryotic peptide transport is carrier-mediated, energy-dependent, restricted to small peptides (2-6 amino acids in length), and has no strict side chain specificity (Becker and Naider, 1980; Payne and Smith, 1994). With regard to the components involved in peptide transport, recent reports suggest that eukaryotic peptide transporters have different structural features from those found in prokaryotes (Perry et al., 1994; Fei et al., 1994). In this section, peptide transport in eukaryotes will be described under three headings; peptide transport in yeast, animals, and plants. This organization is meant to highlight the lack of knowledge concerning peptide transport in plants and emphasize the importance of

characterizing the role of peptide transport in plant growth and development.

Peptide Transport in Yeast

Substrate Specificity: In fungi, peptide transport is best characterized in the yeast *Saccharomyces cerevisiae*. A variety of di- and tripeptides are transported across the plasma membrane, whereas the uptake of tetrapeptides or larger peptides is limited. Peptide transport shows broad substrate specificity with a preference for hydrophobic peptides (Becker and Naider, 1980). Although peptides containing a variety of amino acids can be transported by yeasts, amino acid sequence and composition appears to have a marked effect on peptide transport. As described by Becker et al (1995), "in an early report of peptide transport in *S. cerevisiae* (Naider et al., 1974), tripeptides composed of glycine and methionine were excellent (Met-Met-Met, Gly-Met-Met, Gly-Gly-Met, and Met-Gly-Met), intermediate (Met-Met-Gly and Met-Gly-Gly) or inadequate (Gly-Met-Gly) sources of methionine for a methionine auxotroph, even though these peptides were hydrolyzed to similar extents by intracellular peptidases. However, generalizations can't be made since various studies have reported wide variations in the uptake of specific peptides depending on the organism tested or the assay used to measure peptide transport. For example, the dipeptide Lys-Lys was unable to support the growth of lysine auxotrophic *S. cerevisiae* strains Z1-2D (Naider et al., 1977) and S288C-24 (Becker et al., 1973). In contrast, *S. cerevisiae* strain, Σ 1278b (Nisbet and Payne, 1979) transported this dipeptide well as measured by the disappearance of peptide from an incubation medium". Peptide transport by yeast exhibits stereospecificity. L-amino

acid residue-containing peptides are preferentially transported, while D-residues are not tolerated by the dipeptide transport system (Becker and Naider, 1980). Dipeptides containing D-residues in either the first or second position were not transported by *S. cerevisiae*, but in larger peptides, D-residues were accepted by the transport system depending on their position in the peptide (Becker et al., 1995). D-Met-L-Met-L-Met was demonstrated to be a good transport substrate for *Candida albicans*, a fungal pathogen (Lichliter et al., 1976). However, L-Met-L-Met-D-Met was not transported by *S. cerevisiae* and *C. albicans* (Lichliter et al., 1976; Becker and Payne, 1973).

There are a few reports on modification of the peptide N- and C- termini on peptide transport. Becker et al (1995) described that " some, but not all strains of *S. cerevisiae* tolerate acylated dipeptides and oligopeptides and that esterification of the α -carboxyl group on the C-terminal residue does not interfere with uptake by the peptide transport system" (Becker and Naider, 1980). A different conclusion may be made with regard to peptide transport in *C. albicans* (Becker et al., 1995). α -N-acylated peptides enter some strains, whereas peptide esters are not transported by the peptide transport system (Naider and Becker, 1987). The ability of peptide carriers to transport N-acylated peptides is very useful for drug design. N-acylation can prevent the carrier-toxic agent conjugate from the hydrolysis by aminopeptidase, thereby increasing serum half-lives (Becker et al., 1995). Definitive conclusions concerning the effect of peptide termini on peptide transport need to the further isolation and biochemical analysis of the peptide transport system components under controlled conditions.

S. cerevisiae is sensitive to growth inhibition by a variety of dipeptides containing toxic amino acid analogues, such as ethionine, oxalysine, and DL-m-fluorophenylalanine (Island et al., 1987). This is due to the uptake of toxic peptides by the peptide transport system and subsequent release of the toxic amino acid moiety intracellularly to inhibit the growth of cells. This property has been widely used to determine peptide transport activity and substrate specificity of a transport protein.

Regulation: In both *S. cerevisiae* and *C. albicans*, peptide transport appears to be controlled by the nitrogen source (Island et al, 1987). Transport activity is up-regulated in the presence of poor nitrogen sources, such as proline or allantoin, and down-regulated in the presence of a rich nitrogen source such as ammonium sulfate (Becker and Naider, 1987). In addition, peptide transport is induced by micromolar amounts of certain amino acids (Island et al., 1987). The regulation of peptide transport by the nitrogen source is understandable if one imagines that the cell requires lower levels of nitrogen-containing compounds when growing on a rich nitrogen source, whereas a cell growing on a poor nitrogen source would want to increase the expression of systems that would extract nitrogen from the environment. The induction of peptide transport by small amounts of amino acids may be explained by similar reasoning. When the levels of amino acids in the environment are insufficient for growth, the cell would increase the transport of peptides as a source of amino acids.

Regulation of peptide transport has been demonstrated in both *S. cerevisiae* and *C. albicans*. For example, peptide utilization was repressed in *S. cerevisiae* in the presence of

ammonium ions as a source of nitrogen in comparison to growth on organic sources such as allantoin, isoleucine or proline (Becker and Naider, 1977). The toxicity of polyoxin, a peptidyl-nucleoside transported into *C. albicans* via a peptide transport system, is dependent on the specific nitrogen source in the medium (Becker et al., 1983). Payne et al. (1991) demonstrated that addition of peptide mixtures to culture medium induced a five-fold increase in the transport of dipeptides and oligopeptides. Regulation of dipeptide transport by micromolar amounts of certain amino acids was shown in *S. cerevisiae* by Island et al. (1987). Incubation of cells with 159 μ M tryptophan led to a 25-fold increase in the initial rate of Leu-Leu uptake. This effect was not seen in the presence of the protein synthesis inhibitor cycloheximide. Using allantoin as the nitrogen source, growth inhibition by toxic peptides was increased in the presence of leucine, tryptophan, lysine, isoleucine, threonine, phenylalanine, valine, histidine, alanine, serine, tyrosine, and asparagine. The addition of cysteine, glycine, aspartate, proline, glutamine, glutamate, and arginine did not increase toxicity. The presence of leucine, tryptophan, or lysine resulted in the greatest increase in peptide toxicity in comparison to the other amino acids. Working with *C. albicans* and adding micromolar amounts of amino acids to the growth medium, Basrai et al. (1992) showed increases both in toxicity to di- and tripeptides and in uptake of a radioactive dipeptide. In comparison to *S. cerevisiae*, the addition of arginine, lysine, histidine, glutamine, aspartate, and serine up-regulated peptide transport in *C. albicans*, but alanine, asparagine, cysteine, glutamate, glycine, isoleucine, leucine, phenylalanine, threonine, tyrosine, valine, methionine, proline, and tryptophan did not increase di- and tripeptide toxicity. The different array of amino acids that regulate peptide transport in *S. cerevisiae*,

as compared to *C. albicans*, may reveal some basic metabolic differences between these yeasts or may reflect alternative adaptive mechanisms resulting from adaptation to their different ecological niches (Becker et al., 1995).

Cloning Components of the Yeast Peptide Transport System: The isolation and characterization of peptide transport mutants of *S. cerevisiae* (Island et al., 1991) led to the cloning of the peptide transport genes. Twenty four spontaneous mutants and thirty one N-methyl-N-nitrosoguanidine induced mutants were isolated which were deficient in peptide uptake. Genetic analysis classified these mutants into three complementation groups called PTR1, PTR2, and PTR3, suggesting that at least three genes were involved in the peptide transport process (i.e., *PTR1*, *PTR2*, and *PTR3*; Island et al., 1991). Peptide transport deficient mutants *ptr1* or *ptr2* are not able to utilize a variety of dipeptides as sources of amino acids to satisfy auxotrophic growth, are resistant to the growth inhibiting effect of toxic peptides, and are unable to accumulate radiolabelled dipeptides at wildtype levels (Island et al., 1991). The *ptr3* mutant is characterized by its ability to transport only low levels of radiolabeled dileucine (Island et al., 1991).

The *S. cerevisiae* *PTR2* gene has been cloned by transforming the recessive *S. cerevisiae* mutant strain PB1X-9B with a YCp50-based genomic DNA library (Perry et al., 1994). The *PTR2*-encoding protein, Ptr2p, is a highly hydrophobic polypeptide with 12 putative transmembrane domains and is hypothesized to be the membrane-bound component of the yeast peptide transport system (Perry et al., 1994). The database analysis shows that

Ptr2p shares significant similarity to other transporters, such as *Candida albicans* peptide transporter CaPtr2p (Basrai et al., 1995), *Arabidopsis* nitrate transporter CHL1 (Tsay et al., 1993), rabbit peptide transporter PepT1, and *Lactococcus lactis* peptide transporter DtpT (Hagting et al., 1994). By using the same approach of functional complementation of mutants, *PTR1* was isolated and sequenced. Protein sequence analysis suggests that the *PTR1*-encoding protein, Ptr1p, is identical to UBR1 (Alagramam et al., 1994). UBR1 was previously identified as the recognition component of the N-end pathway, ubiquitin-mediated protein degradation system (Bartel et al., 1990). *ubr1* mutants, like *ptr1* mutants, were unable to transport small peptides into cells. Concomitantly, *ptr1* mutants, like *ubr1* mutants, were unable to degrade an engineered substrate of the N-end-rule pathway. The *ptr1* mutant was also found to be unable to produce *PTR2* mRNA, suggesting that Ptr1p is involved in regulating the expression of *PTR2* (Alagramam et al., 1994). These findings showed that peptide transport and the ubiquitin pathway, two cellular processes universal to eukaryotic cells, share a common component, UBR1/PTR1. The correlation between these two cellular processes is under study. Very recently, *PTR3* has been cloned and characterized by negative selection for toxic dipeptide sensitivity in the presence of ammonium sulfate by functional complementation of the *ptr3* allele (Barnes et al., 1996, personal communication). The *PTR3*-encoded protein is reported to share no significant homology to any previously identified gene. Chromosomal disruption of the *PTR3* open reading frame revealed that *PTR3* is not essential for growth, but is required for normal, induced levels of *PTR2* expression. It is hypothesized that Ptr3p functions, either directly or indirectly, as an amino acid-responsive regulator of *PTR2* expression (Barnes et al., 1996, personal communication).

Further study will be needed to establish the correlation among the *PTR1*, *PTR2*, and *PTR3* genes.

Peptide Transport in Animals

Peptide transport in animal cells is of major physiological importance because of participation in the absorption of an important group of nutrients in the small intestine and the kidney. In the last few years, this process has been well documented at both the physiological and molecular level, especially in brush-border membranes of the small intestines (Ganapathy and Leibach, 1991). An impressive number of peptide transport systems have been identified and shown to be energized by the proton motive force.

Molecular and Biochemical Studies: The PepT1 peptide transporter has been isolated from human and rabbit small intestines (Fei et al., 1994; Liang et al., 1995). The PepT1-mediated uptake pathway is reported to constitute a major mechanism for absorbing peptides from dietary proteins. Rabbit PepT1 mRNA was also found in kidney, liver, and in small amounts in brain. The potential function of the peptide transporter in kidney is to reabsorb filtered peptides, peptide-derived antibiotics, and peptides produced as a result of the action of luminal peptidases (Tiruppathi et al., 1990; Daniel et al., 1993). Another peptide transport-associated protein, HPT1, has been identified in the human intestine. HPT1 appears to facilitate the oral absorption of peptide derivatives such as β -lactam antibiotics and angiotensin-converting enzyme inhibitors into enterocytes lining the luminal wall (Dantzig

et al., 1994). Recently, a kidney-specific H⁺/peptide transporter, PepT2, has been isolated from a human renal cell line (Brandsch et al., 1995).

The intestinal peptide transport system is responsible for the oral absorption of drugs and the renal peptide transport system is responsible for the reabsorption of these drugs from the glomerular filtrate which enhances the half-life of these drugs in the circulation. Therefore, detailed studies on the interaction of drugs such as β -lactam antibiotics with the peptide transporters in these two organs are vital to the understanding of the pharmacodynamics of these drugs. Ganapathy et al. (1995) reported that the human intestinal and renal peptide transporters have marked differences in the recognition of β -lactam antibiotics as substrates. For example, PepT1-mediated uptake of the dipeptide glycylsarcosine was inhibited more effectively by cyclacillin than by cefadroxil, whereas PepT2-induced uptake was inhibited more effectively by cefadroxil than by cyclacillin.

A dipeptide transport system has recently been identified in rat lung type II pneumocytes (Meredith et al., 1995) and ovine enterocyte brush-border membrane vesicles (Backwell et al., 1995). Although most noted for providing the minimal barrier for gas exchange, the lung epithelium has recently been shown to play an active role in controlling the solute composition of the alveolar lining fluid (Meredith et al., 1995). Therefore, this finding of peptide transport activity in the apical surface of the lung type II cell suggests that peptide transport may also play a role in absorbing peptides produced from the breakdown of protein in the alveoli.

It is widely accepted that while a majority of protein-digestion end products enter the enterocytes across the brush-border membrane in the form of small peptides, they exit across the basolateral membranes predominantly as free amino acids. This happens because of the efficient intracellular hydrolysis of peptides by cytosolic peptidases. However, there is substantial evidence in intact tissue preparations that a small, but significant, amount of intact peptides do cross the basolateral membrane (Ganapathy et al., 1991). Dyer et al. (1990) reported that uptake of Gly-Pro in the basolateral membrane vesicles was carrier-mediated and was stimulated by an inwardly directed H^+ gradient. This stimulation was inhibited by protonophores. These results indicate that there is a peptide transporter in the enterocyte basolateral membrane. It is very likely that this transporter plays a role in mediating the exit of intact peptides from cells across the basolateral membrane.

Another report demonstrated that peptide transporters are also present in the lysosomal membrane (Bird and Lloyd, 1990). Lysosomes participate in the breakdown of cellular proteins which enter this organelle by processes involving endocytosis and pinocytosis. It is speculated that breakdown of proteins within the lysosomes may not be complete and that the end products may in fact consist of free amino acids as well as small peptides which exit the lysosomes via specific transporters located in the lysosomal membrane. The investigation of passage of dipeptides across the lysosomal membrane indicated that lysosomes possess a transport mechanism which accepts L-dipeptides as substrates but excludes D-dipeptides. It is very likely that this transporter catalyzes extrusion of dipeptides arising from intra-lysosomal protein breakdown. The H^+ gradient is the most

logical driving force for active extrusion of dipeptides from the lysosome since the interior of the lysosome has an acidic environment with a H^+ gradient across the lysosomal membrane in the direction of lysosome-to-cytoplasm.

Regulation: Recently, information about the expression and regulation of peptide transport systems in animal cells has been emerging. For example, Brandsch et al. (1994) reported that treatment of human intestinal cells with phorbol esters which activate protein kinase C (PKC) resulted in a significant inhibition of peptide transport. This inhibition was specific and could be blocked if treatment was done in the presence of staurosporine, an inhibitor of protein kinases. Kinetic analysis revealed that the inhibition was associated with a decrease in the maximal velocity (V_{max}) of peptide uptake under these conditions, while the K_m was unaffected. The results suggest that the function of the peptide transport system is under the regulatory control of PKC. In Madin-Darby canine kidney cells (MDCK), treatment with the calmodulin antagonists, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) or calmidazolium, inhibited glycylsarcosine uptake by 40-50% in a time- and dose-dependent manner. In contrast, the uptake of alanine, leucine, and glucose was found to be stimulated by treatment with W-7. Kinetic analysis indicated that the inhibition of peptide transport activity was mainly associated with a decrease in the maximal velocity (Brandsch et al., 1995). These results suggest that peptide transport in Madin-Darby canine kidney cells is calmodulin-regulated. In kidney brush border membrane, zinc has been found to stimulate peptide transport, but no effect on the uptake of glutamine or glucose was found (Daniel et al., 1995). In this case, it was postulated that increased

peptide uptake may have resulted from an increased affinity of the membrane transporter for the substrate by a modification of their affinity for the binding site of the membrane transporter.

More recently, regional expression and dietary regulation of peptide transport has been reported in rat small intestine (Erickson et al., 1995). An even distribution profile of steady state levels of mRNA for two identified peptide transporters was observed along the longitudinal axis of the small intestine. Both were upregulated in the distal regions of intestine by a high protein diet, suggesting that the distal regions of small intestine may play an important role in the absorption of peptides. Furthermore, this area appears to be a primary site where dietary-induced changes in peptide transport occurs.

Regulatory studies of peptide transport in animal cells are still in the initial stage. Intensive research is needed to understand this process in order to use the system for pharmaceutical application.

Clinical Consideration: Biochemical and molecular studies of peptide transport in animal cells have a significant clinical value. In addition to playing a nutritional role in animal cells, peptide transporters are also of pharmacological interest, since they provide a route of entry for a wide range of biologically active peptides [e.g., peptide-derived antibiotics (Okano et al., 1986a; 1986b), the anticancer agent bestatin (Inui et al., 1990), and the angiotensin-converting enzyme inhibitors (e.g., captopril, Hu et al., 1988)]. The

phenomenon of using peptides as carriers of molecules that otherwise could not enter the cellular cytoplasm has been termed illicit transport (Gilvarg, 1981). This strategy has been considered in drug design. There are two approaches for the use of peptides for drug delivery by peptide transport: (1) Peptides can be used to target a toxic moiety to a pathogen based on differential substrate specificity between the host and pathogen transporters and (2) The peptides can themselves be therapeutically active. Zlokovic (1995) reviewed strategies to manipulate peptide transport processes, including lipidization, chemical modifications of the N-terminal end of peptides such as N-acylation, coupling of transport with post-metabolism and formation of potent therapeutic-active peptides, up-regulation of the peptide transporter, and use of chimeric peptides in which a non-transportable peptide is chemically linked to a transportable peptide. It is also suggested that future work should be directed towards the development of molecular strategies to up-regulate specific peptide transporters to enhance delivery of peptide pharmaceuticals or to down-regulate transport of peptides with a potential role in pathogenesis.

Peptide Transport in Higher Plants

In comparison to the organisms described above, peptide transport in higher plants has received little research attention. This is due to the fact that reduced nitrogen, assimilated from inorganic nitrogen sources, such as ammonia and nitrate, by plants, is transported mainly in the form of amino acids within the vascular system. Amino acids are distributed within plants via both the xylem and the phloem to heterotrophic tissues, such as

reproductive organs and developing seeds, that are dependent upon amino acid import for growth (Gifford et al., 1984; Thorne et al., 1985). Thus, it appears that the amino acid transport systems play a key role in nitrogen distribution within plants (Frommer et al., 1993; Hsu et al., 1993), whereas peptide transport systems seem unessential for this process.

However, several lines of evidence have demonstrated that peptide transport not only exists in plants, but may also participate in several important physiological processes. For example, significant levels of peptides have been found in phloem and xylem exudates (Higgins et al., 1982). The non-protein-derived peptides, alanylaminobutyric acid and glycylketoglutaric acid, have been identified in the phloem. Evidence has also been presented suggesting a role for glutathione, a tripeptide (described later), in the transport of reduced sulphur in the phloem and xylem (Higgins et al., 1982). The xylem contains lower levels of nitrogen than the phloem, yet peptides have been reported in the xylem exudates of several species, such as the vegetative organs and berries of grape plants (Khachidze, 1975) and the sap of corn (Fejer and Konya, 1958). Higgins and Payne (1982) suggested that the transport of peptides may be a more efficient way for nitrogen distribution than the transport of individual amino acid, especially for long-distance transport during the bulk movement of protein-degradation products (e.g., leaf senescence, seed germination). Peptide transport may also protect amino acids from catabolism by enzymes known to be present in the phloem during the transport process within plants (Higgins et al., 1980).

Glutathione (γ -Glu-Cys-Gly), a tripeptide, occurs in a reduced or in an oxidized

(disulphide) form. These two forms are readily interconverted by the enzyme glutathione reductase. Glutathione and glutathione reductase have been identified in monocotyledonous and dicotyledonous plants and occur in chloroplasts, mitochondria, and the cytoplasm (Higgins et al., 1982). One function of glutathione is to maintain optimal redox conditions within the cell. Glutathione may also act as a cofactor in the reduction of inorganic sulphate, in the production of numerous γ -glutamyl derivatives found in plant cells, and in the storage and transport of reduced sulphur (Higgins et al., 1982).

γ -Glutamyl peptides frequently occur in considerable quantities, especially in seeds and storage organs (Higgins et al., 1982). For example, 34% of the non-protein amino nitrogen of kidney bean seeds is present as γ -glutamyl-S-methyl-L-cysteine. Upon seed germination, this peptide is degraded, suggesting that the peptide plays an important role in the storage of nitrogen and/or sulphur.

Another interesting finding is that plant growth factors, such as auxin and gibberellin, are frequently bound to small peptides and the peptide-hormone conjugates are present in many tissues such as the vascular system and the endosperm of plant seeds (Bewley et al., 1986; Klambt 1960; Salisbury et al., 1991; Thurmann et al., 1962). The conjugation of peptides to these plant hormones may be involved in the regulation of their activity in response to plant growth and development, as well as facilitating the transport of these hormones (Bewley et al., 1986; Salisbury et al., 1991). Peptide transport systems may be involved in the movement of such hormone-peptide conjugates across the cellular membrane

of a plant cell.

In addition to endogenous plant peptides, several phytotoxins produced by plant pathogens are modified peptides (Walton 1990; Willis et al., 1991). Tabtoxin, produced by *Pseudomonas tabaci*, is a dipeptide and causes wildfire disease of tobacco plants (Taylor et al., 1972). Phaseolotoxin, from *Pseudomonas phaseolicola*, is a tripeptide and causes halo blight of beans (Mitchell 1976). Radiography studies showed that [³⁵S]labelled phaseolotoxin can be absorbed intact into plants and distributed into different parts of the plant (Mitchell et al., 1977). The transported phytotoxins are hydrolyzed by intracellular peptidases releasing a toxic moiety to cause plant diseases (Mitchell et al., 1977; Uchtyl et al., 1980). Another possible example of peptide uptake by plants is the uptake of a peptide-based herbicide, bialaphos. Bialaphos is a phosphinothricin-dialanine peptide that, upon cleavage, releases the toxic moiety phosphinotricinyl (PPT), an inhibitor of glutamine synthetase (Damm et al., 1993). It seems likely that these peptides are transported into host cells by peptide transport systems and release impermeant toxic moieties to kill plants by interrupting metabolic processes (Willis et al., 1991). Indeed, evidence in favor of this view has been reported for phaseolotoxin which can be transported via the oligopeptide permease (Opp) system in *E. coli* and *S. typhimurium* (Staskawicz et al., 1980).

Further direct evidence for the existence of peptide transport and its nutritional role in plants comes from studies of germinating barley seeds. The growing embryo in germinating barley seeds transports intact peptides released through the scutellum tissue.

These peptides are subsequently hydrolyzed into amino acids by intracellular peptidases (Higgins and Payne, 1977a; 1978b; 1980; Salmenkallio and Sopanen, 1989). The scutellum present in monocot seeds is a membrane carrier separating the endosperm from the embryo and serves as an absorptive structure highly specialized for transport (Walker and Payne, 1983). The products of endosperm breakdown (sugars, amino acids, peptides) are transported into the vascular system through the scutellum to be further transferred to the growing parts of the embryo (Walker and Payne, 1983). Thus, it is presumed that the peptide-transport proteins are located in the plasmalemma of cells of the scutellum forming the surface epithelium (Walker and Payne, 1983). Two proteins, of approximately 66 kD and 41 kD, were identified by thioaffinity labelling as components of the plant peptide transport system from the scutellum of germinating barley seeds (Payne and Walker, 1987). In addition, another protein of 54 kD was identified by photoaffinity labelling from the scutellum of barley seeds (Hardy and Payne, 1991). Peptide transport in barley seeds shares a number of similarities to that in yeast. The peptide transport system in barley can transport di-, tripeptides and some oligopeptides (Higgins and Payne, 1978a; Sopanen et al., 1977). Peptide transport has little regard to amino acid side chain composition, but has preference for hydrophobic residues and strong stereospecificity for L-amino acids (Higgins and Payne, 1978c). Moreover, peptide transport is pH-dependent and exhibits saturable kinetics (Higgins and Payne, 1977b).

Recently, Jamai et al. (1994) has reported that a peptide transporter also exists in the plasma membrane of mesophyll cells in mature broad bean leaves. This peptide transporter

exhibited pH-dependent and saturable kinetics with a preference to di- and tripeptides. It was proposed that this transporter may be responsible for the exchange of nitrogenous compounds in mature leaves (e.g. leaf senescence).

Taken together, the above data support the belief that peptide transport systems are not only present in plants, but may also play an important role in biological processes of plants by transporting peptides and/or peptide derivatives. Identification and characterization of plant peptide transport systems are essential to understand the biological role(s) of peptide transport in plant growth and development.

Rationale for Present Work

The first phase of my project was to isolate peptide transport genes from *Arabidopsis thaliana*. Functional complementation of yeast mutants has been used successfully to clone a number of plant transport genes including the potassium ion transporters (Sentenac et al., 1992), a sucrose transporter (Riesmeier et al., 1992), and two amino acid transporters (Frommer et al., 1993; Hsu et al., 1993). This method allows the identification of plant genes functionally homologous to yeast genes in well characterized yeast mutants. Expression of plant genes in yeast also affords greater flexibility in analyzing the functional characteristics of the protein product. Thus, the functional complementation strategy and the availability of yeast peptide transport mutants enabled us to identify components of the plant peptide transport system present in *Arabidopsis thaliana*. Expression of identified plant peptide

transport genes in yeast cells further allowed determination of the peptide transport activity and the characteristics of these new peptide transporters.

The second phase of my project was to study the physiological function of the identified peptide transport genes *in planta*. To further elucidate the *in vivo* roles of the peptide transport genes with regard to plant growth and development, *Arabidopsis* transgenic plants expressing antisense or sense *AtPTR2-B* were constructed. In general, to understand the *in vivo* role of a specific gene in plants, it is often desirable to modify or eliminate this gene and to study the resulting phenotypic effects. The use of antisense expression of mRNA is a powerful approach to reduce expression of an endogenous gene. The antisense mRNA suppresses the expression of the target gene resulting in a "mutant" phenotype (Tabler, 1993). The exact mechanism of antisense RNA inhibition is still uncertain. Various studies have suggested that a double-strand RNA duplex is formed between the antisense and the target sense RNA and is rapidly degraded by double-strand RNA-specific RNases or inefficiently transported from the nucleus to the cytoplasm for translation (Eguchi, 1991; Tabler, 1993). It is thus possible that lowering the mRNA and subsequently the protein level of the target gene will produce a distinct "mutant" phenotype which will shed light on the role of this gene.

Co-suppression of gene expression can also be applied for the same purpose. It is reported that the introduction of the sense gene into the plant genome causes the reduction or inhibition of expression of the endogenous gene. Cosuppression is common in plants, but

the mechanism for this phenomenon is still unclear (Jorgensen, 1995; Dougherty and Parks, 1995). Four different mechanisms have been proposed to explain co-suppression. Grierson and colleagues (1991) speculated that nuclear transcription of the transgene from a fortuitous proximal host promoter could result in the formation of an antisense RNA that would suppress the endogenous and transgene-derived sense transcript. In this case, co-suppression would be a fortuitous case of antisense suppression. Sense suppression of the *cab140* gene in *Arabidopsis* was demonstrated by Brusslan et al., 1993 and shown to be due to a decrease in transcription, reminiscent of gene suppression observed during paramutation in corn (Patterson et al., 1993). DNA-DNA interactions between the endogenous gene and the transgene have been suggested to result in the suppression of *cab140* gene expression. Reduction in gene transcription due to DNA methylation has been suggested as the cause for gene inactivation, regardless of the level of homology between the interacting genes (Matzke et al., 1994; Ingelbrecht et al., 1994). There are a number of cases where the suppression of a particular gene is mediated post-transcriptionally (Seymour et al., 1993). The explanation for this mechanism is that the plant cell may have a cytoplasmic threshold-sensitive system that destroys foreign transcripts when produced in excessive quantities (Dougherty and Parks, 1995). Another explanation for post-transcriptional co-suppression is a dynamic regulatory network based on positive autoregulation of coding sequence-specific turnover of homologous transcripts. A diffusible activator molecule, whose function is to maintain stability of the transcripts, is assumed to be degraded in a first-order process; thus, the balance between synthesis and degradation of activator molecules determines concentration. This positive feedback loop, in which an activator is derived from the transcript that it

stabilizes, produces multiple stable states of activator concentration and corresponding states of transcript concentration ((Dougherty and Parks, 1995). Each of the mechanism to explain co-suppression focuses on different aspects of the problem and none offers a complete explanation.

CHAPTER II

MATERIALS AND METHODS

Part I: Isolation and Characterization of two Peptide Transporter cDNAs from *Arabidopsis thaliana*

Bacterial and Yeast Molecular Biology

Bacteria, Yeast Strains and Plasmids: Table 1 lists the strains and plasmids used in this study.

Media and Growth Conditions: *Escherichia coli* was grown in LB medium (Luria-Bertani) (Maniatis et al., 1982) (10g tryptone, 5g yeast extract and 10g NaCl per liter) at 37°C. Transformed *Escherichia coli* strains were routinely maintained on LB medium plus 100 µg/ml ampicillin at 37°C. Yeast strains S288C, PB1X-9B, PB1X-2A, and PB1X-2AΔ were routinely maintained on YEPD agar (Sherman et al., 1986) containing (W/V) 1% yeast extract, 2% bacto-peptone (Difco Laboratories, Detroit, MI), 2% glucose, and 2% agar. Experiments with yeast were performed at 30°C in synthetic complete medium (SC) containing yeast nitrogen base (Difco) without amino acids and ammonium sulfate, with 1 mg of allantoin per mL, 2% glucose, and amino acid supplements (Sherman, 1986). Amino acids were supplied at 150 µM unless indicated otherwise. Solid medium contained 2% agar

Table 1. Bacteria and Yeast Strains and Plasmids

Designation	Characteristics	Source or Reference
Strain <i>E. coli</i>		
DH5 α	F ϕ 80d <i>lacZ</i> Δ m15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR recA1</i> <i>endA1 phoA hsdR17</i> (r^+ , m^+) <i>supE44</i> <i>thi-1 gyrA96 relA1</i>	Hanahan, D., 1983
XL1-Blue	<i>recA⁻ (recA1 lac⁻ endA1 gyrA96 thi hsdR17 supE44 relA1/F^{pro}AB lacI^q lacZΔM15 Tn10)</i>	Bullock et al., 1987
<i>S. cerevisiae</i>		
S288C	MAT α SUC2 <i>mal mel gal2 CUP1</i>	Sherman et al., 86
PB1X-9B	MAT α <i>ura3-52 leu2-3,112 lys1-1 his4-38 ptr2-2</i>	.
PB1X-2A	MAT α <i>ura3-52 leu2-3,112 lys1-1 his4-38 PTR2</i>	.
PB1X-2AA	MAT α <i>ura3-52 leu2-3,112 lys241-1 his4-38 ptr2-2Δ::LEU2</i>	Perry et al., 1994
Plasmid/phagemid		
pFL61	<i>E. coli</i> /yeast shuttle vector URA3 Amp ^R 2 μ /pUC19 PGK 3' promoter/PGK 5' terminator	Minet et al., 1992
pPTF4	pFL61::2800bp NotI fragment containing <i>AtPTR2-A</i> cDNA	This study
pDTF1	pFL61::1930bp NotI fragment containign <i>AtPTR2-B</i> cDNA	This study
pJP9	Ycp50:: <i>PTR2</i>	Perry et al., 1994
pBlue.SK	A cloning vector carrying <i>lacZ</i> and fl phage intergenic region for ssDNA generation	Stratagene, 1990

or 2% Noble agar in the case of disc assays.

***E. coli* Transformation:** The preparation of *E. coli* competent cells and DNA transformation were performed as described previously (Sambrook, 1982).

Yeast Transformation and Selection: Transformation of yeast cells was described by Gietz et al. (1992). Yeast cells (100 ml) were grown to a concentration of $1-2 \times 10^7$ cells/ml in YEPD medium at 30°C and diluted to 2×10^6 cells/ml with fresh and warm YEPD medium. Cells were then regrown to $1-2 \times 10^7$ cells/ml. Cells were harvested, washed in sterile water, resuspended in 1 ml of sterile water and transferred to 1.5 ml eppendorf tubes. Cells were pelleted, washed in 1 ml of 1 × TE/LiAc solution [made freshly from 10 × filter sterile stocks: 10 × TE buffer (100 mM Tris and 10mM EDTA) and 10 × LiAc (1M)], and resuspended in 1 ml of 1 × TE/LiAc at a cell density of 2×10^9 cells/ml. The competent cells (50 µl) were mixed with approximately 1 µg of DNA, 50 µg of sonicated, boiled salmon testes DNA, and 300 µl of sterile 40% PEG 4000 (polyethylene glycol) in 1 × TE/LiAc (made fresh from sterile 50% PEG stock in TE buffer). Cells were incubated for 30 minutes at 30°C with agitation. Subsequently, cells were heat-shocked for 15 minutes at 42°C and centrifuged for 5 seconds at 3500 rpm. The pellet was resuspended in 1 ml of 1 × TE buffer, plated on selective medium and incubated at 30°C.

The mutant strains PB1X-2A Δ and PB1X-9B were transformed with a cDNA expression library derived from *Arabidopsis* seedlings (Minet et al., 1992). This cDNA

library was made from mRNA obtained from complete young (stage two leaves) seedlings, including roots, and constructed in the yeast/*E. coli* expression vector, pFL61, which carries the constitutive phosphoglycerate kinase promoter. The cDNAs ligated to CACA adapters were inserted into the *Bst*XI sites flanked by *Not*I sites in the vector. Primary transformants were selected on SC medium supplemented with L-leucine, L-lysine, and L-histidine (150 μ M). Transformants were washed from these plates with sterile distilled H₂O and plated on dipeptide medium (SC minus uracil plus 80 μ M His-Leu and/or Lys-Leu in place of the required amino acids). To show that the peptide transport phenotype was plasmid borne, transformants were subcultured in nonselective liquid YEPD medium for 5 days and plated on solid YEPD medium to isolate individual colonies. For each transformant, 10 colonies were picked at random and resuspended in sterile water at 2×10^7 cells/ml and then spotted simultaneously (5 μ l) on both SC minus uracil and dipeptide medium and scored for growth. Loss of the ability to grow on medium without uracil always occurred concomitantly with the loss of ability to grow on peptides as a source of required amino acids. Plasmid DNA was isolated from colonies that appeared on the dipeptide medium and transformed into *E. coli* for plasmid amplification. The isolated plasmid DNA was used to transform the mutant strains PB1X-2A Δ or PB1X-9B. Only plasmids restoring the growth of the mutants on dipeptide medium were further analyzed.

Plasmid DNA Isolation: *E. coli* plasmid DNA was isolated by the alkaline lysis method (Maniatis, 1982). Yeast plasmid DNA was isolated by the guanidine hydrochloride method (Holm, 1986).

Growth of Yeast Transformants on Peptide Substrates: The yeast peptide transport mutant, PB1X-2A Δ or PB1X-9B, containing plasmids encoding either of the *Arabidopsis* cDNA clones (*AtPTR2-A* or *AtPTR2-B*) or the yeast peptide transport gene (*PTR2*), were grown on various peptides to determine the substrate specificity of the peptide transporters. In addition, untransformed PB1X-9B, PB1X-2A Δ and PB1X-2A were also assayed. Each strain was grown overnight in SC medium with amino acids plus uracil (nontransformed) or minus uracil (transformed) to stationary phase and harvested by centrifugation, washed twice with sterile water, and resuspended in 5×10^6 cells/ml. Five microliters of each suspension was applied as a small spot to a 2 ml volume of solid medium in a 24-well plate and incubated at 30°C for 72 hr. Growth was scored as uniform colony formation compared to negative controls that showed no growth. The medium for each assay consisted of SC medium plus or minus uracil for vector selection and was supplemented with 80 μ M of a specific peptide and auxotrophic-required amino acids except amino acids contained in the added peptide.

Disc Assays: Sensitivity to toxic amino acids and peptides was measured as described previously (Island, 1987). Briefly, a filter disc containing 0.38 μ mol of the toxic compound was placed on a lawn containing 5×10^6 cells and the plate was incubated at 30°C for 1 to 2 days. Resulting halos were measured across their diameter.

Radiolabeled Peptide Transport Assays: Uptake of L-leucyl-L-³H-leucine was done as previously described (Island et al., 1987). Yeast cultures were grown overnight in

SC medium without amino acids and ammonium sulfate, supplemented with 2% (w/v) glucose and 1 mg/ml allantoin plus amino acids to supplement auxotrophic markers. The cell culture was diluted to 3×10^6 cells/ml in fresh medium and regrown to 6×10^6 cells/ml. The cells were pelleted, washed twice with cold sterile water, and resuspended in 2% glucose to reach a final cell concentration of 2×10^8 cells/ml. This mixture was placed on ice.

The assay solutions were mixed in an eppendorf tube as follows: 400 μ l water, 50 μ l 20% glucose, 40 μ l 0.1M citric acid/ KH_2PO_4 buffer, pH 5.5, and 15 μ l [^3H]-Leu-Leu (1.5 mM, specific activity 30 mCi/mmol). Metricel filters (GN-6, 0.45 μ m from Baxter, Chicago, USA) were prewet with citrate/phosphate buffer and placed on a 10-well manifold. With the vacuum applied, 500 μ l of the cell suspension was added to each assay tube. This represents time zero. At 25 seconds, 1 minute, 2 minutes, 3 minutes, and 4 minutes time intervals, 190 μ l of the assay solution/cell suspension mixture was removed and applied to the prewet Metricel filters. The filter was washed three times with 2 ml of cold sterile water followed by 2 ml of room-temperature water. After washing, the vacuum was removed and the filter was pulled off. The filtered cells were placed into a scintillation vial containing 5 ml of scintillation fluid and counted (liquid scintillation counter; LS-7000; Beckman Instruments, Inc., Fullerton, Calif.). With the remaining cell suspension, 500 μ l of the cell suspension was added to 500 μ l of water and 190 μ l of this mixture solution was transferred to dried and preweighed filters for determination of the cell dry weight by drying and weighing it. Uptake was expressed as nanomoles per minute per milligram dry weight by calculating the amount of isotope incorporated using the specific activity of ^3H -Leu-Leu. All

competitors were supplied at 10-fold molar excess, except for dileucine, which was supplied at 100-fold excess.

Peptide Transport Kinetics: Radiolabeled uptake assays were performed as described (Island et al., 1987). [^3H]dileucine concentrations were 1, 5, 10, 20, 50, and 100 μM with the specific activity of [^3H]dileucine held constant (20 mCi/mmol). Individual samples were taken at 20 second intervals. The kinetic parameters, K_m representing the Michaelis constant, and $V_{\max}$ representing the maximum enzyme velocity, were determined using the Michaelis- Menten equation: $v_i = V_{\max}[S] / K_m + [S]$, in which v_i indicates the initial velocity of uptake in nanomoles per milligram of dry weight of cells per second and $[S]$ indicates the concentration of the [^3H]dileucine substrates in micromolar. Using the same data of $[S]$ and initial velocity, the Eadie-Hofstee plot was drawn ($v_i/[S]$ versus v_i) (Tinoco et al., 1985).

DNA Sequencing and Computer Analysis

DNA Sequencing: The DNA sequence of the cDNA clone was determined on both strands by the dideoxynucleotide chain termination method (Sanger, 1977) using Sequenase version 2.0 (United States Biochemicals, U.S.A.). A *NotI* cDNA fragment (1.96 kb *AtPTR2-B* and 2.8 kb *AtPTR2-A*) was inserted into the pBluescript II SK+ plasmid (Stratagene, U.S.A.). Universal T₇ and T₃ primers in the pBluescript Sk plasmid were used for double-strand DNA sequencing. To sequence the full-length cDNA fragment, the clone was digested

and subcloned into the pBluescript II SK+ plasmid (Stratagene, U.S.A., 1990). An exonuclease III-mungbean nuclease deletion kit (Stratagene, U.S.A., 1990) was applied to generate a set of deletions of the subclones. The general rules for deletion were (1) to create 3' blunt end or 5' overhangs of double stranded DNA near the insert site; (2) the linearized DNA was treated with *ExoIII* nuclease to delete the single stranded DNA of the double-stranded DNA template from 3' to 5' starting at the 3' blunt end or 5' overhang. The time interval was 1 minute (100-200 nucleotides deleted per minute); (3) The generated single-stranded DNA-containing template was digested with the DNA nuclease S1 to remove the single-strand DNA; (4) The blunt-ended and linear DNA template was religated using *T₄* DNA ligase and then transformed into *E. coli* cells. The deleted subclones were recovered and analyzed by plasmid DNA isolation and restriction digestion.

Sequence analysis: The University of Wisconsin Genetics Computer Group GCG program (Devereux et al., 1984) was used for nucleic and amino acid sequence analysis. The hydrophobicity of the predicted proteins was analyzed using the program PepPlot in the GCG software package. The program Bestfit was used to search the similarity of amino acids among the different proteins. The dendrogram plot was drawn by the program Pileup. The program Pretty, using the output from Pileup, was used to construct multiple sequence alignments and a consensus sequence. The consensus was generated at a minimum plurality of 2.00. The NCBI BLAST algorithm search program (Altschul et al., 1990) was used for database searches.

Synthesis of L-leucyl-L-³H-Leucine Trifluoroacetic Acid and Toxic Dipeptides

L-leucyl-L-³H-leucine trifluoroacetic acid and toxic peptides were synthesized by standard solution phase techniques (Naider et al., 1974) and generously provided by Dr. F. Naider (City University of New York).

Chemicals and Reagents

All nontoxic peptides were purchased from Sigma or Bachem BioSciences, Inc. (Philadelphia, PA, U.S.A.).

GeneBank Accession Number

Sequence data have been submitted to GeneBank under the accession number U01171 for *AtPTR2-A* and L39082 for *AtPTR2-B*.

Part II: Construction and Characterization of Transgenic *Arabidopsis thaliana* Plants Expressing Sense or Antisense *AtPTR2-A* or *AtPTR2-B*

Bacterial Molecular Biology

Bacteria and Plasmids: Strains and plasmids used in this study are listed in Table

2.

Growth of Bacteria: The growth of *E. coli* strains has been described in Part I. *Agrobacterium tumefaciens* strain EHA105 (Hood, 1993) was grown in YEP medium (Koncz et al., 1994) (10 g Bacto-peptone, 10 g Bacto-yeast extract, 5 g NaCl, and 15 g agar per liter) at 30°C. Antibiotics were used in the following concentrations (µg/ml) for *E. coli*, ampicillin 100, tetracycline 3, kanamycin 10; For *A. tumefaciens*, kanamycin 50, timentin 400, rifampicin 25.

***Agrobacterium* Electroporation:** A 500 ml culture of *A. tumefaciens* was grown to an OD₆₀₀ of 0.5-0.8 and harvested by centrifugation for 10 minutes at 8000rpm. The cell pellet was washed three times with sterile dH₂O and resuspended in 15% sterile glycerol. At this point the cells can be directly used for electroporation or aliquoted and stored at -80°C. Competent cells (40 µl) were mixed with 1-3 µl of DNA (approximately equal to 1 µg) and transferred to a cold 0.2 cm-wide cuvette. The cuvette was placed in the pulse chamber of the electroporator. The conditions for electroporation of *A. tumefaciens* was 25 µF of capacity, 400 • of resistance, and 2.5 kV of voltage. Under these conditions, the time constant was around 4.6 milliseconds.

Plasmid DNA Isolation From *A. tumefaciens*: The alkaline lysis method (Sambrook et al., 1989) can be used for plasmid DNA isolation from *A. tumefaciens*, but the yield is too low to be detected by ethidium bromide (EtBr) staining. In order to obtain sufficient DNA for analysis, plasmid DNA isolated from *A. tumefaciens* was transformed into *E. coli*.

Table 2. Bacterium Strains and Plasmids

Designation	Characteristics	Source or Reference
<u>Strain</u>		
XL1-Blue	Described previously	
JM101	<i>lac proAB supE thiF' traD36 lacI^s ZM15</i>	Messing et al., 1981
MC1000	<i>araD139Δ(ara-leu)7679 galU galKΔ(lac)174 rpsL thi-1</i> Preferable host strain of pGA941	Casadaban et al., 1980
EHA105	Super-efficient infectious agrobacterium (octopine) carrying a helper plasmid pEHA105 Rif ^R Gm ^R	Hood et al., 1993
<u>Plasmid/phagemid</u>		
pBlueSK(+)	Described previously	
PRR217	Contains a full length of rice rDNA clone	Takaiwa et al., 1984
pUC19	High copy-number cloning vector, Amp ^R	Viera et al., 1982
pGA941	Binary vector carrying 35S promoter and <i>npt</i> gene conferring kanamycin resistance	An et al., 1991
pGAAS	pGA941::2600bp <i>XbaI</i> fragment containing sense <i>AtPTR2-A</i> with a 330bp deletion of 5' uncoding region	This study
pGAAA	pGA941::2600bp <i>XbaI</i> fragment containing antisense <i>AtPTR2-A</i> with a 330bp deletion of 5' uncoding region	This study
pGABS	pGA941::1600bp <i>XbaI</i> fragment containing sense <i>AtPTR2-B</i> with a 320bp deletion of 3' region	This study
pGABA	pGA941::1600bp <i>XbaI</i> fragment containing antisense <i>AtPTR2-B</i> with a 320bp deletion of 3' region	This study

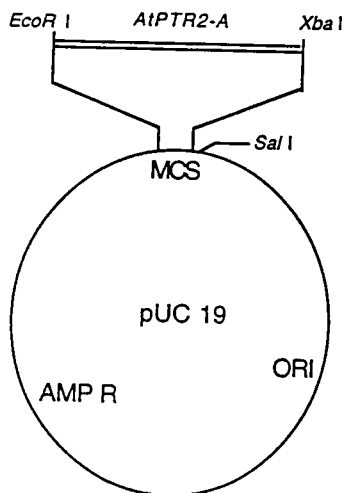
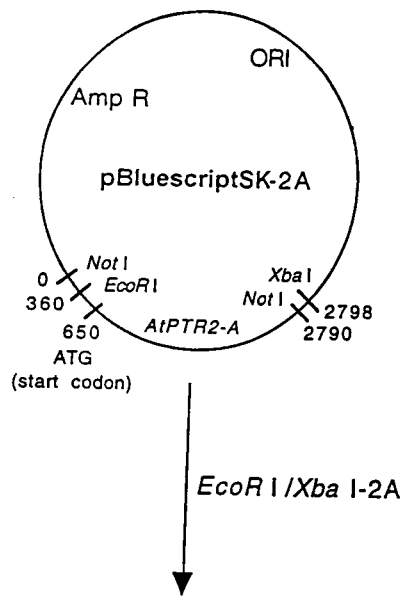
Subsequently, sufficient DNA could be isolated from *E. coli* for analysis.

DNA Cloning and Manipulation: Figure 1a shows the procedures used for cloning *AtPTR2-A* or *AtPTR2-B* into the plant expression vector, pGA941, that carries the 35S promoter and kanamycin resistance gene (*npt*). The plasmid pSK2-A (pBluescript SK containing *AtPTR2-A* at the *NotI* site) was digested with *EcoRI* and *XbaI*. The *EcoRI-XbaI* fragment containing the full-length coding region of *AtPTR2-A* with a 360 bp deletion from the 5' noncoding region, was inserted into the pUC 19 plasmid. The recombinant plasmid was then digested with *EcoRI* and *SalI*, and the isolated *EcoRI-SalI* fragment was cloned into plasmid pBluescript SK. The recombinant plasmid was then cut with *XbaI* and the DNA fragment released was inserted into pGA941 at the *XbaI* site. Orientation of the cloned fragments was determined by restriction digestion. The sense gene construct was designated pGA-2AS and the antisense gene construct designated pGA-2AA.

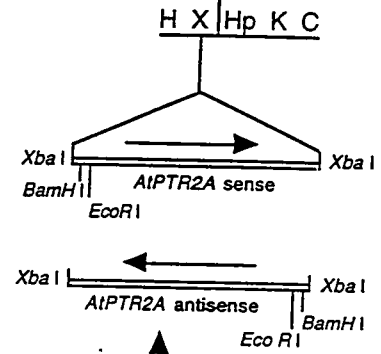
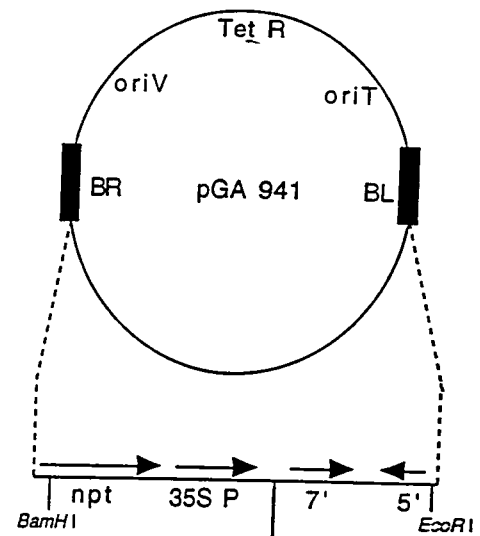
To clone *AtPTR2-B* into pGA941, a similar strategy was used (Figure 1b). The plasmid pSK2-B was digested with *SacI* and *XbaI*. The *SacI-XbaI* fragment containing a truncated *AtPTR2-B* gene, deleted of 100bp of coding region and 230 bp of the noncoding region at the 3' end, was inserted into pUC19. The newly-constructed plasmid was cut with *EcoRI* and *SalI* and the released fragment was cloned into pBlue. SK. A 1.63 kb fragment was liberated from the recombinant pBluescript SK by digesting with *XbaI* and inserted into pGA941. The orientation of *AtPTR2-B* in pGA941 was determined by restriction digestion analysis. The sense gene construct was designated pGA-2BS and the antisense gene construct

Figure 1. Construction of antisense and sense *AtPTR2-A* (a) and *AtPTR2-B* (b) vectors used to transform *Arabidopsis thaliana*. The drawing is not to scale. RB and LB are the right and left border sequences, respectively, of the Ti plasmid. The *npt* gene is the neomycin phosphotransferase gene conferring kanamycin resistance. 35S P is the 35S promoter of the cauliflower mosaic virus. 7' and 5' are the terminator sequences in opposite orientations from the genes 7 and 5, respectively (An et al., 1991).

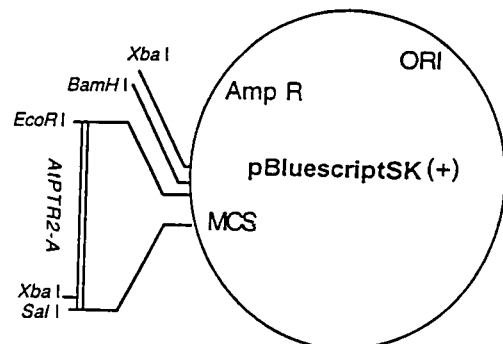
a



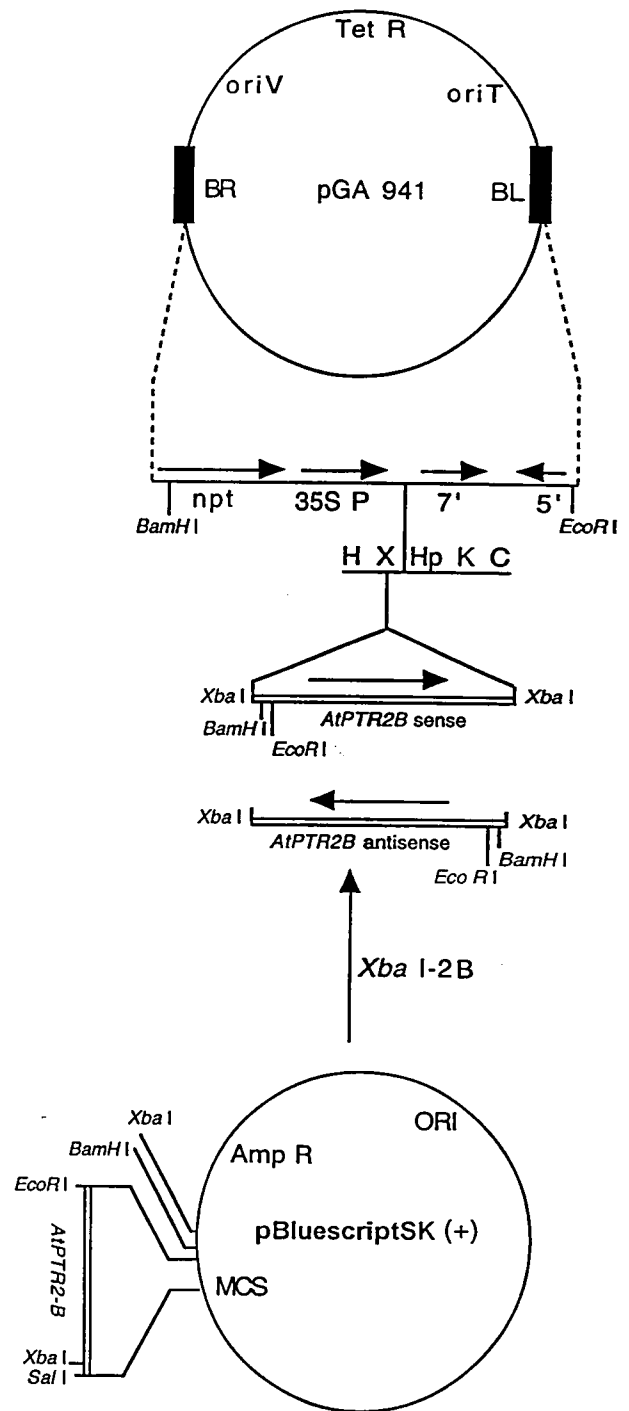
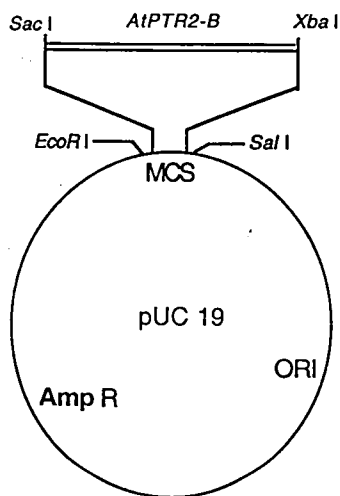
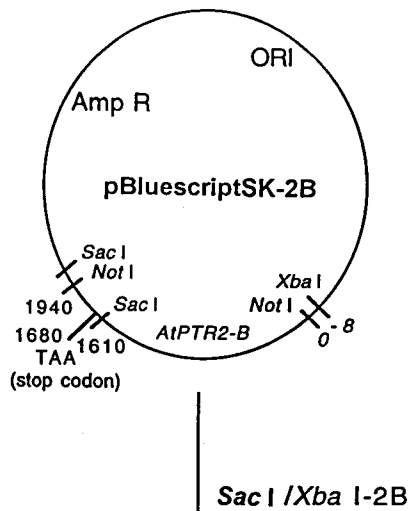
EcoRI / *SalI* 1-2A



XbaI 1-2A



b



designated pGA-2BA. To obtain a high transformation efficiency of the plasmid pGA941 into *E. coli*, strain MC1000 was used and the transformants were selected on the plates containing kanamycin (10 µg/ml) and tetracycline (3 µg/ml).

Plasmid constructs were transformed into *A. tumefaciens* strain EHA105 (pEHA105) by electroporation and the transformants were screened on agar plates containing 3 µg/ml tetracycline, 10 µg/ml kanamycin and 25 µg/ml rifampicin. The plasmids were isolated from the *Agrobacterium* transformants by the alkaline lysis method and then digested with *Xba*I, *Bam*HI and *Eco*RI, respectively, to confirm that no changes had occurred in the plasmid DNA.

Plant Molecular Biology

Genomic DNA Isolation and Southern Hybridization: Plant genomic DNA isolation was accomplished by the cetyltrimethylammonium bromide (CTAB) method (Rogers, 1988). Genomic Southern blot analysis was done as described in Sambrook et al.(1982). A ³²P-labelled DNA probe was obtained using a random hexamer-primed labelling kit (Promega, Madison, Wisconsin, USA).

Total RNA isolation and Northern Hybridization: Plant total RNA was extracted by using either the Trizol extraction method (GIBCO, BRL, Grand Island, New York, USA) or the guanidine thiocyanate method (Chirgwin, 1979). A TRIzol Reagent Kit was used to

isolate total RNA from all tissues, except siliques. The TRIzol extraction was found unsuitable for isolation of total RNA from siliques. Therefore, total RNA was isolated from siliques by the guanidine thiocyanate isolation procedure followed by LiCl precipitation. Twenty micrograms of total RNA were electrophoresed in a 1.0% agarose gel and transferred to a neutral nylon membrane (Sigma, Cleveland, Ohio, USA). A DNA probe was prepared by random hexamer-primed [α - ^{32} P]dATP labelling as described above and hybridized to blots at 42°C in a solution containing 50% formamide, 5 × SSC, 5% SDS, 6 × Denhardt, 100 µg/ml SSDNA). Blots were washed for 30 minutes to 1 hour under high stringency conditions (0.1% SDS, 0.1% SSC, 65°C) for one or two times depending on the intensity of the signal. To confirm approximately equal amounts of total RNA were loaded in each lane, an *Arabidopsis* ubiquitin gene-specific probe (Takaiwa et al., 1984) was hybridized to the membrane and the density of the bands was measured by a computing densitometer (Molecular Dynamics, ImageQuant™, Model 325 E; Quantitation by volume integration).

Reverse-Transcription-Polymerase Chain Reaction (RT-PCR): To measure low amounts of *AtPTR2-A* mRNA in different tissues, reverse transcription and PCR were performed as described previously (Sambrook, 1982). Briefly, 10 µg of total RNA was pretreated with RNase-free DNase (Promega, Madison, Wisconsin, USA) and reverse transcribed with moloney murine leukemia virus (M-MLV) reverse transcriptase (United States Biochemicals,) using an oligo(dT) primer. The first-strand synthesized cDNA (treated with RNase A) was amplified by PCR (94°C, 1 min; 55°C, 1.5 min; 72°C, 1.5 min; 30 cycles) using Taq polymerase (Promega, Madison, Wisconsin, USA). The primers used were as

follows: an upstream primer starting at base 1975, 5'-GCTTCCATGATTTACGCTG-3' (20mer) and a downstream primer starting at base 2528, 3'-GGCCATCCGCAAATACG-5' (17mer). These primers generate a 569-bp fragment from the *AtPTR2-A* cDNA. The amplified DNAs were electrophoresed and Southern blotted to nitrocellulose filters (Optitran, Schleicher & Schuell, Keene, NH, USA). The filters were probed with an α -³²P-labeled-*AtPTR2-A*-specific fragment.

Plant Transformation

Sterile Techniques: Items utilized for root-explant transformation were sterile. Disposable sterile plasticware was used when available. Forceps and scalpels were frequently sterilized by ethanol flaming and allowed to cool prior to handling tissue. Cleanliness of the work area was found to be absolutely essential. Interior surfaces of the transfer hood were cleaned with full-strength commercial bleach (5.25% chlorite), rinsed with water, and ethanol dried.

Media Preparation: Table 3 outlines the components of the plant-tissue culture media used in these studies (Marton et al., 1991). Chemicals were purchased from Sigma Chemicals Co., St. Louis, MO except that the timentin was obtained from the pharmaceutical division of University of Tennessee Hospital. To purchase the timentin, special permission for drug use was requested, that could be obtained by applying to the Drug Research Committee of the University of Tennessee.

Table 3. Plant Tissue Culture Media

Type ^a	Components
ARC	contains the basic components (see "a")
ARM I	3 mg/l indoleacetic acid, stock 2 mg/ml ^b 0.15 mg/l 2,4-dichlorophenoxyacetic acid, stock 0.5 g/ml 0.6 mg/l benzyladenine, stock 1 mg/l 0.3 mg/l isopentenyl adenine, stock 1 mg/ml
ARM II	0.2 mg/l naphthaleneacetic acid, 1 mg/ml 4 mg/l isopentenyl adenine, stock 1 mg/ml 400 mg/l Timentin ^c , stock 100mg/ml 75 mg/l kanamycin, stock 50 mg/ml
ARM IV	0.2 mg/l naphthaleneacetic acid, 1 mg/ml 0.2 mg/l benzyladenine, stock 1 mg/ml 400 mg/l Timentin, stock 100mg/ml 75 mg/l kanamycin, stock 50 mg/ml

^a Each medium type contains the same basic components as follows: for one liter of medium, 4.3 g Murashige and Skoog salts, 30 g sucrose, 0.2 g *myo*-inositol, 3 ml Miller's solution (6% w/v KH₂PO₄), and 2 ml 500 × vitamix (all chemicals purchased from Sigma Chemicals Co., unless otherwise noted). The pH was adjusted to 5.6-5.8 with 1 N NaOH prior to the addition of 7 g agar and autoclaving. The 500 × vitamix contains 5 mg/ml vitamin B1, 0.5 mg/ml vitamin B6, 1 mg/ml glycine, 0.5 mg/ml nicotinic acid, 0.25 mg/ml folic acid, and 0.5 mg/ml biotin(vitamin H).

^b Stock solution were made in distilled water and filtered unless otherwise noted.

^c Pharmaceutical division of the University of Tennessee, Hospital, TN.

Agrobacterium Preparation: *Agrobacterium* transformants were grown at 30°C to an OD₆₀₀ of 0.5-0.8 in YEP medium for approximately two days with antibiotic selection of tetracycline and kanamycin. Rifampicin selection was omitted since it inhibits plant transformation by *Agrobacterium*.

Root Explant Preparation:

(1) Seed sterilization and growth: Approximately 200 seeds of *Arabidopsis thaliana* ecotype RLD were placed in an eppendorf tube and incubated with 1 ml of 0.1% HgCl for 15 minutes with occasional shaking. The seeds were washed 3-4 times with sterile dH₂O to remove the HgCl₂ solution. The sterilized seeds were transferred to G-H medium (Marton et al., 1991) (3.1 g Gamborg's B₅ salts, 3 ml Miller's solution [6% w/v KH₂PO₄], 30 g sucrose, and 10 g agar, pH 5.6-5.8) and spread to cover the plate. The plate was sealed with parafilm and placed in a growth chamber set at a photoperiod of 16 hr light/25°C and 8 hr night/22°C. The seeds were allowed to grow for three to four weeks in order to generate enough roots (Note: don't allow seedlings to grow too old and turn yellow).

(2) Root explant pretreatment: The parafilm was removed and the petri dish was opened in the sterile hood. The leaves of seedlings were cut and removed using sterile forceps and scalpels. The petri dish was placed upside down in order to expose the roots and 4 ml of ARC liquid medium (Table 3) was added to wet the roots. The exposed roots were

transferred to ARM I medium (Table 3) (Note: remove the leaves and agar from root explants as much as possible). The roots were separated as much as possible. The plate was sealed and incubated in the growth chamber under the same conditions as described above for six days. Precautions should be taken to avoid condensation in the petri dish. During the period of incubation, root primordia appeared along the roots (Figure 2). These are most likely sites for *Agrobacterium tumefaciens* infection.

Root Explant Transformation: The pretreated roots prepared as above were transferred to a sterile petri dish containing 4 ml ARC liquid medium (Note: all green leaves remaining along the roots must be discarded). The roots were injured by cutting with the sterile scalpel or squeezing with sterile forceps. Approximately 200 μ l of *Agrobacterium* culture ($OD_{600} = 0.5-0.8$) was added to the plate and mixed well with the roots by gently shaking. The plate was sealed and placed in a dark cabinet at room temperature for two days. As a negative control, no *Agrobacterium* culture was incubated with the roots. Two days later, the *Agrobacterium* culture was removed and the roots were washed with ARC liquid medium at least three-times to clean the *Agrobacterium* cells as much as possible. The roots were transferred to the ARM I medium plus 400 μ g/ml timentin and spread uniformly over the plate. The plate was incubated in the growth chamber for one day.

Tissue Culture/Plant Regeneration: Following incubation, roots were transferred to shoot-inducing medium, ARM II (Table 3), containing 400 μ g/ml timentin and 75 μ g/ml kanamycin. Because overlapping of roots inhibits shooting, the roots must be transferred

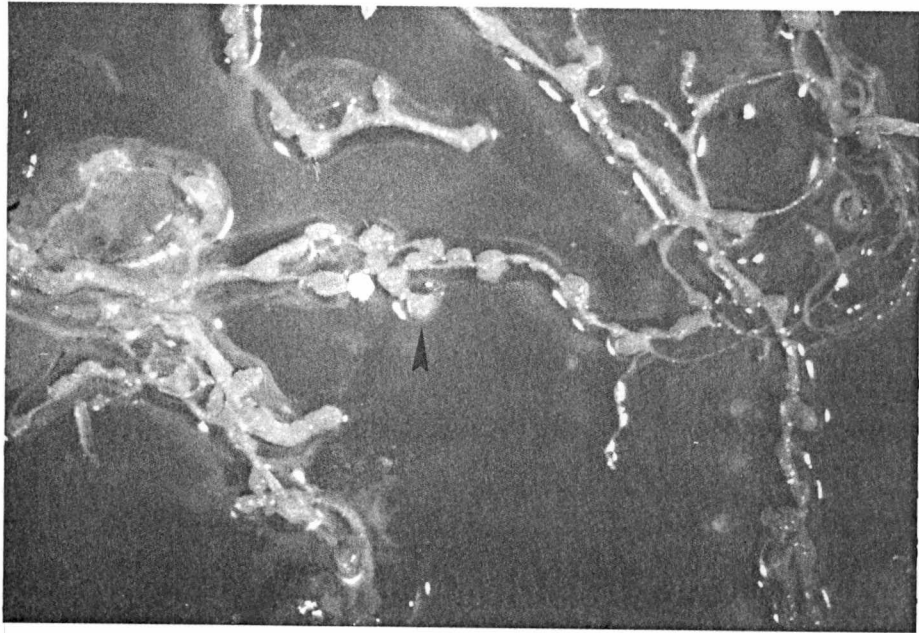


Figure 2. Root primordium formation after roots were pretreated with ARM I medium.

piece-by-piece. As a positive control for shooting, kanamycin was omitted from the ARM II medium, but 400 µg/ml timentin was added to inhibit *Agrobacterium* growth. Plates were sealed with parafilm and incubated in the plant tissue culture room (23°C-25°C and continuous light) for about two weeks (Note: any condensation in the plate must be avoided). After 10 to 15 days, green sectors (embryo-like tissues) appeared on the roots and then developed into shoots. Negative control roots, untreated with *Agrobacterium tumefaciens*, subjected to selection, turned brown and appeared dead within one month. The small and young shoots were transferred to shooting-enhancing medium, ARM IV (Table 3), plus 400 µg/ml of timentin and 75 µg/ml of kanamycin. One week after transfer, multiple shoots were produced from the original transplant. These shoots were separated using sterile forceps and scalpel. The shoots were imbedded in rooting medium, ARC (Table 3), containing 0.1 µg/ml of naphthaleneacetic acid (NAA) in a tissue culture test tube. Within 10 days, most shoots were rooted and started elongating. However, several shoots never formed roots.

Acclimation: The young plants with or without roots were removed carefully from the tubes and washed gently with water to remove any remaining agar. The plants were placed into pots filled with sterile Pro-mix BX (Premier Brands Inc. New Rochelle, NY) and watered with *Arabidopsis* nutrient solution (5 mM KNO₃, 2.5 mM KH₂PO₄, 2 mM MgSO₄, 2 mM Ca(NO₃)₂, 0.05 mM FeEDTA, pH 6.5) (Genetic sources in *Arabidopsis*, 1987). A tent constructed out of drinking straws and syran wrap was fitted around each pot to provide humidity. The pots were placed in the growth chamber at a photoperiod of 16 hr light/24°C and 8 hr night/22°C. The tents were gradually opened over a period of one week to allow for

acclimation. At the end of one week, noticeable growth had occurred and the tents were removed.

Nomenclature

Nomenclature of the transgenic plants is as follows; *AtPTR2-A* sense or antisense gene transformed plants are designated as AS or AA followed by numbers, respectively. *AtPTR2-B* sense and antisense gene transformed plants are designated as BS and BA, respectively, followed by numbers.

Plant Genetics

Seed Sterilization: *Arabidopsis* seeds (100-200) were surface sterilized as described above.

Seed Germination and Segregation of Kanamycin Resistance: Seeds were sown to a density of 50 seeds per plate on G-H medium with or without 50 µg/ml of kanamycin. Parafilm-sealed plates were placed at room temperature in the dark for two days before transferring to the growth chamber with a photoperiod of 16 hr/24°C light and 8 hr dark/22°C. Plates lacking kanamycin selection were used to measure germination frequency. Seedlings were individually transferred aseptically to plates containing 50 µg/ml kanamycin, sealed and placed back in the light. After 10 days, plates were scored for kanamycin

sensitivity as characterized by chlorosis and death (Figure 4). Seeds directly sown onto plates containing kanamycin were scored for sensitivity after 10 days in the light.

Phenotypic Analysis of Transgenic Plants

Leaf size was determined by measuring the length and width of 10 individual leaves taken from three plants of each line. The leaves were chosen from the top to bottom of the basal leaves (not from the stem). The fresh weight of these leaves was also measured.

Average weight per seed (μg) was determined by weighing five samples of 100 seeds each. Average seed number per silique was measured by counting the seed number of 30 siliques. The length of siliques was determined by measuring 30 siliques for each line.

Analysis of Seed maturation: Seed development in siliques was analyzed by dissecting the siliques and examining them under a dissecting stereo microscope. Different magnifications (2.5-8 fold) were used in order to observe seeds in siliques and for photography.

Scanning Electron Microscopic Analysis

For scanning electron microscopy (SEM), green siliques were dissected under the stereo microscope and fixed in 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH

7.0) at 4°C for 12 to 16 hours. The samples were then rinsed in the same buffer briefly and postfixed in 1% osmium tetroxide (OsO₄) in 0.05 M sodium cacodylate buffer (pH 7.0) at 4°C for 12 to 24 hours. After rinsing with 0.025 M sodium phosphate buffer (pH 7.0), the samples were dehydrated in a graded ethanol series (25% to 100%) at 4°C. The treated siliques were critical point dried using liquid CO₂ and mounted on SEM stubs. The mounted specimens were coated with gold:palladium(6:4) and observed with an ETEC Autoscan scanning electron microscope at 20kV. Photographs were taken using Polaroid type 55 film.

Stereo Microscopic Analysis of Root Morphology

To observe the root morphology of plants, seeds were germinated on G-H medium. The agar plates were placed in an upright position and incubated in the plant growth chamber. Two weeks after germination, plant roots were observed under the dissecting stereo microscope. Lateral roots and root hairs were examined using the magnification of 0.5×7.5 and 1.5×7.5 , respectively.

Statistical Analysis

Chi-square analysis was performed on the genetic data according to the methods of Sokal and Rohlf (Steel and Torrie, 1980). Two-sample testing (t-test) procedures were used to test for significant differences between sample means (Steel, 1980).

RFLP Mapping

Genomic DNA was isolated from *Arabidopsis thaliana* ecotypes Landsberg *erecta* and Colombia, and digested with the restriction enzyme *Bam*HI, *Bcl*II, *Hpa*I, or *Xba*I, respectively. Electrophoresis was performed before Southern hybridization. Southern blots were hybridized with a ³²P-labelled *AtPTR2-B*-specific cDNA probe to determine the polymorphism of *AtPTR2-B* between these two ecotypes. The *Xba*I polymorphism detected was used for RFLP mapping.

AtPTR2-B was mapped using 98 recombinant inbred lines generated from the cross between the Col-0 and Landsberg *erecta* ecotypes (Lister and Dean, 1993). The same probe used as above detected the *Xba*I polymorphism between the Col-0 and Landsberg *erecta* ecotypes. Blots with *Xba*I digested DNA from the 98 recombinant inbred lines were prepared as described previously (Ausubel et al., 1995). The filters (GeneScreen Plus, Du Pont-New England Nuclear) were hybridized and washed according to the manufacturer's recommendations, and then exposed to Phosphorimager screens and developed by using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The segregation of the marker was followed by Lister and Dean (1993). The RFLP mapping was performed by Eliana Drenkard from Harvard University.

CHAPTER III

RESULTS

Part I: Isolation and Characterization of Two Peptide Transporter cDNAs from *Arabidopsis thaliana*

(These experiments were performed in conjunction with Dr. Henry York-Steiner.
Data attributable to Dr. Steiner is acknowledged in the respective figure legends)

Isolation of Two *Arabidopsis* Peptide Transporter cDNAs by Functional Complementation of Yeast Mutants

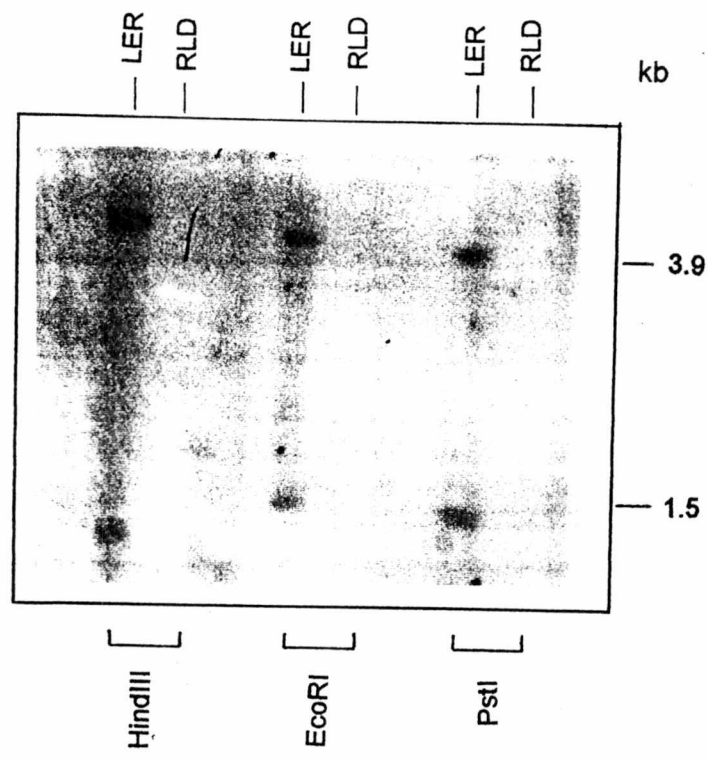
S. cerevisiae strains PB1X-9B and PB1X-2AΔ are unable to grow on minimal medium supplemented with dipeptides containing amino acids to satisfy their leucine, lysine, or histidine auxotrophic requirements. Strains PB1X-9B and PB1X-2AΔ have a point mutation and deletion, respectively, in the *PTR2* gene. To isolate a plant gene that encodes a peptide transporter, an *A. thaliana* cDNA library was transformed into both mutant strains and the transformants were screened for growth on dipeptide medium containing His-Leu and/or Lys-Leu. A total of 21 primary transformants were recovered (15 from PB1X-9B and 6 from PB1X-2AΔ). The plasmids isolated from the transformants were transformed into the *E. coli* strain DH5α for amplification and the recovered plasmids were then analyzed by

restriction digestion. Based on the restriction digestion patterns, the plasmid DNAs were classified into two groups; the first group included those with the expected vector and cDNA inserts of different band sizes (1.96 kb and 2.8 kb). The second group included those showing two or more DNA bands but without the expected vector. The two unique plasmids from the first group, one isolated from PB1X-9B (designated pPTF4) and one from PB1X-2AΔ (designated pDTF1), were reintroduced back into their respective mutant strains and plated on dipeptide medium. The two plasmids were able to restore both growth on peptides and peptide transport to the mutants. The plasmids selected from the second group of plasmids when reintroduced into the mutant strains did not restore growth on peptides. Restriction digestion of pDTF1 with *NotI* released a 2.0-kb insert, while *NotI* digestion of plasmid pPTF4 released a 2.8-kb insert.

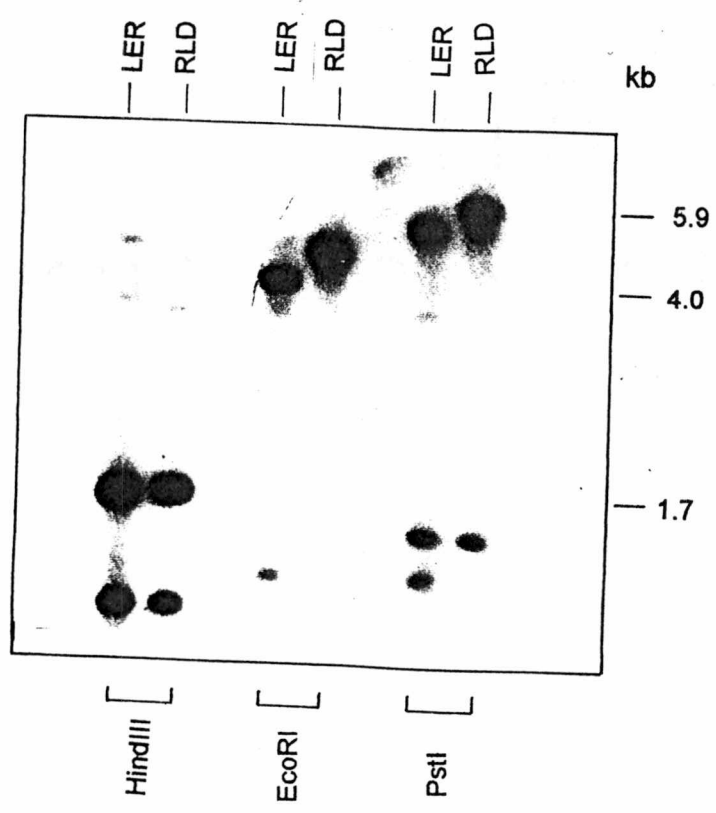
An α -³²P-labeled probe consisting of the entire *NotI* 2.8-kb insert from pPTF4 did not hybridize under low-stringency conditions to uncut or *NotI*-digested pDTF1 plasmid DNA (data not shown). These data indicate that two unique cDNA clones were isolated in plasmid pDTF1 and pPTF4. The 2.8-kb clone was designated *AtPTR2-A* and the 2.0-kb clone designated *AtPTR2-B*. Southern blot analysis of *Arabidopsis* genomic DNA digested with *EcoRI*, *PstI*, *HindIII*, or *BamHI* showed one or two bands in response to these different restriction enzymes when a *AtPTR2-A*- or *AtPTR2-B*-specific probe was used, respectively (Figure 3). These data suggest that there is a single copy of *AtPTR2-A* and *AtPTR2-B* in the *Arabidopsis thaliana* genome. The pattern of restriction digestion was compared between *Arabidopsis thaliana* ecotype Landsberg erecta and RLD. The results showed that *AtPTR2-A*

Figure 3. Southern blot analysis of genomic DNA isolated from *Arabidopsis thaliana* ecotype Landsberg (LER) and RLD when digested with *Hind*III, *Eco*RI, or *Pst*I. a, the blot was hybridized with a *AtPTR2-A*-specific cDNA probe. Detailed labels are shown in the figure; b, the blot was hybridized with a *AtPTR2-B*-specific cDNA probe. Detailed labels are shown in the figure.

a



b



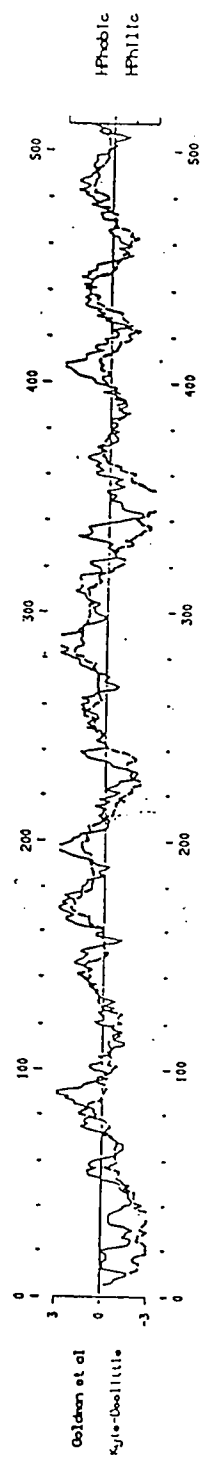
only hybridized to genomic DNA from the ecotype Landsberg erecta, while the *AtPTR2-B* probe hybridized to DNA from both ecotypes. However, the size of the hybridizing bands were different between the Landsberg and RLD indicating an apparent polymorphism in restriction pattern (Figure 3).

DNA Sequencing and Sequence Analysis of *AtPTR2-A* and *AtPTR2-B*

The DNA sequences of the *AtPTR2-A* and *AtPTR2-B* cDNA clones were determined. Computer analysis of the DNA sequence predicted that *AtPTR2-A* contained an 1830-bp open reading frame encoding a putative polypeptide of 610 amino acids (approximately, 68 kD). Similar analysis predicted that *AtPTR2-B* is a 1755-bp open reading frame encoding a putative protein of 585 amino acids (approximately, 64 kDa) (data not shown). Hydropathy analysis (Kyte and Doolittle, 1982) predicted that both proteins are highly hydrophobic (Figure 4) and suggested twelve putative transmembrane segments. A precise model of transmembrane domain topology will require further biochemical evidence. A search of the protein sequence database using the NCBI BLAST algorithm (Altschul et al., 1990) revealed a high degree of similarity of *AtPTR2-A* and *AtPTR2-B* to the yeast peptide transporter Ptr2p (Perry et al., 1994), the *A. thaliana* nitrate transporter AtCHL1 (Tsay et al., 1993), the rabbit intestinal peptide transporter PepT1 (Fei et al., 1994), the *Candida albicans* peptide transporter CaPtr2p (Basrai., 1995), and the *Lactococcus lactis* peptide transporter DtpT (Hagting., 1994) (Table 4). Comparison of these protein sequences to a number of other transport proteins (Figure 5 and Table 5) led to the suggestion that these peptide transporters

Figure 4. Hydropathy plots of AtPTR2-A and AtPTR2-B. A plot of the hydrophilic and hydrophobic moments as predicted by Kyte and Doolittle (1982) for the entire AtPTR2-A and AtPTR2-B amino acid sequences are presented. Positive numbers indicate hydrophobicity.

AtPTR2-B



AtPTR2-A

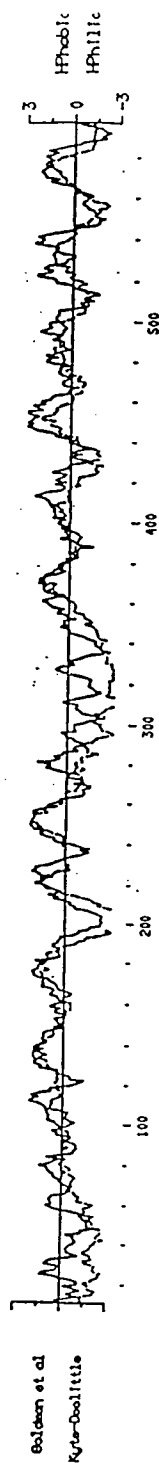


Table 4. Comparison of the AtPTR2-B Protein Sequence to Other Peptide Transporters and the *Arabidopsis* Nitrate Transporter

Pairs of comparison	% identity	% similarity
AtPTR2-A ^a : Ptr2p ^b	45.2	64.8
AtPTR2-A : AtPTR2-B ^c	28.2	52.9
AtPTR2-A : AtCHL1 ^d	24.7	52.9
AtPTR2-A : CaPtr2p ^e	32.4	54.6
AtPTR2-A : PepT1 ^f	28.6	56.8
AtPTR2-A : DtpT ^g	19.0	51.0
AtPTR2-B : Ptr2p	26.7	56.0
AtPTR2-B : AtCHL1	39.2	66.0
AtPTR2-B : CaPtr2p	21.7	48.1
AtPTR2-B : PepT1	34.0	58.6
AtPTR2-B : DtpT	24.0	46.0

^a, AtPTR2-A represents *A. thaliana* peptide transporter previously designated AtPTR2 (Steiner et al., 1994); ^b, Ptr2p represents *S. cerevisiae* peptide transporter encoded by the *PTR2* gene (Perry et al., 1994); ^c, AtPTR2-B represents *A. thaliana* peptide transporter AtPTR2-B (this paper); ^d, AtCHL1 represents *A. thaliana* nitrate transporter 1 (Tsay et al., 1993); ^e, CaPtr2p represents *Candida albicans* peptide transporter (Basrai et al., 1995); ^f, PepT1 represents *Rabbit* peptide transporter (Fei et al., 1994); ^g, DtpT represents *Lactococcus lactis* peptide transporter (Hagting et al., 1994).

Figure 5. Phylogenetic tree of peptide transport and other transport protein sequences. Protein sequences were fetched from the Genebank database and aligned using the Pileup program of the GCG software to create the phylogenetic tree (Altschul et al., 1990). The PTR peptide transporter family is shown bracketed on the right. The length of the line represents the phylogenetic distance between sequence pairs.

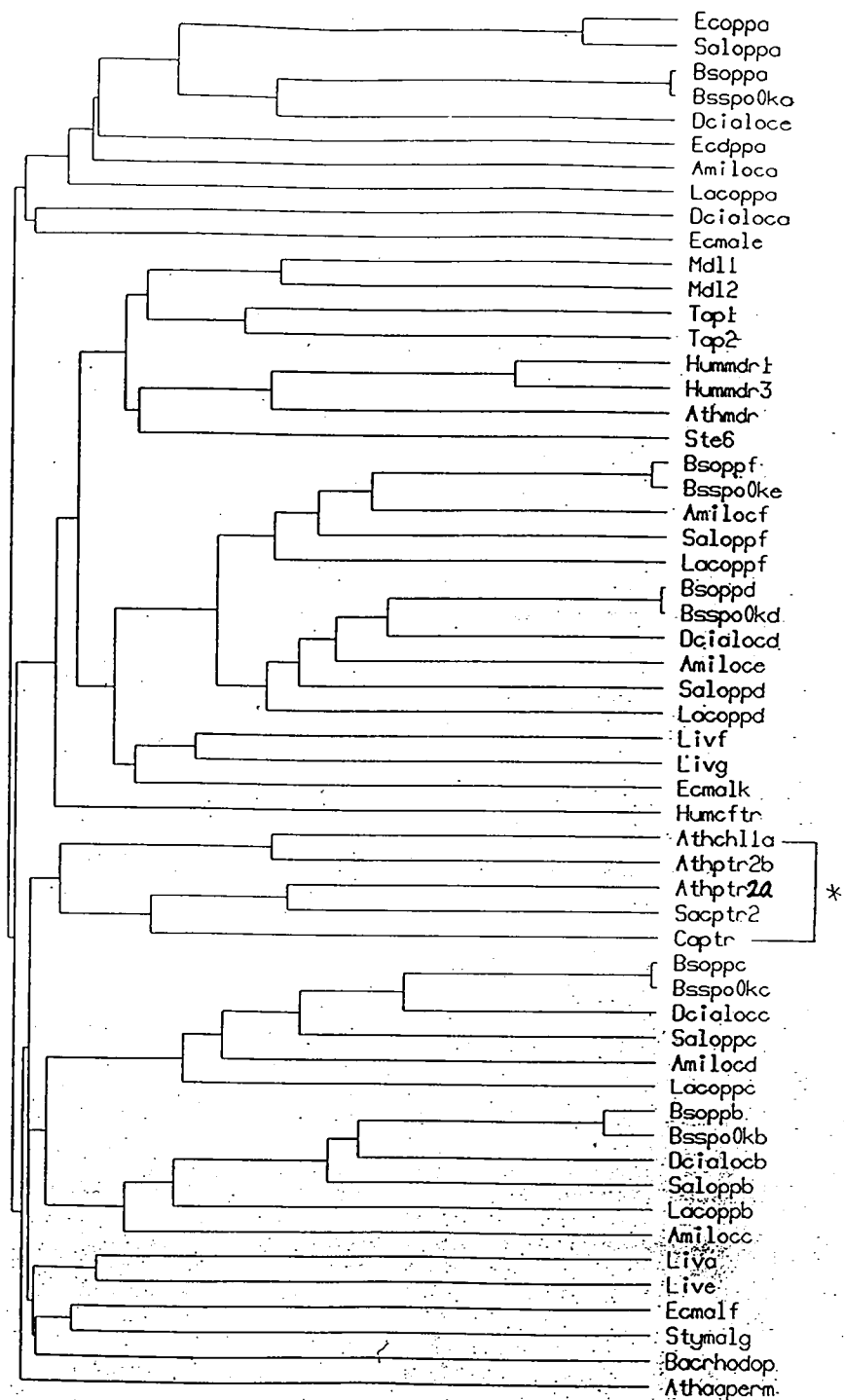


Table 5. List of Protein Sequences Mentioned in Text and Used in Figure 5 Dendrogram

Protein	Definition	Reference
AmiA AmiC AmiD AmiE AmiF	<i>Streptococcus pneumoniae</i> Ami Oligopeptide transport system- AmiA,AmiC, AmiD, AmiE, AmiF	Alloing et al., 1990
AtAap1	<i>Arabidopsis thaliana</i> amino acid permease I	Frommer et al., 1993
AtChl1	<i>Arabidopsis thaliana</i> nitrate transporter	Tsay et al., 1993
AtMdr	<i>A. thaliana</i> p-glycoprotein homolog	Dudler and Hertig, 92
AtPTR2-A AtPTR2-B	<i>A. Thaliana</i> peptide transporters	Steiner et al., 1994; Song et al., 1996
BacRhodop	<i>Halobacterium</i> bacteriorhodopsin	Dunn et al., 1983
BsOppA BsOppB BsOppC BsOppD BsOppF	<i>Bacillus subtilis</i> opp operon for the oligopeptide permease- OppA, OppB, OppC, OppD, OppF	Perego et al., 1991
CaPtr2p	<i>Candida albicans</i> peptide transporter	Basrai et al., 1994
DciaA 1991 DciaB DciaC DciaD DciaE	<i>Bacillus subtilis</i> DciA dipeptide transport system-DciAA, DciAB, DciAC, DciAD, DciAE	Mathiopoulos et al.,
DtpT	<i>Lactococcus lactis</i> di- and tripeptide transporter	Hagting et al., 1994
EcDppA	<i>E. Coli</i> dipeptide binding protein DppA	Abouhamad et al., 91; Olson et al., 1991

EcLacY	<i>E. Coli</i> lactose permease	Buechel et al., 1980
EcMalE EcMalF EcMalK	<i>E. Coli</i> maltose binding and maltose uptake proteins	Gilson et al., 1982; Duplay et al., 1984; Froshauer and Beckwith, 1984
EcOppA	<i>E. Coli</i> oligopeptide binding protein OppA	Kashiwagi et al., 1994
HPT1	Human peptide transporter 1	Dantzig et al., 1994
HumCfr	Human cystic fibrosis transmembrane Conductance regulator CFTR	Riordan et al., 1989
HumMdr1 HumMdr3	Human p-glycoprotein MDR1 Human p-glycoprotein MDR3	Chen et al., 1986 Van der Bliek, 1988
HumPorin	Human porin protein	Ha et al., 1993
HumPtr	Human intestinal peptide transporter	F. Leibach (personal Communication)
LacOppA LacOppB LacOppC LacOppD LacOppF	<i>Lactococcus lactis</i> oligopeptide transport system- OppA, OppB, OppC, OppD, OppF	Tynkkynen et al., 93
LivA LivE LivF LivG	<i>Salmonella typhimurium</i> high affinity branched amino acid transport system	Matsubara et al., 1992
Mdl1 Mdl2	<i>Saccharomyces cerevisiae</i> ATP binding cassette proteins	Dean et al., 1993
PepT 1	Rabbit intestinal peptide transporter	Fei et al., 1994
Ptr2p	<i>Saccharomyces cerevisiae</i> peptide Transporter	Perry et al., 1994
SalOppA	<i>Salmonella typhimurium</i> periplasmic Component of the oligopeptide permease	Hiles and Higgins, 86
SalOppB	<i>Salmonella typhimurium</i> opp operon	Hiles et al., 1987

SalOppC SalOppD SalOppF	for the oligopeptide permease- OppB, OppC, OppD, OppF	
ScPorin	Yeast outer mitochondrial membrane Porin	Mihata and Sato, 85
Ste6	<i>Saccharomyces cerevisiae</i> MDR Homolog	McGrath and Varshavsky, 1989
StyMalG	<i>Salmonella typhimurium</i> inner membrane Maltose transport protein	Francoz et al., 1990
Tap1 Tap2	<i>Homo sapiens</i> MHC class II antigen processing peptide pumps	Beck et al., 1992

The following data base were used in obtaining sequences: GenBank, release 78.0; EMBL (modified), release 35.0; Swiss-Prot, release 26.0.

and the *A. thaliana* nitrate transporter AtCHL1 comprise a new family of membrane transport proteins, called the PTR (Peptide TRansport) family (Figure 6). The same family of transport proteins was identified recently on the basis of a protein database search and called the POT family for proton-dependent oligopeptide transport (Paulsen and Skurray, 1994). However, as it is not known whether all of the members of this family are proton-dependent or that they mediate the transport of oligopeptides, we believe the more generic designation PTR (for Peptide TRansport) is more appropriate at this time than the POT designation. The higher identity of AtPTR2-B to the *Arabidopsis* nitrate-inducible nitrate transporter (AtCHL1) and the rabbit peptide transporter (PepT1) suggests that the PTR family may be composed of two distinct subfamilies.

The predicted protein sequences of AtPTR2-A, AtPTR2-B, Ptr2p, PepT1, and CHL1 were aligned using the Genetics Computer Group (GCG,) program Pileup and a consensus sequence was generated with the GCG program Pretty from the pairwise alignment (Figure 7). The consensus sequence was most conserved in the first and last third of the protein with the middle portion of the proteins, corresponding to an extra-membrane loop, the least conserved. In general, the greatest areas of sequence consensus correspond to the putative hydrophobic segments, which are presumably the transmembrane domains.

The AtPTR2-B is 99.8% identical to the NTR1 protein reported by Frommer et al. (1994). The *NTR1* gene was postulated to encode a histidine transporter. Since the *NTR1* gene was also isolated using the *Arabidopsis* cDNA library constructed by Minet et al.

Figure 6. Phylogenetic relationship between the PTR proteins. Alignment method was the same as in Figure 5. Protein sequences were fetched from the Genebank database and aligned using the Pileup program of the GCG software to create the phylogenetic tree (Altschul et al., 1990). The PTR peptide transporter family is shown bracketed on the right. The length of the line represents the phylogenetic distance between sequence pairs.

NOVEL FAMILY OF TRANSPORT PROTEINS

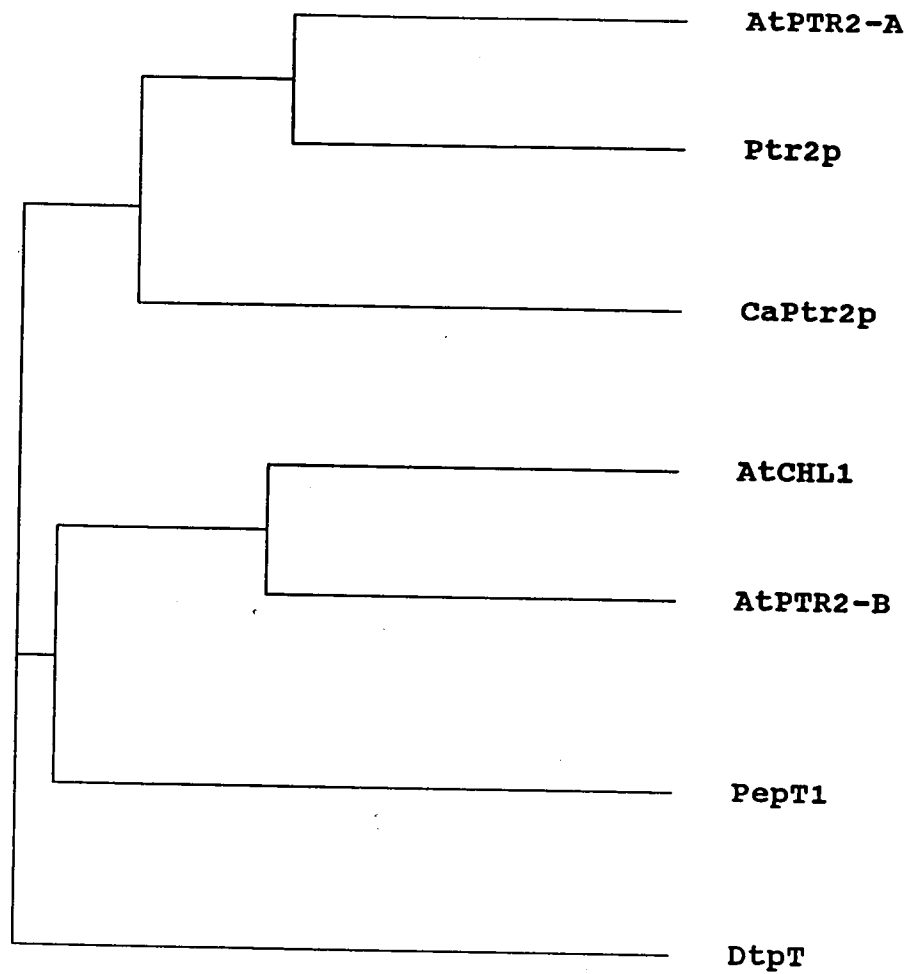


Figure 7. Protein sequence alignment of PTR peptide transporters and the derived consensus sequence. The protein sequences were aligned with the DNA* program MegAlign (DNA*, Madison, WI) using the Clustal method (Higgins et al., 1992). Only amino acids which are present in four or more sequences at any particular position within the alignment are represented in the consensus sequence and boxed in the multiple alignment. Dashed lines indicate gaps within the aligned sequences. Conserved glycosylation and phosphorylation sites are shown by shaded boxes. N-glycosylation site is Asn (N) located within the shaded box covering the residues from 146 to 150. The remained shaded boxes contain the phosphorylation sites of residues, Thr or Ser.

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1
Ptr2
AtPtr2
CaPtr2
AtPtr1
AtChl1
PepT-1
DtpT-1

Consensus #1 - - F -

Consensus #1 - - -

Consensus 'Consensus #1': When 57% (4) match the residue of the Consensus show the residue of the Consensus, otherwise show '-'

Decoration 'Decoration #1': Box residues that match the consensus named 'Consensus #1' exactly.

(1992), it is likely that the *AtPTR2-B* and *NTR1* genes are identical and the differences noted are the result of slight sequencing differences. However, as shown below, our data with *AtPTR2-B* are not consistent with a functional role in histidine transport.

Peptide Utilization in Yeast *AtPTR2-A* or *AtPTR2-B* Transformants

The peptide transport system in the yeast *Saccharomyces cerevisiae* has been shown to transport a variety of peptides with two to three amino acid residues (Perry et al., 1994). Strain PB1X-9B, a *ptr2* mutant of *S. cerevisiae*, is auxotrophic for Leu, His, and Lys. Therefore, uptake of peptides can be measured by growth if these peptides provide one or more of the required amino acids. To test whether these plant transporters have the ability to uptake different peptides varying in composition and length, the yeast *ptr2* mutant (PB1X-9B) was transformed with pPTF4 (i.e., *AtPTR2-A*) or pDTF1 (i.e., *AtPTR2-B*). Table 6 shows that *PB1X-9B* transformants expressing *AtPTR2-A* or *AtPTR2-B* grew on all peptides tested except trilysine and peptides with four or more residues. This growth pattern was similar to that of transformants containing the plasmid pJP9 expressing the yeast *PTR2* gene. The one exception is that the two *Arabidopsis* gene products were able to uptake Met-Met-Leu. These results indicate that *AtPTR2-A* and *AtPTR2-B* have similar, but not identical substrate specificity to the Ptr2p peptide transporter, and also suggest that *AtPTR2-A* and *AtPTR2-B* encode di- and tripeptide transporters.

Table 6. Growth on Peptide Substrates

Peptides	Yeast Mutant/Transformants			
	PB1X-9B (<i>ptr2</i> ⁻)	PB1X-9B[pJP9] (<i>PTR2</i>)	PB1X-9B[pDTF1] (<i>AtPTR2-B</i>)	PB1X-9B[pPTF4] (<i>AtPTR2-A</i>)
<i>Ala-Leu</i>	-	+	+	+ ¹
<i>Ala-Ala-Leu</i>	-	+	+	+
<i>Ala-Lys</i>	-	+	+	+
<i>Lys-Ala-Ala</i>	-	+	+	+
<i>Lys-Leu</i>	-	+	+	+
<i>His-Leu</i>	-	+	+	+
<i>His-Lys</i>	-	+	+	+
<i>Leu-Leu</i>	-	+	+	+
<i>Leu-Leu-Leu</i>	-	+	+	+
<i>Lys-Lys</i>	-	+	+	+
<i>Lys-Lys-Lys</i>	-	-	-	-
<i>tetra-Lys</i>	-	-	-	-
<i>Poly-Lys</i>	-	-	-	-
<i>Leu-Met</i>	-	+	+	+
<i>Met-Met-Leu</i>	-	-	+	+
<i>Gly-Leu-Gly-Leu</i>	-	-	-	-
<i>Thr-Pro-Arg-Lys</i>	-	-	-	-
<i>Tyr-Gly-Gly-Phe-Leu</i>	-	-	-	-

¹ + indicates growth, - indicates no growth. This table was contributed by Dr. Henry Y. Steiner.

Sensitivity of *AtPTR2-A* or *AtPTR2-B* Transformants to Toxic Peptides

Mutations in the yeast *PTR2* gene cause the concomitant loss of sensitivity to peptides containing toxic amino acids (such as ethionine, fluorophenylalanine, and oxalysine) (Perry et al., 1994). Transformation of PB1X-9B with pPTF4 (i.e., *AtPTR2-A*) or pJP9 (i.e., *PTR2*) restored sensitivity to toxic di- or tripeptides as indicated by an apparent inhibition zone (Table 7, Figure 8). This is due to the uptake of the toxic peptides by the peptide transport system and subsequent release of the toxic amino acid moiety intracellularly to inhibit the growth of cells. However, as shown in Table 6, transformants expressing *AtPTR2-B* were partially resistant to these same toxic peptides as reflected by the heavy growth of cells within the zone of inhibition (data not shown). These results indicate that *AtPTR2-A* and *AtPTR2-B* have different abilities to transport these toxic peptides. As shown below, *AtPTR2-B* has a lower uptake capacity for these toxic peptides than *AtPTR2-A* or the yeast *Ptr2p* transporter. This difference in transport kinetics may explain the difference in sensitivity to toxic peptides.

Radiolabeled Dileucine Uptake and Competitive Inhibition

The growth assay on peptide medium and testing for sensitivity to toxic peptides are indirect measures of peptide uptake. A more direct method is to measure the uptake of radiolabeled peptide. Figure 9 shows that the yeast mutant strain PB1X-2AΔ transformed with pPTF4 (*AtPTR2-A*) or pDTF1 (*AtPTR2-B*) incorporated [³H]dileucine to a level similar

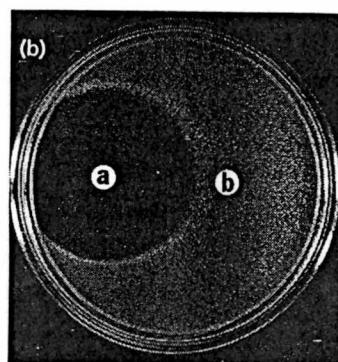
Table 7. Effect of Toxic Peptides on Growth of Mutant and Transformed Yeast Strains

Toxic Peptides	Diameter (mm) of the Zone of Inhibition ¹			
	PB1X-9B (<i>ptr2</i>)	PB1X-9B (<i>PTR2</i>)	PB1X-9B (<i>AtPTR2-A</i>)	PB1X-9B (<i>AtPTR2-B</i>)
f-Phe	14.0±1.0	20.0±0.0	21.0±0.0	22.0±0.0
Leu-f-Phe	0	20.5±3.0	25.0±0.0	0
Ethionine	44.5±3.5	46.5±1.5	37.0±0.0	41.5±1.5
Leu-Eth	0	47.0±1.0	24.0±0.0	25.0±1.0 ²
Lys-Leu-Eth	0	36.0±0.5	35.0±1.0	31.5±1.5 ²
Lys-Leu-Leu-Eth	0	0	0	0
Lys-Leu-Leu-Leu-Eth	0	0	0	0
Ala-Eth	0	42.5±2.5	35.0±1.0	33.5±1.0 ²
Lys-Ala-Eth	0	36.0±1.5	36.0±1.5	30.5±0.5 ²
Lys-Leu-Ala-Eth	0	0	0	0
Lys-Leu-Leu-Ala-Eth	0	0	0	0
Oxalysine	0	0	0	0
Oxalys-Gly	0	24.5±0.5	29.5±0.5	31.0±1.0 ²
Oxalys-Leu-Gly	0	25.0±0.0	29.0±1.0	27.5±0.5 ²

¹ Values are given as mean ± standard error of the mean of at least two independent experiments. ² Although a zone of inhibition was apparent, cells within the zone were apparently growing but at a reduced rate. This table was contributed by Dr. Henry Y. Steiner.

Figure 8. Zone of inhibition of yeast cell growth by Ala-Eth. *S. cerevisiae* transformants expressing *AtPTR2-A* were plated on the minimal medium (SC) (Island et al., 1987) supplemented with amino acids required for auxotrophic growth and treated with a toxic dipeptide contained in a filter disc. After two to three day's incubation at 30°C, the zone of inhibition was formed. a, contained 0.38 µmol of Ala-Eth; b, contained H₂O as a control.

Sensitivity of *S. cerevisiae* Transformants

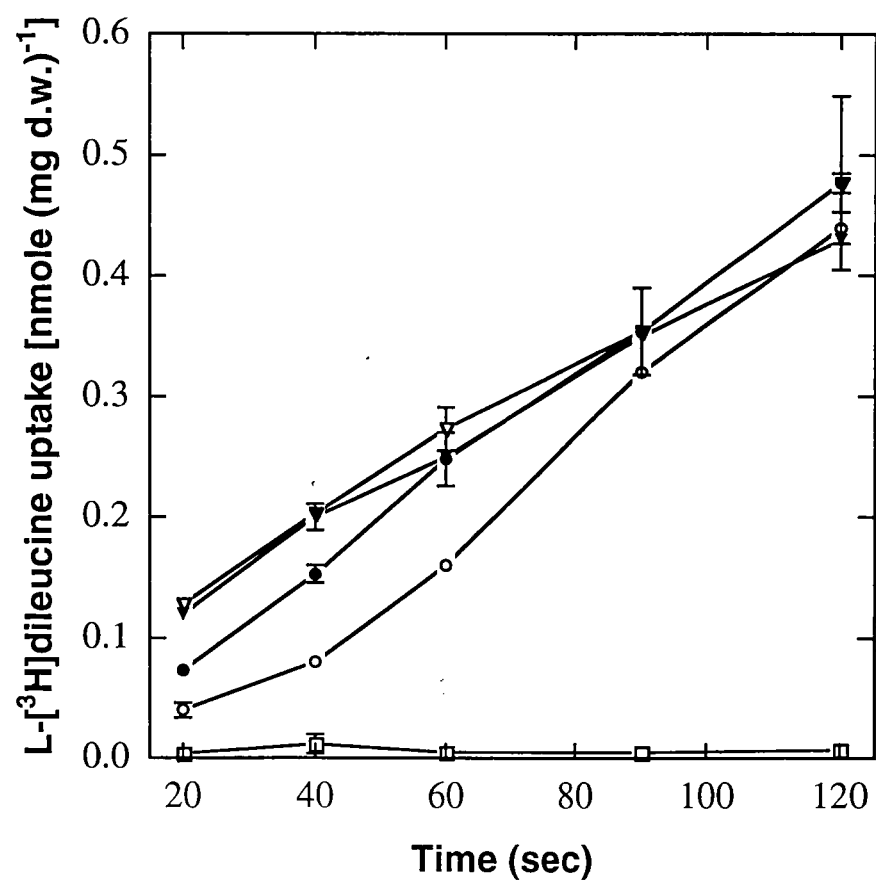


a = Ala-Eth

b = H₂O

Zone of Inhibition

Figure 9. Uptake of L-[³H]dileucine (30 μM, specific activity 10 mCi/mmol) by the following yeast strains and transformants: (•) PB1X-2A•, (•) PB1X-2A• [*AtPTR2-B*], (•) PB1X-2A• [*AtPTR2-A*], (•) PB1X-2A, (•) PB1X-2A• [*PTR2*]. (This figure was contributed by Dr. Henry Y. Steiner).



to that of transformants with pJP9 (*PTR2*) or the wildtype strain (i.e., PB1X-2A) after 120 seconds of uptake. Since the AtPTR2-B transformant exhibited partial resistance to toxic peptides, it was also of interest to determine the effect of these toxic peptides on uptake of [^{3} H]dileucine. As shown in Figure 10, increasing concentrations of Leu-Eth resulted in increasing inhibition of [^{3} H]dileucine uptake by AtPTR2-A or AtPTR2-B. Uptake by AtPTR2-B was completely inhibited by the addition of Leu-Eth at a 7.8-fold excess concentration (234 μ M) over that of [^{3} H]dileucine, and uptake of AtPTR2-A was almost completely inhibited at the same concentration of Leu-Eth.

As indicated previously, it is likely that AtPTR2-B is identical to the previously reported histidine transport protein NTR1 (Frommer et al., 1994). Therefore, in order to test whether AtPTR2-B could also transport histidine, various concentrations of histidine were tested for their ability to competitively inhibit [^{3} H]dileucine uptake in transformants expressing *AtPTR2-B*. Figure 11 indicates that histidine concentrations up to 200-fold excess over that of [^{3} H]dileucine did not inhibit uptake. We also determined whether AtPTR2-B would allow better growth on histidine when expressed in yeast cells. The growth response to various levels of histidine was identical in cells expressing or lacking the *AtPTR2-B* gene (data not shown), demonstrating no physiological role of AtPTR2-B in histidine uptake.

Since AtPTR2-A and AtPTR2-B show significant homology to the *Arabidopsis* nitrate transporter AtCHL1, we also examined the ability of nitrate to compete for [^{3} H]-

Figure 10. Inhibition of uptake of L-[³H]dileucine (30 μ M, specific activity 10 mCi/mmole) by AtPTR2-A or AtPTR2-B with Leu-Eth as a Competitor. (○), AtPTR2-A, 0 μ M Leu-Eth; (●), AtPTR2-A, 78 μ M Leu-Eth; (▽), AtPTR2-A, 234 μ M Leu-Eth; (▼), AtPTR2-A, 390 μ M Leu-Eth; (□), AtPTR2-B, 0 μ M Leu-Eth; (■), AtPTR2-B, 78 μ M Leu-Eth; (△), AtPTR2-B, 234 μ M Leu-Eth. (▲), PB1X-9B. Standard error was < 5% of the mean. d.w., Dry weight. (This figure was contributed by Dr. Henry Y. Steiner).

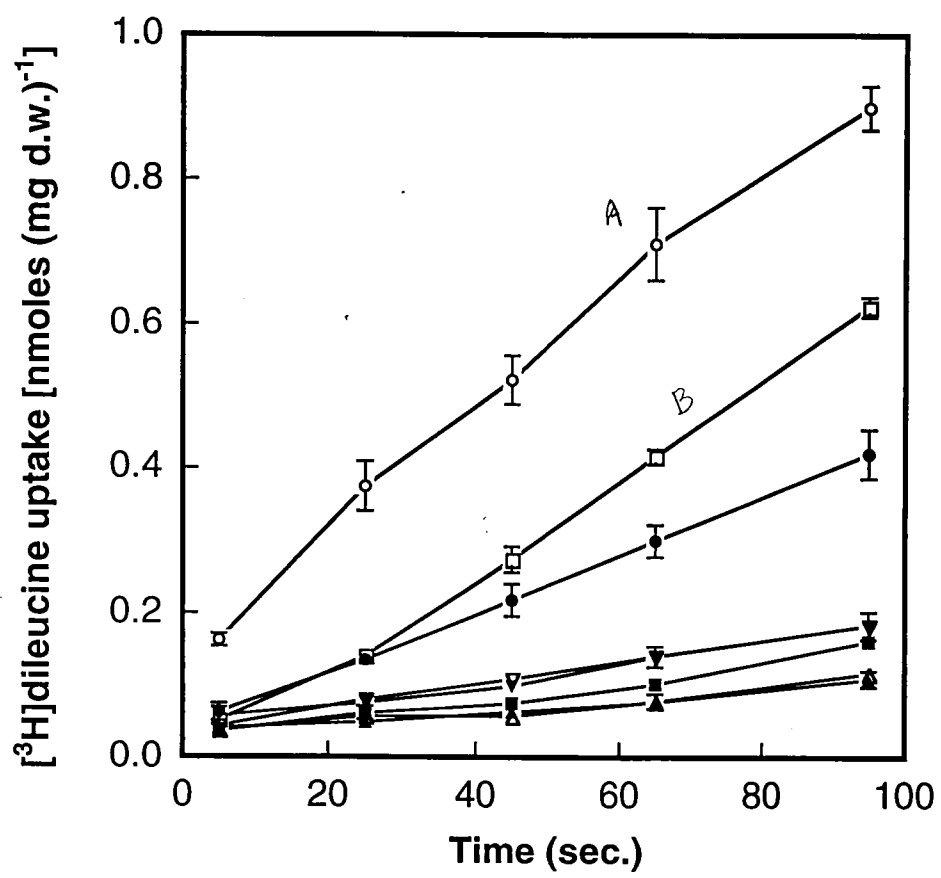
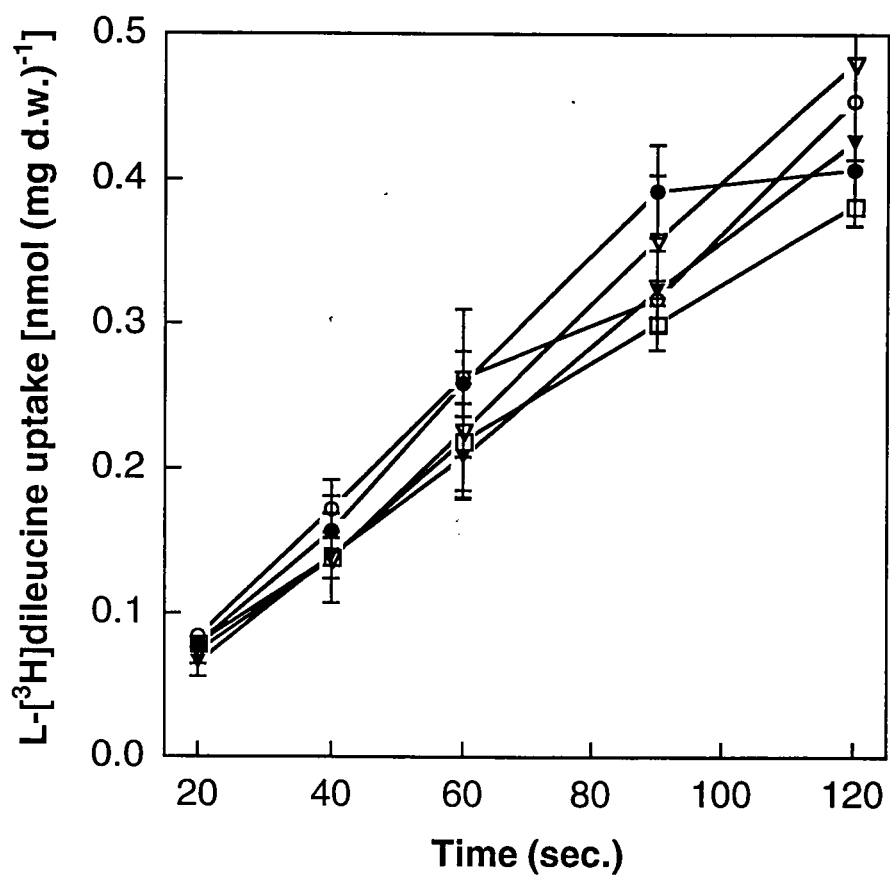


Figure 11. Radiolabeled dileucine uptake by AtPTR2-B with various concentrations of histidine as a competitor. Uptake of L-[³H]dileucine (30 μM, specific activity 10 mCi/mmoles) by PB1X-2A• [pDTF1] with the following concentrations of histidine: (•), none; (•), 0.19 mM; (•), 0.63 mM; (•), 3.17 mM; (•), 6.34 mM. Error bars indicate standard error from two independent experiments. (This figure was contributed by Dr. Henry Y. Steiner).



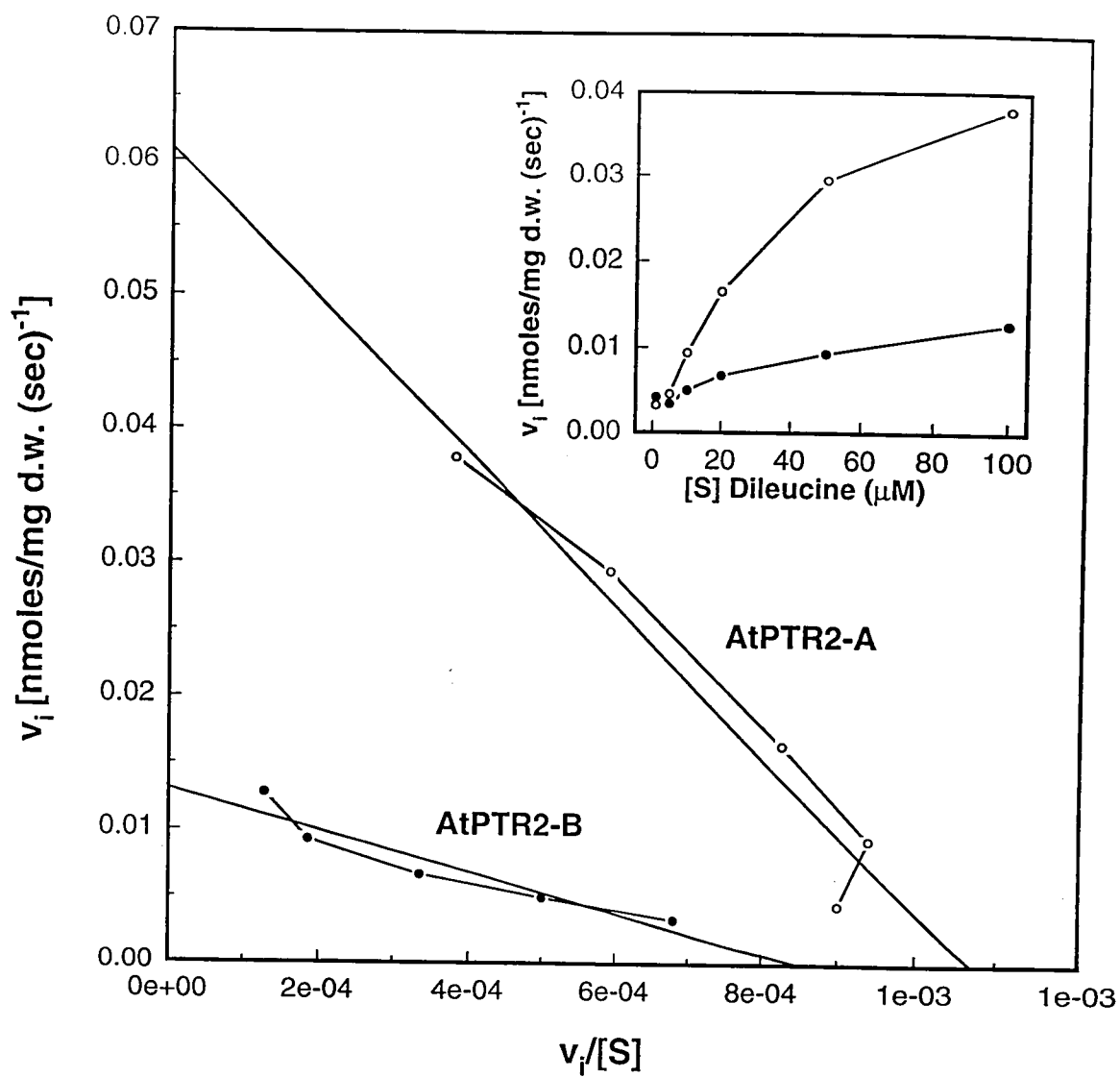
dileucine uptake. No inhibition of uptake was seen when nitrate was added up to 10 mM (333-fold excess to that of [^{3} H]dileucine) (data not shown).

Kinetic Analysis of Peptide Transport mediated by AtPTR2-A or AtPTR2-B

To further confirm that AtPTR2-A and AtPTR2-B indeed encode peptide transporters and not simply transporters of broad specificity, a kinetic analysis of peptide transport was performed (Song et al., 1996). Figure 12 shows that [^{3} H]dileucine uptake by AtPTR2-A or AtPTR2-B transformants exhibits concentration-dependent, saturable kinetics consistent with carrier-mediated uptake. Moreover, the kinetics of uptake suggest the involvement of a single transporter system.

To calculate the kinetic parameters, K_m and V_{max} , the Michaelis - Menten equation was applied. The K_m and V_{max} of AtPTR2-A and AtPTR2-B are 47 μ M, 0.061 nmoles mg^{-1} cell dry wt sec^{-1} and 14 μ M, 0.013 nmoles mg^{-1} cell dry wt sec^{-1} , respectively. Assuming that the concentrations of AtPTR2-A and AtPTR2-B are the same when expressed in the yeast cells, these kinetic measurements thus suggest that relative to each other, AtPTR2-A is a lower affinity, higher capacity transporter, whereas AtPTR2-B is a higher affinity, lower capacity transporter. Based on these results, the partial resistance of AtPTR2-B to toxic peptides described above can be explained by its low capacity for these toxic peptides. The K_m values of AtPTR2-A and AtPTR2-B are lower but in the same μ M range as those reported for the rabbit intestinal peptide transporter PepT1 ($K_m = 137 \mu$ M) (Fei et al., 1994)

Figure 12. Kinetics of L-[³H]dileucine uptake by AtPTR2-A and AtPTR2-B as a function of concentration (*Inset*). [³H]dileucine uptake rate of PB1X-2AΔ expressing AtPTR2-A or AtPTR2-B was measured in the presence of concentrations ranging 1 μM to 100 μM. Uptake data represent the saturable kinetics. The results are given as an Eadie-Hofstee plot ($v_i/[S]$ versus v_i) where v_i is the initial velocity of uptake in nanomoles per mg of dry weight of cells per second and [S] the [³H]dileucine concentration in micromolar. (○), AtPTR2-A; (●), AtPTR2-B. (This figure was contributed by Dr. Henry Y. Steiner).



and human kidney peptide transporter PepT2 ($K_m = 67 \mu\text{M}$) (Brandsch et al., 1995). These low K_m values show that these transporters have a high affinity for peptides and support the belief that AtPTR2-A and AtPTR2-B do function physiologically in transporting peptides and/or peptide derivatives.

Germination Assay of *Arabidopsis* Seeds

As indicated by Higgins and Payne (1977a,b, 1978), the germinating barley embryo has the ability to uptake peptides from the endosperm through the scutellum tissue. Because *Arabidopsis* seeds do not have an apparent scutellum and also are too small to be dissected ($< 1 \text{ mm}$), peptide transport was characterized by germinating *Arabidopsis* seeds in the presence of toxic peptides. The term germination, as used here, is defined as the process that begins with imbibition (water uptake by the seed) and ends with the start of elongation of the radicle (Bewley and Black, 1985). Ethionine, oxalysine(Oxalys), Ala-Eth, Leu-Eth, and Oxalys-Gly did not inhibit germination of *Arabidopsis* seeds as reflected by the appearance of primary roots. The lack of inhibition may be due to an inability of the toxic peptides to penetrate through the seed coat. Alternatively, the embryo may lack a peptide transport system for these toxic peptides. However, root growth beyond 1 to 3 mm was inhibited by the toxic peptides (data not shown). The addition of the competitor peptides Ala-Met and Leu-Met reversed the inhibition of root growth due to Ala-Eth and Leu-Eth, respectively. Adding the amino acids leucine, alanine, and methionine as individual competitors for Leu-Eth or Ala-Eth had no effect on the inhibition of root growth. These results show that root

inhibition is due to the uptake of the toxic peptides and not due to uptake of ethionine after peptide hydrolysis.

Assay for Root Growth Inhibition

To further assess whether *Arabidopsis* roots were sensitive to toxic peptides, 4-day-old seedlings were exposed to peptides containing toxic amino acids. In this assay, toxicity was measured by inhibition of root elongation. Figure 13A and 13B show the inhibitory effects of Leu-Eth and Ala-Eth, respectively, on growing *Arabidopsis* seedlings. Root growth remained inhibited in the presence of Ala-Eth or Leu-Eth plus the following compounds: Met, Ala-Ala, and Ala-Ala-Ala. As in the germination assay, Ala-Met and Leu-Met successfully competed with and reversed the toxicity of Leu-Eth and Ala-Eth, respectively (Figure 13D and 13E). Met-Met was able to reverse the toxicity of both Leu-Eth and Ala-Eth, although less efficiently (Table 8). Inhibition of root growth was identical with ethionine or peptides containing ethionine. These results show that *Arabidopsis* roots retain the ability to take up peptides long after the events of seed germination have occurred and this uptake is independent of the amino acid uptake system.

mRNA Expression Patterns of *AtPTR2-A* and *AtPTR2-B* in *Arabidopsis thaliana*

Consistent with the above observation of the root inhibition by toxic dipeptides, *AtPTR2-A* and *AtPTR2-B* mRNAs were detected in roots of *Arabidopsis thaliana*. Northern

Figure 13. Inhibition of *Arabidopsis* seedling root growth by Ethionine-containing peptides. *Arabidopsis* seeds were sown on solid *Arabidopsis* growth media (see Methods). After five days of growth, filter discs were placed on the agar surface and 5 μ l of an aqueous solution of dipeptides was applied to the discs. Roots are shown 6 days after the addition of toxic peptides. (A) Leu-Eth (0.19 μ M). (B) Ala-Eth (0.19 μ M). © Control (H_2O). (D) Leu-Eth (0.19 μ M) plus Leu-Met (4.00 μ M). (E) Ala-Eth (0.19 μ M) plus Ala-Met (4.00 μ M).

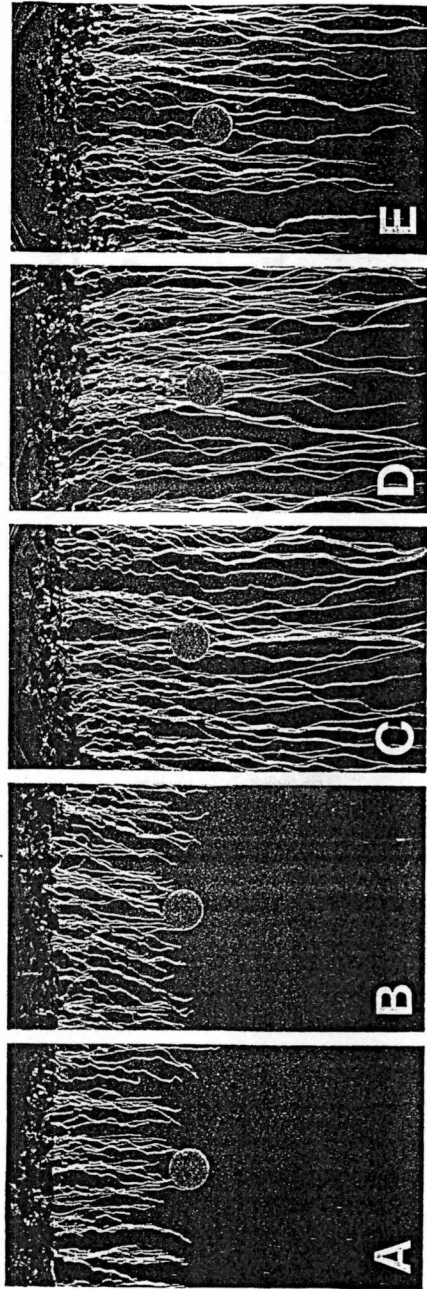


Table 8. Reversal of Toxic Peptide Inhibition of Root Growth by Competitors^a

Competitors	Toxic Peptide	
	Ala-Eth	Leu-Eth
Met	0	0
Ala-Ala	0	0
Ala-Ala-Ala	0	0
Met-Met	40%	50%
Ala-Met	70%	N.D.
Leu-Met	N.D.	100%

^a Reversal inhibition measured by measuring root growth of untreated control to treated root in the presence of toxic peptides and competitors. Competitors were supplied in 10-fold excess over toxic peptides, except Met (20-fold) and Ala-Ala (25-fold). Ala-Eth and Leu-Eth were supplied with 5 ul of 0.19 uM per disk.

blot analysis showed that *AtPTR2-B* is highly expressed in roots, leaves, germinating seeds, moderately expressed in stem and flower, and at a slightly lower level in siliques (Figure 14). In contrast, *AtPTR2-A* mRNA could not be detected by Northern blot analysis, but is detectable in roots after RT-PCR amplification (Figure 15). The results suggest that *AtPTR2-B* is constitutively expressed in all *Arabidopsis* organs with slightly differing levels of expression. *AtPTR2-A* may be expressed in a tissue- or cell type-specific manner or may be inducible in response to developmental and/or environmental stimuli. Further studies (e.g. *in situ* hybridization and immunolocalization) will help elucidate the precise expression pattern of *AtPTR2-A*. It is known that the yeast *PTR2* gene is inducible by micromolar amino acid concentrations (Island et al 1987).

Part II: Construction of Transgenic Plants Expressing the *AtPTR2-A* or *AtPTR2-B* Sense or Antisense cDNA and Their Phenotypic Analysis

Binary Vector Construction and Plant Transformation

A full length *AtPTR2-A* cDNA clone or a truncated *AtPTR2-B* cDNA clone with a 330 bp deletion in the 3' end were inserted into the plant expression vector pGA941 in both the sense and antisense orientations (for details, see "Materials and Methods"). Transformation of *Arabidopsis thaliana* ecotype RLD was accomplished by root explant transformation using *Agrobacterium tumefaciens* containing pGA-2AS, pGA-2AA, pGA-2BS, pGA-2BA (the nomenclature can be found in "Materials and Methods) or the pGA941

Figure 14. High-stringency northern blot analysis of *AtPTR2-B* gene expression using total RNA from various *Arabidopsis* organs. The *AtPTR2-B*-specific probe was labelled with [³²P]dATP and hybridized to 20 µg total RNA extracted from different organs . (A): Lane 1, flower; lane 2, stem; lane 3, mature leaf; lane 4, silique; lane 5, young leaf; lane 6, root; lane 7, 3 day-germinating seed. (B): rRNA bands stained with methylene blue were used as standards to confirm that roughly equal amounts of total RNA were added to each lane. RNA markers show the size of bands.

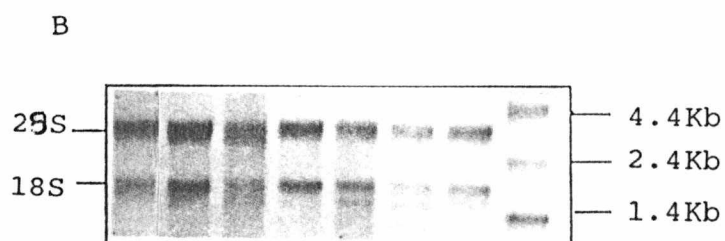
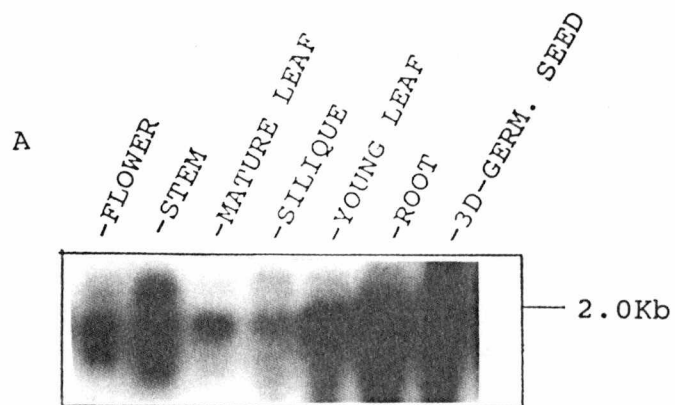
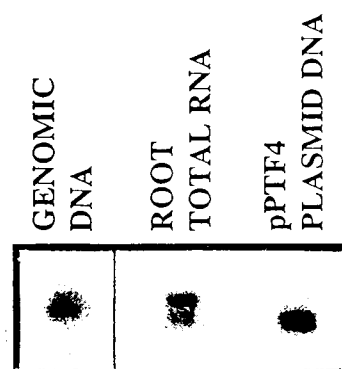


Figure 15. Expression of *AtPTR2-A* detected by RT-PCR. Hybridization of an α - ^{32}P -dATP-labeled *AtPTR2-B* probe to PCR-generated products from *Arabidopsis* genomic DNA (left), total RNA from seedling roots (middle), and plasmid DNA containing the *AtPTR2-A* gene (right) is shown.



Expression of *AtPTR2-A*

binary vector (Figure 1). Root explants were embedded in ARC medium to allow growth of the *Agrobacterium* strain to facilitate transformation of the plant cells. By the third day, a ring of *Agrobacterium* growth was evident around the transformed roots indicating efficient inoculation. These roots were transferred to ARM II medium to induce the formation of embryo-like tissue in transformed kanamycin resistant (Kan^R) cells.

Positive control roots were transferred to ARM II medium without timentin and kanamycin selection for embryo-like tissue induction. Negative control roots were transferred to ARM II containing timentin and kanamycin. These roots turned yellow and died within two weeks (Figure 16a).

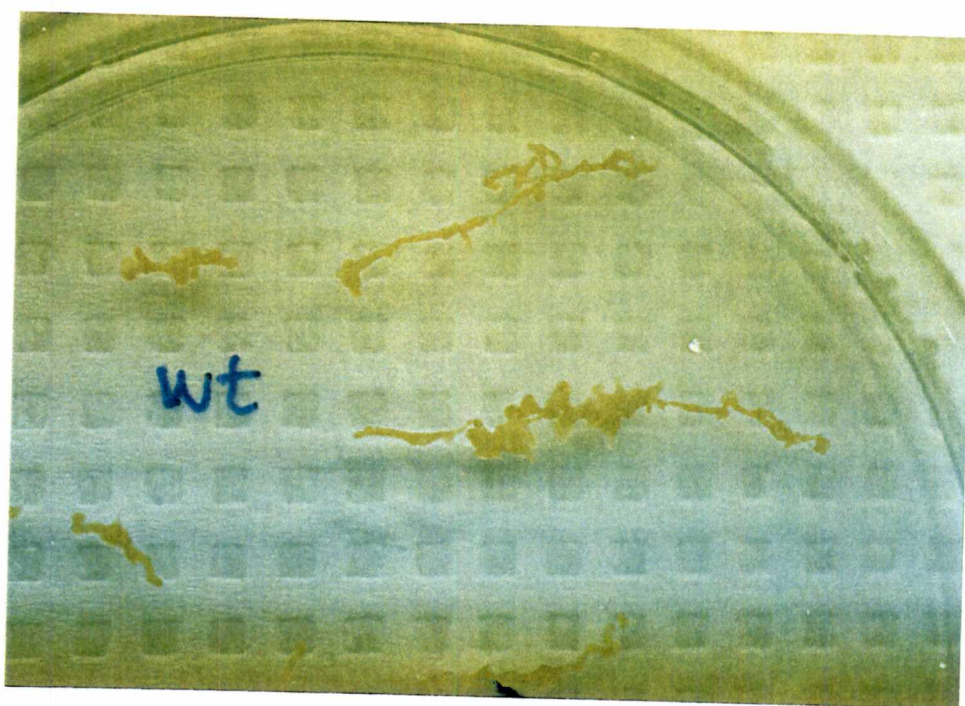
A few small green sector tissues appeared on the transformed roots by the tenth day post-transformation. Figure 16b shows a picture of typical Kan^R sectors obtained within 15 days after *A. tumefaciens* inoculation. In general, the positive control roots had larger green sectors than the transformed roots. Within seven days after the appearance of the green sectors, shoots developed from the sectors. These small shoots were transferred to ARM IV medium plus timentin and kanamycin for multiplication (Figure 17). The positive control was also transferred to ARM IV, but without kanamycin selection.

At twenty-five days post-transformation, the shoots were separated and transferred to test tubes containing ARC medium plus 0.1 µg/ml NAA as described in methods for root initiation and elongation . Under the growth conditions used, most shoots did not generate

Figure 16. Emergence of transgenic shoots along the roots on kanamycin-containing plates.

a, represents the roots incubated without the *Agrobacterium* strain as a negative control (wildtype); b, represents the roots incubated with the *Agrobacterium* strain containing the recombinant pGA vector carrying the kanamycin resistance gene (see Methods).

a



b



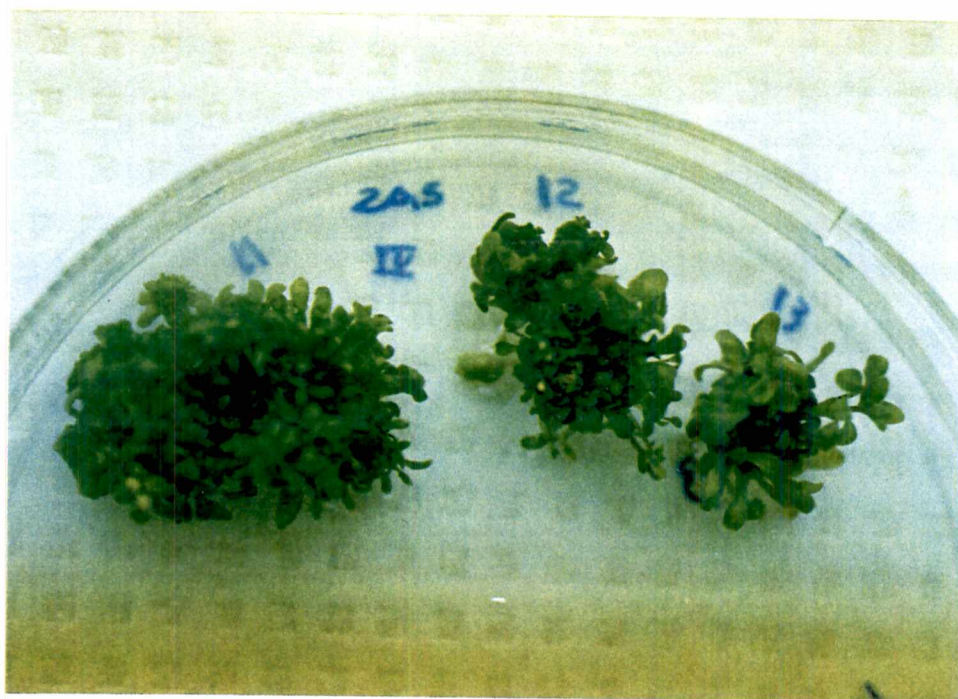


Figure 17. Multiplication of transgenic shoots grown on ARM IV medium containing 75 $\mu\text{g/ml}$ of kanamycin for selection.

roots. By the fifth week post-transformation, fifteen *AtPTR2-A* sense, fifteen *AtPTR2-A* antisense, fifteen *AtPTR2-B* sense and twenty seven *AtPTR2-B* antisense plants were transferred to pots and acclimated to the growth chamber. Due to the lack of roots and transfer shock, plants in the pots grew weakly and slowly. Only about 50% of the transferred plants survived and grew under these conditions. The surviving plants (T_1) reached maturity and set seeds after 20-30 days in the growth chamber. Flowers and seeds were collected and analyzed (see below). Because the *Arabidopsis thaliana* ecotype RLD does not have an endogenous *AtPTR2-A* gene (as judged by Southern hybridization), analysis was focused only on the *AtPTR2-B* transgenic plants.

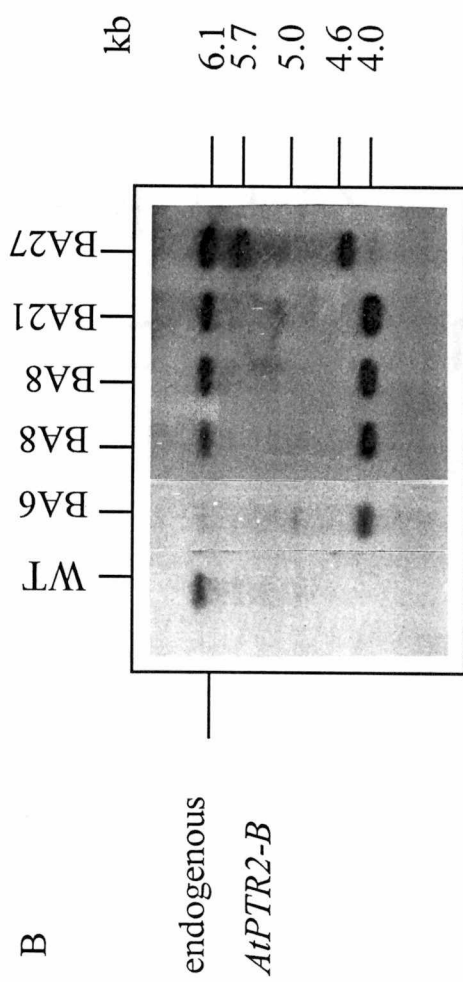
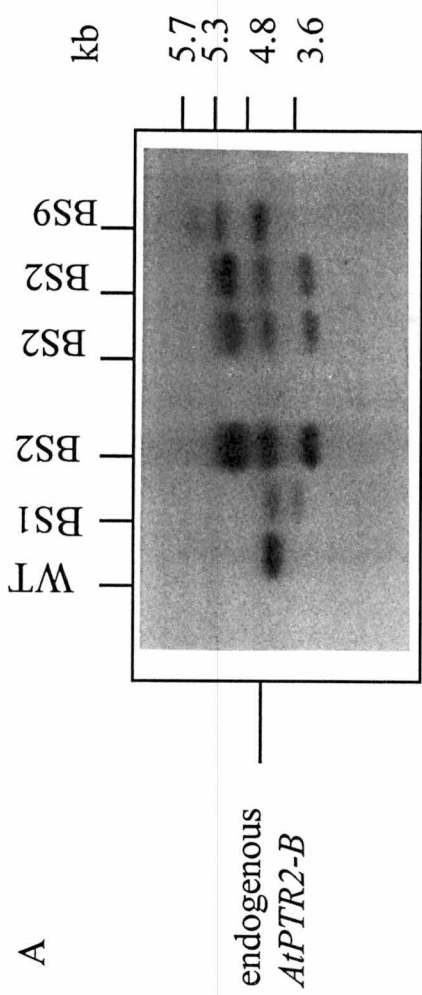
Southern Blot Analysis

DNA isolated from young leaves of T_1 plants was used to confirm T-DNA integration and copy number by Southern hybridization (Sambrook et al., 1989). Southern blots prepared with *Bam*HI-digested genomic DNA of *AtPTR2-B* sense plants were screened with a randomly labeled 1.9 kb *AtPTR2-B* cDNA probe. Figure 1 is a pictorial representation showing the restriction sites used and the origins of the hybridization bands detected. *AtPTR2-B* sense-containing T-DNA as shown in Figure 1b was cut once by *Bam*HI on the 5' end of the *AtPTR2-B* cDNA. Therefore, a second *Bam*HI site in the case of integrated T-DNAs would occur outside the T-DNA within the flanking chromosomal DNA. A *AtPTR2-B*-specific probe would hybridize only to fragments containing the *AtPTR2-B* gene. Therefore, if there were more than one *AtPTR2-B* insert in the genome, each copy of the

AtPTR2-B gene would likely be on a separate *Bam*HI fragment. Furthermore, the number of hybridizing *Bam*HI fragments should correspond to the *AtPTR2-B* T-DNA copy number plus the endogenous *AtPTR2-B* gene. The *Bam*HI fragment containing the endogenous *AtPTR2-B* gene should differ in size from the one(s) containing the transgene *AtPTR2-B*. As shown in Figure 18a, *Bam*HI-digested genomic DNA of various transgenic sense plants yielded hybridizing fragments of different sizes (e.g., 5.7 kb, 5.3 kb, 4.8 kb, and 3.6 kb), whereas hybridization to DNA from wildtype plants yielded only the expected 4.8 kb band when probed with *AtPTR2-B* DNA. These results indicate that T-DNA insertion occurred at random into different loci of plant genome in each plant line. These hybridization results also indicated that one T-DNA copy was present in line BS1, while two copies were present in lines BS2 and BS9. These observation was also supported by the genetic segregation analysis (see later). Based on the Southern blot results, three independent transgenic *AtPTR2-B* sense lines, named BS1, BS2, and BS9, were identified.

To determine the integration of T-DNA and the copy number of the *AtPTR2-B* antisense gene, the same strategy was used. *AtPTR2-B* antisense-containing T-DNA contains only one *Eco*RI site at the 3' end of *AtPTR2-B*. Therefore, the genomic DNA of *AtPTR2-B* antisense plants was digested with *Eco*RI and hybridized with a *AtPTR2-B*-specific probe. As shown in Figure 18b, there were four independent transgenic lines identified, BA6, BA8, BA21, and BA27. BA8 and BA21 contain one copy of T-DNA corresponding to the bands of 4.0 kb and 4.3 kb, respectively. Lines BA6 and BA27 have two copies of T-DNA insertion corresponding to the *Eco*RI bands of 5.0 kb and 4.0 kb for BA6, and 4.6 kb and 5.7 kb for

Figure 18. Southern blot analysis of antisense and sense *AtPTR2-B* plant DNAs hybridized with a *AtPTR2-B*-specific probe. a, shows the genomic Southern blot of sense plants digested with *Bam*HI. Lane 1 is the wildtype; Lane 2 is BS1; Lane 3 is BS2; Lane 4 is BS5; Lane 5 is BS6; Lane 6 is BS9. b, shows the genomic Southern blot of antisense plants digested with *Eco*RI. Lane 1 represents the wildtype; Lane 2 is BA6; Lane 3 is BA7; Lane 4 is BA8; Lane 5 is BA21; Lane 6 is BA27.



BA27. Southern blot of wildtype DNA digested with *EcoRI* showed a 6.1 kb fragment containing the endogenous *AtPTR2-B*.

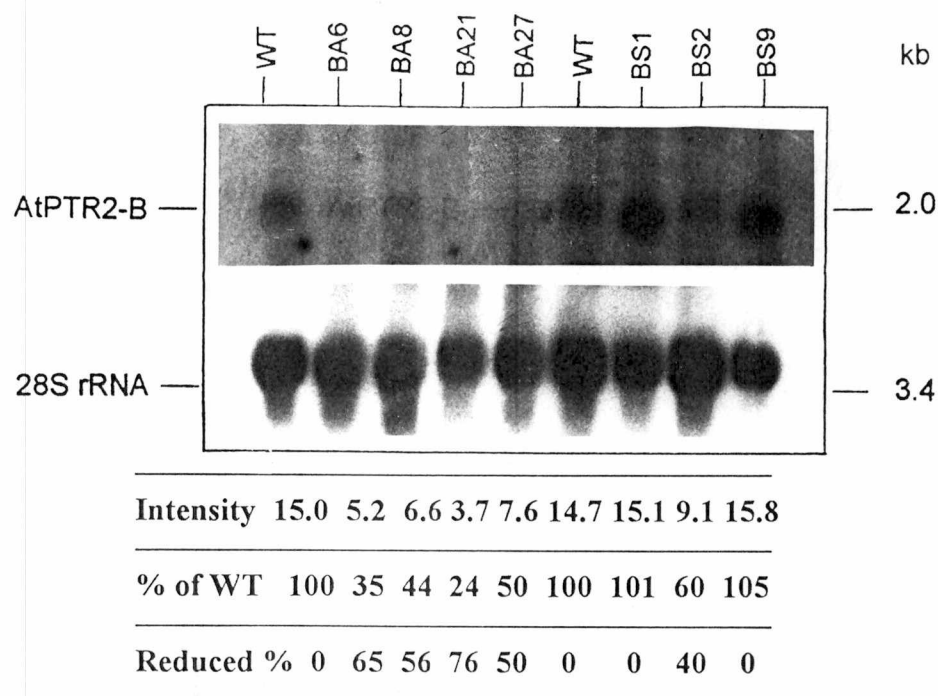
Northern Blot Analysis

To determine the effect of *AtPTR2-B* transgene expression upon the steady-state levels of the endogenous *AtPTR2-B* mRNA, total RNA was extracted from leaves, roots, and flowers of transgenic plants, Northern blotted, and hybridized with a 330bp-*SacI/NotI* fragment of *AtPTR2-B* cDNA clone. This fragment is lacking in the transgene so hybridization will only detect the endogenous mRNA. As shown in Figure 19, *AtPTR2-B* mRNA levels were significantly (30-80%) reduced in leaves and flowers of the transformed lines containing the antisense construct. The reduction in mRNA was not significant in transgenic roots (around 10%). The reduced mRNA levels varied among these transgenic lines with the BA21 line showing the most significant reduction in *AtPTR2-B* mRNA (60-80%).

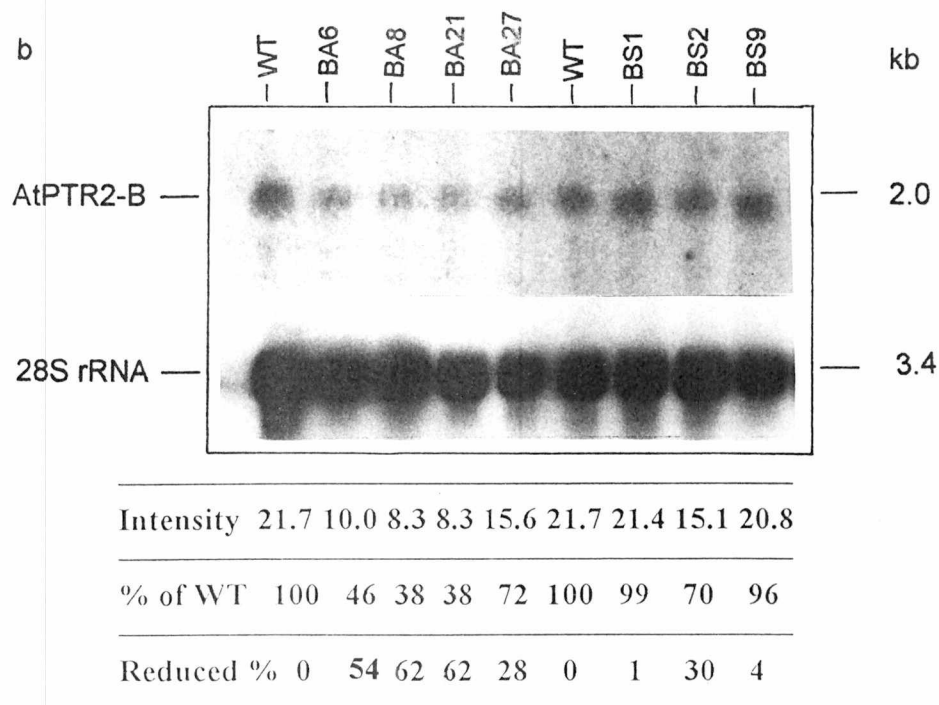
Among the transgenic plants expressing *AtPTR2-B* in the sense orientation, the endogenous levels of *AtPTR2-B* mRNA also varied. The Northern blot data (Figure 19) indicated that the endogenous *AtPTR2-B* mRNA level varied from 60% of the wildtype level in line BS2 to 100% of the wild-type level in lines BS1 and BS9. We attribute this reduction in endogenous *AtPTR2-B* mRNA levels in line BS2 to co-suppression (Dougherty and Parks, 1995). As shown below, consistent with this idea, the phenotype of the BS2 line resembles

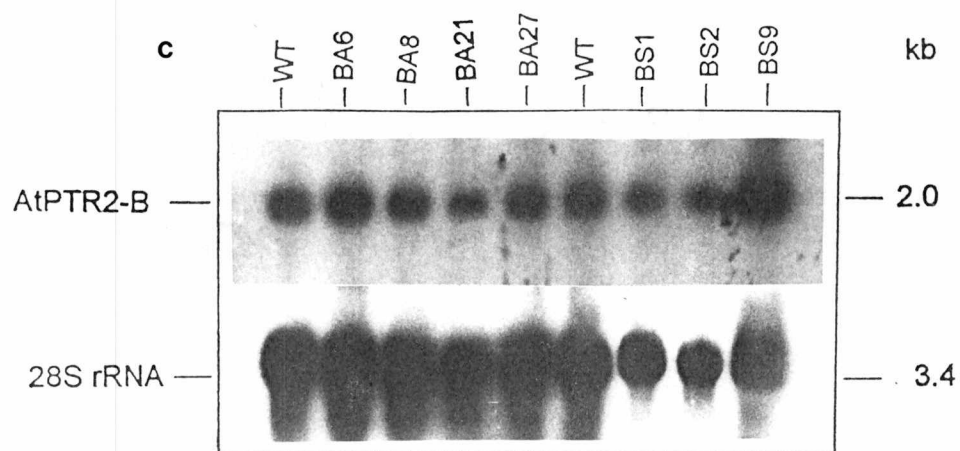
Figure 19. Northern Blot analysis of endogenous *AtPTR2-B* mRNA in leaves, flowers, and roots from antisense and sense *Arabidopsis* plants. 20 µg of total RNA from the wildtype and the transgenic plants were separated on formaldehyde/agarose gels, blotted onto nitrocellulose membranes, and probed with a ³²P-labeled 330 bp of *AtPTR2-B* cDNA. Approximate transcript size is given to the right. The membrane was reprobed with a 28S rRNA-encoding DNA probe to ensure equal loading in each well. The intensity of signals was measured using a densitometer and the data were normalized in order to compare the difference in signal intensity among the samples. a, represents the northern blot of leaf RNA. Lane 1 is the wildtype; Lane 2 is BA6; Lane 3 is BA8; Lane 4 is BA21; Lane 5 is BA27; Lane 6 is the wildtype; Lane 7 is BS1; Lane 8 is BS2; Lane 9 is BS9. The labels of lanes in all below panels are the same as described above. b, represents the northern blot of flower RNA. c, represents the northern blot of root RNA.

a



b





Intensity	11.1	11.1	10.1	9.6	11.0	11.5	11.4	12.0	12.2
% of WT	100	100	90	86	100	103	102	105	106
Reduced%	0	0	10	14	0	0	0	0	0

that of the antisense transgenic plants.

Segregation of Kanamycin Resistance

In order to further confirm the presence of T-DNA and its copy number in the genome of transgenic plants, the segregation of the T-DNA was followed by selecting for kanamycin resistance. The T-DNA encodes for the expression of neomycin phosphotransferase (*npt*). The T₂ seeds produced from self-pollinated T₁ plants were germinated on kanamycin-containing medium. Three to four days after imbibition, seedlings were transferred to the kanamycin-containing medium. Seven to ten days post-germination, the seedlings sensitive to kanamycin turned yellow and died, whereas the resistant seedlings remained green and healthy (Figure 20). The number of the resistant and sensitive seedlings was scored and used to calculate the segregation ratio of Kan^R:Kan^S. As shown in Table 9, two independent *AtPTR2-B* antisense lines, BA6 and BA27, showed an 11:1 ratio of Kan^R:Kan^S not very close to the expected ratio of 15:1 when two copies of T-DNA segregate independently. This value may be the result of using an insufficient number of seeds for this analysis. Due to the lack of sufficient T₂ seeds of BA8 and BA21, T₃ seeds from self-pollinated T₂ plants were used instead to determine the segregation pattern. The actual ratio of BA8 and BA21 was 2.5:1 and 2.7:1, respectively, close to the predicted ratio of 3:1 when one copy of T-DNA segregates. Similarly, the *AtPTR2-B* sense T₂ seeds were examined on the kanamycin-containing plates. The three independent *AtPTR2-B* sense lines had actual Kan^R:Kan^S ratios, 3.7:1 for BS1, 12.3:1 for BS2, and 13.7:1 for BS9, which are consistent



Figure 20. Growth of *Arabidopsis* seeds harvested from T_1 self-fertilized transgenic plants on kanamycin-containing plates. Seeds were sterilized and grown on the G-H medium. Five to seven days after germination, the kanamycin sensitive seedlings turned yellow and died, whereas the kanamycin resistant seedlings appeared healthy.

Table 9. T-DNA Segregation in *AtPTR2-B* Transgenics: Self-fertilized T₁ Plants

Lines	Germinate (%) ^a	segregation ----- Kan ^R /Kan ^{Sb} Ratio ^c	χ ² ----- 3:1 ^d 15:1 ^e	Conclusion
<i>AtPTR2-B</i> antisense				
BA6	96	44/4 11.0:1	7.8 (0.005<P<0.01)	15:1 ^f
BA8	98	35/14 2.5:1	0.4 (0.5<P<0.9)	3:1 ^g
BA21	96	35/13 2.7:1	0.3 (0.5<P<0.9)	3:1 ^g
BA27	99	45/4 11.2:1	7.8 (0.005<P<0.01)	15:1
<i>AtPTR2-B</i> sense				
BS1	93	22/6 3.7:1	0.2 (0.5<P<0.9)	3:1
BS2	93	86/7 12.3:1	15.1 (P<0.001)	15:1
BS9	98	41/3 13.7:1	7.8 (0.005<P<0.01)	15:1

^a % Germination was derived from dividing the number of the germinating seeds, including Kan^R and Kan^S seedlings, by the total seed number.

^b Kanamycin resistant to kanamycin sensitive (Kan^R : Kan^S) ratios was observed with these seeds germinating.

^c Derived Kan^R : Kan^S ratios from actual observations.

^d Chi-square value and probability obtained from testing if the derived ratio is the same as 3:1 Kan^R : Kan^S ratio.

^e Chi-square value and probability obtained from testing if the derived ratio is the same as 15:1 Kan^R : Kan^S ratio.

^f Kan^R : Kan^S ratio accepted as significant according to the chi-square analysis.

^g Due to the lack of enough T₂ seeds, T₃ seeds from self-pollinated T₂ plants were used for the segregation analysis.

with one or two copies of T-DNA segregating independently. These genetic results are consistent with the Southern hybridization data described previously.

Germination Assay in the Absence of Kanamycin Selection

During the course of the segregation assay, the germination frequency of the transgenic seeds was also determined. A germination frequency of 93-99% shown in Table 9 indicates that the transgenic seeds were viable, suggesting that the expression of the *AtPTR2-B* sense or antisense gene did not result in lethality. However, the germination of transgenic seeds appeared slower than the wildtype seeds.

In order to document that the transgenic seeds had a slower germination rate, the germination of T₂ *AtPTR2-B* sense seeds were analyzed. Table 10 shows that three days post-imbibition, 77% of the wildtype seeds had root emergence, whereas only 9-42% of the transgenic seeds had root emergence. By day four post-imbibition, 91% of the wildtype seeds showed root emergence. In contrast, six days were required before the transgenic seeds reached the same percentage of root emergence (87-91%). A similar situation was observed with regard to cotyledon emergence in the wildtype and transgenic seeds (Table 9). The transgenic seeds were approximately one to two days late for cotyledon emergence when compared to the wildtype. Due to the lack of sufficient T₂ *AtPTR2-B* antisense seeds, the germination assay was not performed.

Table 10. Germination Rate of T₂ *AtPTR2-B* Sense Seeds^a

Lines	seed No.	% Root Emergence				% Cotyledon Emergence				% Germinate
		2d	3d	4d	6d	3d	4d	6d	7d	
Wildtype	50	0	77	91	100	0	68	91	100	100
BS1	20	0	9	65	91	0	40	80	91	91
BS2	80	0	28	68	91	0	46	89	95	95
BS9	45	0	42	69	87	0	33	80	91	91

^a Seeds were from self-fertilized T₁ plants and germinated on the plant growth medium (G-H) without addition of kanamycin. After two day-imbibition in the dark, the seeds were exposed to light and scored for germination.

^b Represents the total seed number plated on the medium.

^c The percentage of seeds showing the root elongation.

^d The percentage of seeds having cotyledon appearance.

^e Germination frequency was calculated from the number of germinating seeds including root-appearing and/or cotyledon- appearing seeds divided by the total seed number.

Leaf Analysis and Late Flowering

One of the earliest phenotypic changes observed among the transgenic lines was late-flowering (Figure 21). Wildtype and pGA941 vector-transformed plants starting flowering when 6 to 8 rosette leaves were produced after three-weeks vegetative growth. In contrast, the antisense lines and the sense BS2 line were delayed in flowering by seven to fifteen days. The sense lines BS1 and BS9 flowered at the same time as the controls. Moreover, prior to initiating flowering, the late-flowering plants produced rosette leaves three-fold larger than the controls. The number of the rosette leaves were the same as that of the controls (Table 11). After transition to flowering, the late-flowering lines generated bigger inflorescence stems and flowers, as well as more rosette leaves (data not shown). However, the flower structure of the late-flowering plants was similar to that of the controls.

Measurements were made of 10 rosette leaves taken from three plants per line and statistically analyzed by the t-test method. At the time of measurement, the control plants had started flowering, but the antisense and BS2 lines were still growing vegetatively after three to four weeks. The average size of wildtype or control (vector alone) leaves was determined to be $4.0 \text{ cm}^2 \text{ leaf}^{-1}$, compared to the average size of BS1 or BS9 ($3.85 \text{ cm}^2 \text{ leaf}^{-1}$). There was no significant difference between the controls and the sense lines BS1 and BS9 ($t=0.970$, $\text{d.f.}=18$, $0.5 < P < 0.999$). Thus, these data indicate that the expression of the *AtPTR2-B* sense gene in BS1 and BS9 did not affect the leaf size, which is consistent with the normal levels of endogenous *AtPTR2-B* mRNA found in these lines (Figure 19b). In contrast, the *AtPTR2-*

Figure 21. The late flowering phenotype of *AtPTR2-B* antisense lines when compared to the wildtype and vector alone plants. Upper panel: the left side shows the wildtype plant, while the right side shows the antisense BA8 line; Lower panel: the first column represents the wildtype (the first row) and vector alone (the second row) plants, while the rest of columns represent the antisense *AtPTR2-B* lines.

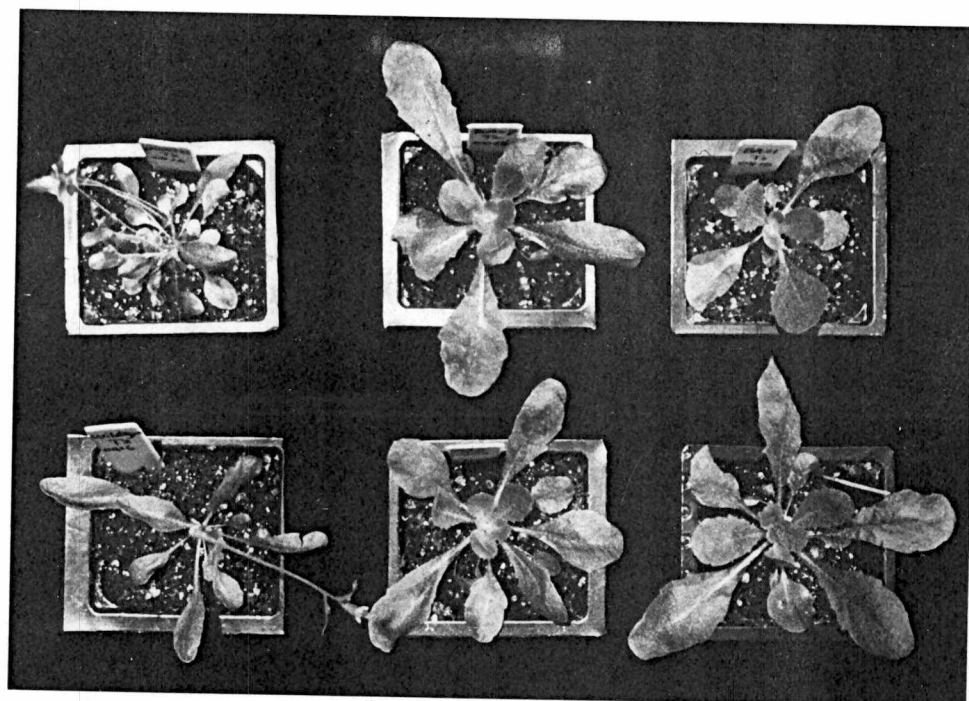


Table 11. Leaf Analysis of *AtPTR2-B* Transgenics: T₂ Plants

Lines ^a	Mean Size /Leaf (Length × Width)(cm ²) ^b	Mean Weight/Leaf (mg) ^b
Wildtype	4.0 ± 0.9 ^d	45.2 ± 6.5 ^d
pGA941	4.0 ± 0.9	35.9 ± 14.8
<i>AtPTR2-B</i> antisense		
BA6	9.6 ± 0.5	141.4 ± 21.6
BA8	6.6 ± 0.4	106.3 ± 20.7
BA21	8.2 ± 0.9	123.7 ± 22.2
BA27	8.1 ± 0.9	106.3 ± 16.5
<i>AtPTR2-B</i> sense		
BS1	3.7 ± 0.9	41.2 ± 7.2
BS2	8.4 ± 0.8	135.2 ± 20.5
BS9	4.0 ± 0.9	42.5 ± 8.5

^a T₂ transgenic plants were obtained from T₂ seeds germinated on kanamycin-containing medium and then transferred to the soil. Therefore, all T₂ transgenic plants analyzed were homozygous or heterozygous and resistant to kanamycin.

^b Mean leaf size of *AtPTR2-B* transgenic lines, BA6, BA8, BA21, BA27 and BS2, are significantly different from the mean leaf size of wildtype and pGA941 vector controls ($t=30.896$, d.f.=18, $P<0.001$), as well as different from transgenic lines BS1 and BS9 ($t=0.970$, d.f.=18, $0.5<P<0.999$).

^c Mean leaf weight of *AtPTR2-B* transgenic line, BA6, BA8, BA21, BA27 and BS2, are significantly different from the mean leaf weight of wildtype and pGA941 vector controls ($t=19.016$, d.f.=18, $P<0.001$), as well as different from transgenic lines BS1 and BS9 ($t=0.336$, d.f.=18, $0.1<P<0.999$). ^d Standard deviation was derived from the average of ten leaves taken from at least two independent plants each line.

B antisense and sense BS2 leaf size (average 8.5 cm² leaf⁻¹) differed significantly from the controls ($t=30.896$, d.f.=18, significant at $P<0.001$), indicating that expression of the *AtPTR2-B* transgene in all antisense lines and the sense BS2 affected leaf size. The results are consistent with the reduction in endogenous *AtPTR2-B* mRNA levels found in these lines (Figure 19a).

The leaf average weight was compared between the transgenic lines and the controls. The average weight of wildtype and vector controls (40.5 mg leaf⁻¹) was significantly different from that of the antisense lines and BS2 line (122.6 mg leaf⁻¹) ($t=19.016$, d.f.=18, significant at $P<0.001$), but no significant difference was observed between the controls and the BS1 and BS9 lines (41.8 mg leaf⁻¹) ($t=0.336$, d.f.=18, significant at $0.1<P<0.999$).

Seed Analysis

As described above, peptide transport has been suggested to play an important nutritional role in supplying nitrogen to the plant embryo (Higgins et al., 1978). Our previous results indicate that *AtPTR2-B* is highly expressed in young siliques (Song et al., 1996), suggesting its role in seed development. Therefore, reduction of *AtPTR2-B* mRNA levels might affect seed maturation. To test this hypothesis, the silique length, seed number per silique and seed weight per seed were determined. Measurements were made using 30 siliques and five samples of 100 seeds per line. Table 12 shows that the transgenic lines produced siliques the same length as the controls, indicating that the reduced *AtPTR2-B*

Table 12. Seed Analysis of *AtPTR2-B* Transgenics: T₂ Plants

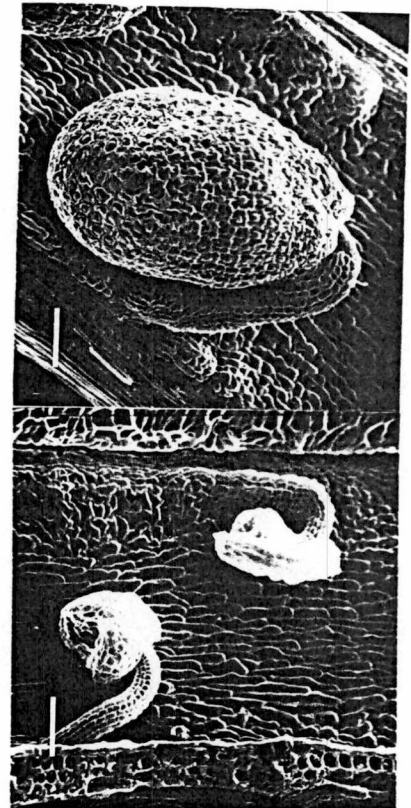
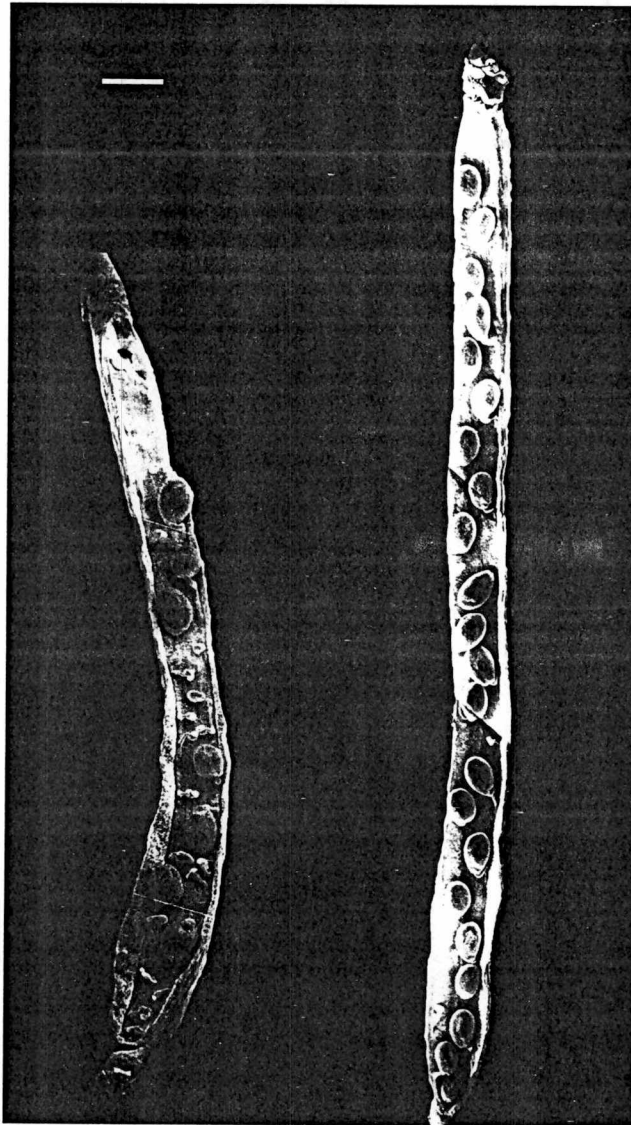
Lines ^a	Mean Length per Silique (cm) ^b	Mean Seed No per Silique ^c	Seed No/ Silique Length (cm) ^d	Mean Weight per Seed (ug) ^e
Wildtype	1.2 ± 0.2	40 ± 8	35	14.4 ± 0.9
pGA941	1.2 ± 0.2	45 ± 8	38	15.3 ± 0.7
<i>AtPTR2-B</i> antisense				
BA6	1.2 ± 0.1	22 ± 5	18	35.6 ± 0.6
BA8	1.2 ± 0.2	16 ± 7	13	36.2 ± 0.7
BA21	1.2 ± 0.2	21 ± 6	17	37.4 ± 0.6
BA27	1.2 ± 0.1	17 ± 7	14	34.0 ± 0.8
<i>AtPTR2-B</i> sense				
BS1	1.2 ± 0.2	34 ± 9	29	15.2 ± 0.8
BS2	1.2 ± 0.2	22 ± 7	18	27.6 ± 0.6
BS9	1.2 ± 0.2	37 ± 7	32	14.6 ± 0.6

^a Seeds resulting from self-pollination of T₂ plants. ^b Mean silique length of *AtPTR2-B* transgenic lines are not significantly different from the mean silique length of wildtype and pGA941 vector controls ($t=0$, d.f.=58, $0.5 < P < 0.999$). ^c Mean seed number per silique of *AtPTR2-B* antisense transgenic lines and BS2, is significantly different from the mean seed number of wildtype and pGA941 vector controls ($t=8.884$, d.f.=58, $p < 0.001$), as well as different from transgenic lines BS1 and BS9 ($t=1.546$, d.f.=58, $0.5 < P < 0.999$). ^d Mean seed number per silique length of *AtPTR2-B* antisense transgenic lines and BS2, is significantly lower than the mean seed number per silique of wildtype and pGA941 vector controls, as well as different from transgenic lines BS1 and BS9. ^e Mean seed weight per seed of *AtPTR2-B* antisense transgenic lines and BS2, is significantly different from the mean seed weight per seed of wildtype and pGA941 vector controls ($t=27.848$, d.f.=8, $p < 0.001$), as well as different from the transgenic lines, BS1 and BS9 ($t=0.844$, d.f.=8, $0.5 < P < 0.999$). ^f Standard deviation was derived from average of 30 siliques. ^g Standard deviation from average of five samples of 100 seeds each line.

mRNA did not appear to affect silique formation and growth. However, the seed number per silique was significantly decreased in the antisense plants and the sense plant BS2 (20 seeds per silique) when compared to that of the controls (40 seeds per silique) ($t=8.884$, d.f.=58, significant at $P<0.001$), implying that reduced *AtPTR2-B* mRNA expression affects seed development. In contrast, the sense plants BS1 and BS9, expressing normal levels of *AtPTR2-B* mRNA, produced a similar number of seeds per silique (36 seeds per silique) as the controls ($t=1.546$, d.f.=58, $0.5<P<0.999$). The seed weight per seed was significantly greater in the antisense and BS2 lines (34.5 μg per seed) when compared to the controls (16 μg per seed) or the BS1 and BS9 lines (15.5 μg per seed) ($t=27.848$, d.f.=8, significant at $P<0.001$). This suggests a similar import of nutrients into the transgenic and control siliques which is then utilized in the antisense transgenic lines to produce fewer seeds.

The fact that the silique formed by the transgenics and the controls were of the same length, but that fewer seeds were produced in the transgenics, suggests that silique elongation and seed development are independent events with regard to *AtPTR2-B* expression. To further address the role of *AtPTR2-B* in seed development, ten green and matured siliques per line were dissected and seeds were examined under the stereo microscope and by scanning electron microscopy. As expected, a large amount of aborted seeds were observed in the antisense and BS2 transgenic siliques. Figure 22a shows that the transgenic mature siliques contained healthy and aborted seeds which were easily distinguished by the differences in seed shape and size. The aborted seeds (shrinkage likely due to dehydration) were significantly smaller than the healthy seeds (Figure 22b). The early-developed seeds,

Figure 22. Scanning electron microscopic examination of siliques. a, siliques formed on the wild-type (upper) and transgenic line (lower). The magnification is of 13 $\times$. b, wild-type seeds (left) and aborted seeds (right) formed by the transgenic line BA21. The magnification is of 125 $\times$. (This photograph was provided by Serry Koh)



such as heart-stage embryo-containing seeds in young siliques, did not show a significant difference in size and shape (data not shown). Table 13 summarizes the number of the healthy and aborted seeds per silique. The control plants contained an average of 45 healthy seeds and 2 aborted seeds and the ratio was 18:1 of healthy : aborted seeds. This small portion of aborted seeds could be considered as a normal physiological phenomenon. However, approximately 50% of the seeds from the *AtPTR2-B* antisense lines and BS2 line aborted (average 25 aborted seeds per silique), and the ratio was significantly different from that of the controls, as well as the BS1 and BS9 lines. These results strongly suggest that the expression of the *AtPTR2-B* antisense gene disrupts normal seed development.

Previous studies have shown that antisense expression results in a 40 to 100% reduction in endogenous mRNA levels of the target gene and that this reduction sometimes exhibits tissue-specificity (Jones et al., 1995). Thus, it is not possible to attribute the ratios of healthy : aborted seeds in siliques to simple Mendelian segregation. Indeed, the normal seeds formed in the siliques of the antisense transgenic plants showed the predicted segregation for the T-DNA (Kan^R : Kan^S, Table 8).

Root Growth and Morphology

Another striking phenotypic observation was the change in root growth and morphology of the antisense and BS2 transgenic plants. When seeds were germinated on petri dishes and the plates were placed in the upright position, root growth and morphology

Table 13. Seed Maturation in Siliques of T₂ *AtPTR2-B* Transgenics

Lines ^a	Healthy Seed No per Silique	Aborted Seed No per Silique	Ratio (Healthy:Aborted)
Wildtype	45 ± 6 ^b	2 ± 2	22.5 : 1
pGA941	49 ± 2	3 ± 2	16.3 : 1
<i>AtPTR2-B</i> antisense			
BA6	25 ± 4	23 ± 4	1 : 1.1
BA8	13 ± 2	35 ± 5	1 : 2.6
BA21	14 ± 4	39 ± 8	1 : 2.9
BA27	21 ± 2	18 ± 5	1.2 : 1
<i>AtPTR2-B</i> sense			
BS1	38 ± 6	3 ± 3	12.5 : 1
BS2	21 ± 5	27 ± 9	1 : 1.2
BS9	34 ± 2	2 ± 2	19.1 : 1

^a Siliques were generated from T₂ self-fertilized plants.

^b Standard deviation was derived from ten siliques analyzed taken from three independent plants each line.

could be easily examined. In comparison to the controls, the anti-sense transgenic lines and the sense BS2 line produced significantly more lateral roots and root hairs (Figure 23). the BS1 and BS9 lines also generated more lateral roots and root hairs than the controls, but greatly fewer than the other transgenic lines (data not shown).

As described previously, the *Arabidopsis* roots exhibited sensitivity to toxic dipeptides. To determine if the transgenic plants were sensitive to these toxic peptides, the inhibition assay of root growth was performed. As shown in Table 14, all transgenic lines exhibited similar sensitivity to the toxic peptides as the wildtype. These results leave open the question of whether the AtPTR2-B transporter mediates toxic dipeptide uptake in roots. As shown in Figure 19, antisense expression of *AtPTR2-B* did not significantly reduce the endogenous level of gene expression in roots. Therefore, it is likely that the slightly reduced expression of endogenous *AtPTR2-B* by antisense inhibition in roots was insufficient to prevent transport of the toxic peptides.

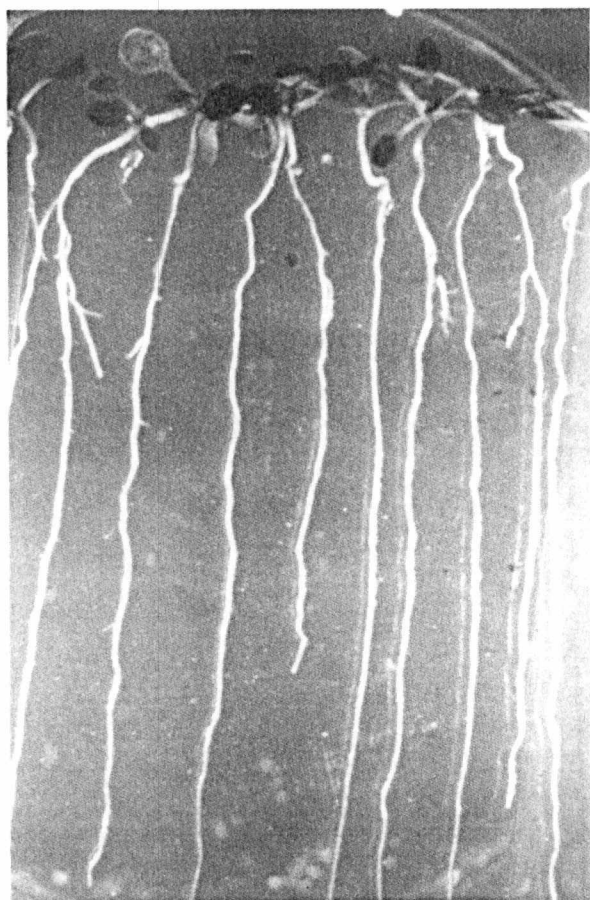
RFLP Mapping of *AtPTR2-B*

Restriction digestion of genomic DNA by *HpaI*, *BclI*, or *XbaI* generated distinguishable polymorphisms between *Arabidopsis thaliana* ecotype Landsberg and Columbia (Figure 24). The *XbaI*-generated polymorphism was used to map the *AtPTR2-B* gene using the recombinant inbred lines generated by Lister and Dean (1993). *AtPTR2-B* maps to chromosome 2 at 10.5 centimorgans (Figure 25), (data are accessible at

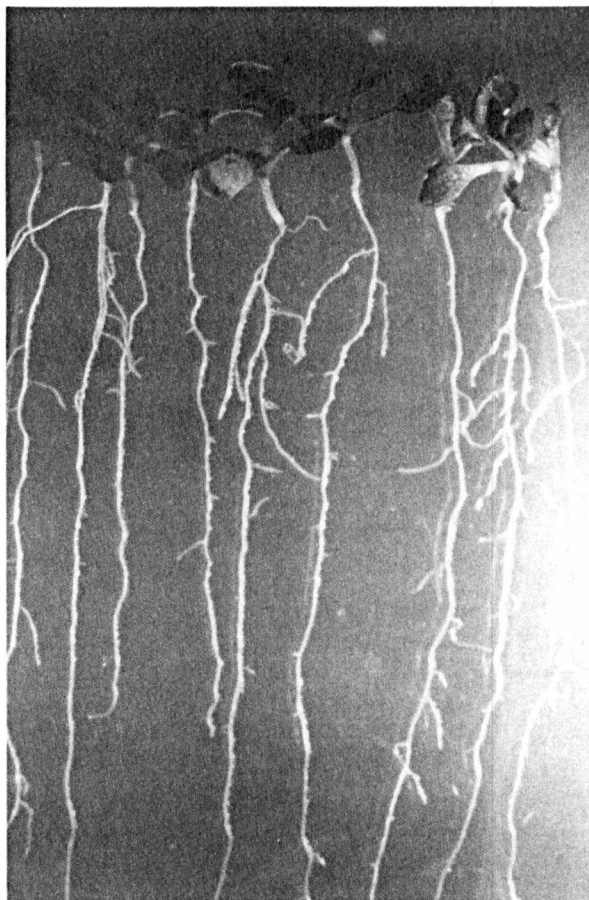
Figure 23. Lateral root and root hair formation. a, shows the difference in the number of lateral roots formed by the wild-type (left) and the BA21 transgenic line (right). b, shows the difference in the number of root hairs formed by the wild-type (left) and the BA21 transgenic line (right). The magnifications are 4 × and 10 ×, respectively.

a

Wild-type

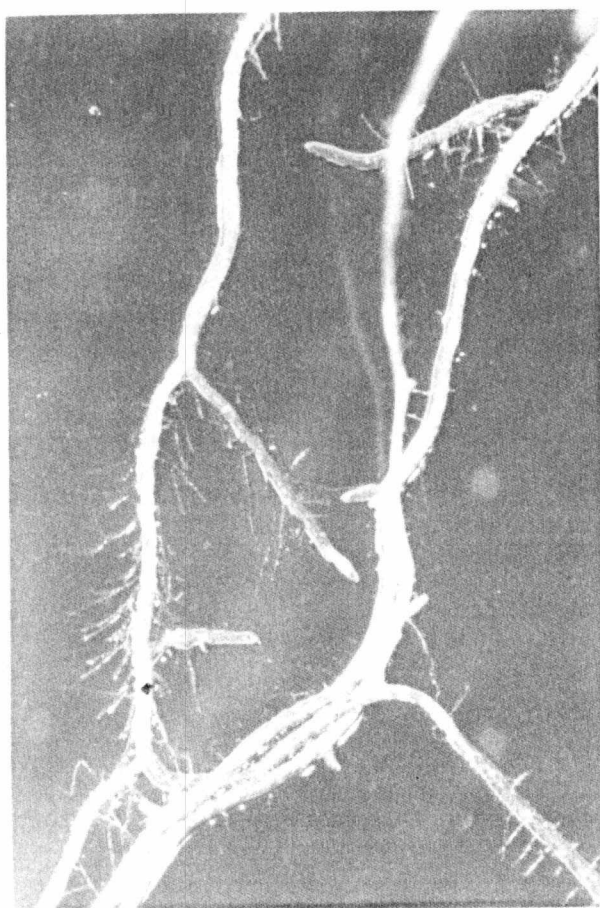


Transgenic



b

Wild-type



Transgenic

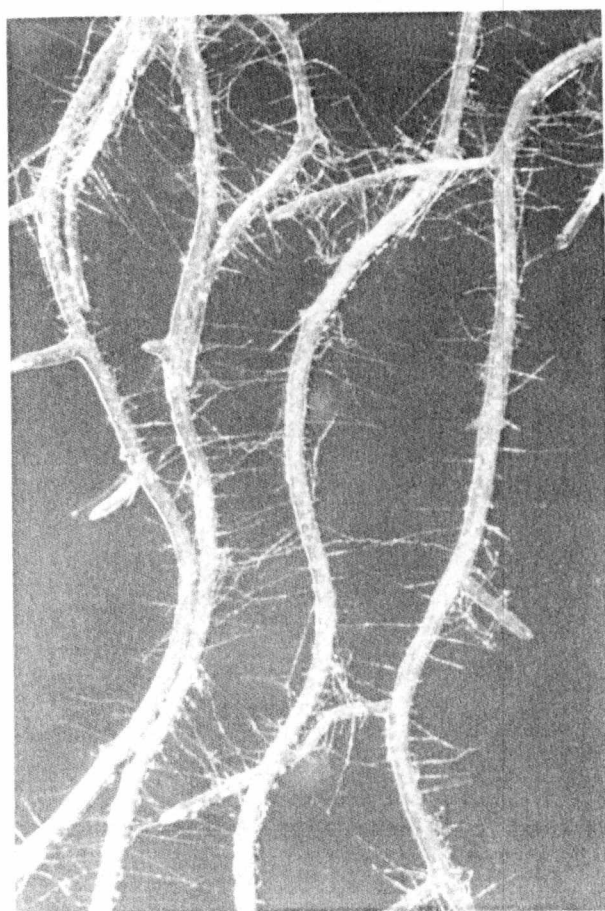


Table 14. Inhibition of Root Growth by Toxic Dipeptides in Transgenic Plants

Lines	H ₂ O	Toxic Peptides ^a	
		Leu-Eth	Ala-Eth
Wildtype	+ ^b	-	- ^c
<i>AtPTR2-B</i> antisense			
BA6	+	-	-
BA8	+	-	-
BA21	+	-	-
BA27	+	-	-
<i>AtPTR2-B</i> sense			
BS1	+	-	-
BS2	+	-	-
BS9	+	-	-

^a 5 µl of 7.6 mM of toxic peptides was applied to the disc, which is the minimal concentration of inhibition of root growth in the wildtype(MIC= 38 nmoles). 5 µl of H₂O was used as a control.

^b represents the growth of roots.

^c represents the inhibition of root growth.

Figure 24. Polymorphism of *AtPTR2-B* between *Arabidopsis thaliana* ecotype Landsberg and Columbia shown by Southern blot analysis. 20 µg of genomic DNA was digested with *Bam*HI, *Hpa*I, *Bcl*II, or *Xba*I respectively, electrophoresed, and hybridized with a *AtPTR2-B*-specific cDNA probe. High stringency conditions (0.1 % SDS, 0.1 × SSC, and 60°C) were used to wash the membrane.

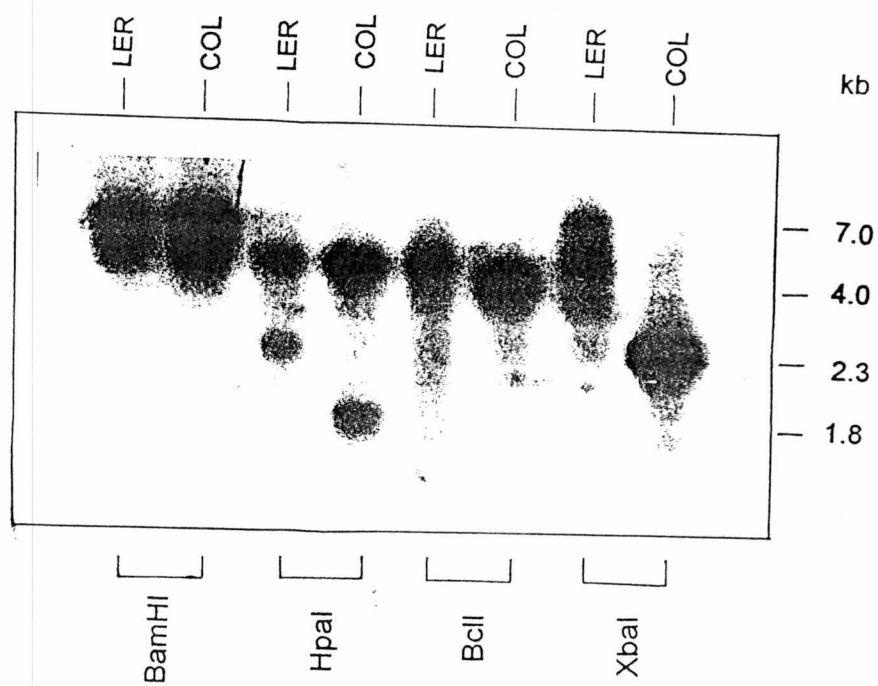
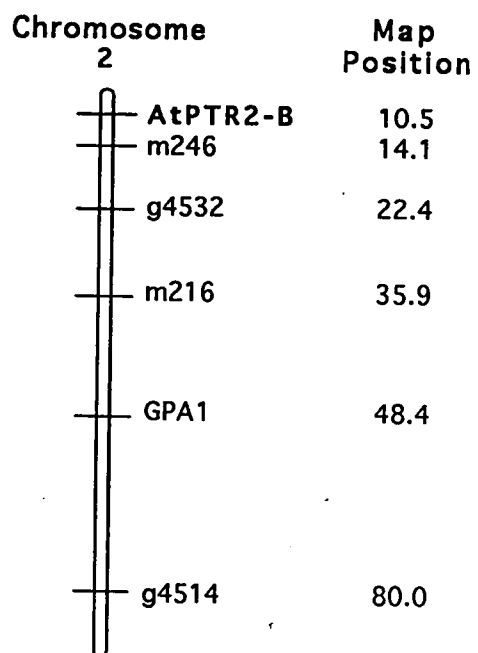


Figure 25. Map position of *AtPTR2-B* on chromosome II of *Arabidopsis thaliana*. The map position of the *AtPTR2-B* gene was determined using 98 recombinant inbred lines as described in methods. Distances between loci are shown in centimorgans. Mapping was done by Eliana Drenkard, Harvard University.



http://probe.nalusda.gov:8300/cgi-bin/dbrun/aatdb?find+map+Recombinant_inbred*). An *Arabidopsis thaliana* database search reveals that the *AtPTR2-B* gene maps close to the emb34 locus that defines an embryo defective mutant.

CHAPTER IV

DISCUSSION

Part I: Isolation and Characterization of *Arabidopsis* Peptide Transporter cDNAs

Peptide transport represents a basic phenomenon likely to be common to all organisms. The availability of yeast peptide transport mutants has allowed us to begin to dissect the components of the plant peptide transport system by functional complementation of yeast mutants. This strategy has been used successfully to clone other plant transport genes (Anderson et al., 1992; Riesmeier et al., 1992; Sentenac et al., 1992; Frommer et al., 1993; Hsu et al., 1993). This method allows rapid identification of plant genes functionally homologous to yeast genes in well-characterized yeast mutants.

Characterization of the plant peptide transporters reveals striking similarities to those of yeast. The specificity conferred by *AtPTR2-A* or *AtPTR2-B* is most likely a result of a chain length limitation because the tripeptide Lys-Ala-Eth and Lys-Leu-Eth were toxic to both the wildtype and transformants, whereas longer peptides of similar composition were not toxic to the cells (Table 7). This confirms earlier reports that peptide uptake by isolated barley scutella was limited to dipeptides and tripeptides (Sopanen et al., 1977; Higgins and Payne, 1978b).

Another similar characteristic shared by the plant and yeast transport systems is the lack of a strict side chain specificity. An exception to the observed broad substrate specificity is the inability of highly basic peptides to be transported. Peptides containing three or more lysines did not support the growth of the yeast mutant (PB1X-9B) transformed with either *AtPTR2-A*, *AtPTR2-B* or *PTR2*. This is also consistent with previous work on yeast (Lichliter et al., 1976; Marder et al., 1977) and plants (Higgins and Payne, 1978b, 1978c; Sopanen et al., 1978).

Whereas there appears to be little side chain specificity for uptake, both plant transporters transport peptides that, in general, have a hydrophobic character. These peptides contain one or more of the hydrophobic amino acids alanine, leucine, or methionine. In general, this was also the case with yeast and barley (Higgins and Payne, 1978a; Becker and Naider, 1980). Consistent with this observation, transport of radiolabeled dileucine was inhibited by a number of peptides containing hydrophobic residues (Figure 11). This also supports earlier work on germinating barley seeds, which showed a strong bias toward transport of peptides composed entirely or in part of hydrophobic residues (Higgins and Payne, 1978a, 1978b; Sopanen et al., 1978). Furthermore, the hydrophobic peptides Ala-Met, Leu-Met, and Met-Met reversed the growth inhibition of Ala-Eth and Leu-Eth in roots of *Arabidopsis* seedlings (Table 8).

Considering the number of similarities between *AtPTR2-A*, *ATPTR2-B*, and *Ptr2p*, it was surprising to find that *AtPTR2-B* conferred only partial sensitivity to toxic peptides

when expressed in yeast. Yeast transformants expressing either the yeast Ptr2p or AtPTR2-A were sensitive to toxic peptides. Competition experiments indicated that [^3H]dileucine uptake by AtPTR2-A or AtPTR2-B was completely inhibited by a 7.8-fold excess of Leu-Eth. Therefore, the partial sensitivity of the pDTF1 transformants is most likely explained by the difference in kinetic parameters between AtPTR2-A and AtPTR2-B. The K_m and V_{max} values for dileucine uptake by AtPTR2-A indicate that this transporter has a 3-fold lower affinity for dipeptides than AtPTR2-B, but a 5-fold higher capacity. This higher capacity likely results in the transport of greater amounts of toxic peptide and, therefore, transformants show a high level of sensitivity. In contrast, the kinetic parameters for AtPTR2-B indicate that this transporter has, relative to AtPTR2-A, a higher affinity, but a reduced transport capacity. This might explain the presence of some growth even in the presence of toxic peptide. Such differences between AtPTR2-A and AtPTR2-B lead us to believe that these two peptide transporters likely play different functional roles in the plant. This suggestion is further supported by analysis of the expression patterns of *AtPTR2-A* and *AtPTR2-B*. We found that *AtPTR2-A* mRNA could not be detected by northern blot analysis but could be detected in flowers, stems, leaves, and roots after PCR amplification. In contrast, Northern analysis showed a reasonably high level of *AtPTR2-B* expression in all plant organs examined. It is possible that AtPTR2-B supplies the plant with a basal level of peptide uptake capacity, while AtPTR2-A may be an inducible uptake system. It is known that the yeast Ptr2p transporter is inducible by amino acids (Island et al., 1987). Moreover, the Southern blot analysis of genomic DNAs revealed that *AtPTR2-A* was present only in *Arabidopsis thaliana* ecotype Landsberg, but not present in the ecotype RLD, whereas *AtPTR2-B* was present in

both ecotypes. These results suggest the ecotype-specific localization of *AtPTR2-A*. Further research will be required to explore the role of *AtPTR2-A* *in planta*.

Frommer et al. (1994) reported the isolation of a putative histidine transporter, *NTR1*, from *Arabidopsis thaliana*. The DNA sequence reported for the *NTR1* gene and that given here for *AtPTR2-B* are virtually identical. Since these genes were both isolated from the cDNA library constructed by Minet et al. (1992), we feel that it is likely that these two genes are indeed identical and that the small amount of sequence divergence is a result of sequencing differences. However, a 200-fold excess of histidine had no effect on [³H]dileucine uptake by yeast transformants expressing *AtPTR2-B*. Indeed, Frommer et al. (1994) were unable to detect [¹⁴C]histidine uptake in yeast transformants expressing *NTR1*. Although the expression of *NTR1* in a yeast histidine auxotroph allowed for growth on medium containing 6 mM histidine, it was noted that this growth was quite slow. Therefore, we conclude that high level expression of *NTR1* (*AtPTR2-B*) in yeast can provide a low level of histidine uptake. This level appears sufficient to support marginal growth, but insufficient to be detected by the measurement of [¹⁴C]histidine uptake. It is our contention that our and Frommer's results provide compelling evidence that *NTR1* (*AtPTR2-B*) is actually a component of the peptide transport system in *Arabidopsis* plants and not a histidine transporter.

Consistent with the presence of *AtPTR2-A* and *AtPTR2-B* mRNA in roots, toxic peptides (e.g., Ala-Eth) inhibited root growth. Nontoxic peptides tested for their abilities to

compete with the ethionine-containing peptides showed different relative affinities to that seen in competition experiments using the radiolabeled uptake assay and transformed yeast cells. For example, Ala-Ala and Ala-Ala-Ala did not reverse the growth inhibition of Ala-Eth or Leu-Eth in root assays, but were effective competitors of radiolabeled dileucine in the uptake assay. However, these apparent differences may reflect the relative expression levels of *AtPTR2-A* and *AtPTR2-B* and their functional products in their native genetic background versus that in yeast. There may also be considerable differences in the levels of competitor necessary to achieve strong reversal of toxicity in the seedling assay versus the radiolabeled uptake assay. Moreover, this difference in competitor specificity may indicate that other factors or components are present in roots versus yeast and these may determine to a great degree the specificity by which peptides are transported in plants. More studies are required to clarify these possibilities. Surprisingly, methionine did not reverse the root growth inhibition of ethionine. Ethionine may be very potent in this *in vivo* assay of root growth such that, if a small percentage of ethionine is transported, root growth is inhibited. Alternatively, ethionine and methionine may have different transporters in the root and thus transport competition would not occur.

When toxic peptides were applied to *Arabidopsis* seedlings in an experiment analogous to the disc assays performed with yeast, peptides containing ethionine, as well as ethionine itself, inhibited elongation of seedling roots on an agar medium (Figure 13). This inhibition did not appear to affect other parts of the plant because the leaves looked comparable to the untreated controls. Seeds germinated in the presence of peptides

containing ethionine or oxalysine, as well as ethionine and oxalysine themselves, also were inhibited. The seeds were able to germinate and roots grew to 1 to 3 mm in length after which they died. This occurred within three to four days after imbibition. Consistently, *AtPTR2-B* mRNA was detected in germinating 3-day-old imbibed seeds although no *AtPTR2-A* mRNA was detected. These results suggest that the inhibitory effect of these toxic peptides occurs after germination of the seed and growth of root primordia. These data also imply that the inhibition by toxic peptides occurs after the radicle emerges from the seed, RNA synthesis begins, and the peptide transport system is expressed, perhaps via the newly synthesised *AtPTR2-B* transporter system.

Sequence comparison revealed that *AtPTR2-A*, *AtPTR2-B*, and *Ptr2p* show considerable similarity at the amino acid level. Further comparison with the *AtCHL1*, *CaPTR2*, the rabbit *PepT1*, and *DtpT* sequences revealed that these proteins comprise a novel family of transport proteins (PTR) which share significant similarity throughout their length. As a group, *AtPTR2-A*, *AtPTR2-B*, *Ptr2p*, *CaPTR2*, *AtCHL1*, *PepT1*, and *DtpT* are distinct from other transporters from bacteria, yeast, mammals and plants. These other transporters include the ATP binding cassette family of transport proteins, the maltose transport proteins, amino acid transporters, *Escherichia coli* peptide binding proteins, and the membrane and nonmembrane-bound components of bacterial peptide transporters (Henry et al., 1995). Furthermore, the higher identity of *AtPTR2-B* to the *Arabidopsis* nitrate-inducible nitrate transporter *AtCHL1* and the rabbit peptide transporter *PepT1* suggests that the PTR family may be composed of two distinct subfamilies.

The PTR family of transport proteins was also recently identified by computer analysis and called the POT family for proton-dependent oligopeptide transport by Paulsen and Skurray, 1994. However, because it is not known whether all of the members of this family are proton dependent or whether they mediate the transport of oligopeptides, we believe that the more generic designation PTR (for peptide transport) is more appropriate at this time.

Part II: Construction and Characterization of Transgenic *Arabidopsis* Plants Expressing Sense or Antisense AtPTR2-B

Despite their potential importance in plants, peptide-like compounds and the corresponding transport systems has been paid relatively little attention in plants. However, several lines of evidence have demonstrated that peptide transport not only exists in plants, but may also participate in central physiological processes such as nutrition. The importance of peptides for long distance transport in the phloem and xylem is still a matter of debate but would require the existence of peptide transporters in the plasma membrane of these cells (Higgins and Payne, 1980). Moreover, the plant cells must have different peptide transport systems in order to correspond to various peptides or peptides analogues. Such analogues could include peptide-hormone conjugates (Bewley et al., 1986; Klambt, 1969; Salisbury et al., 1991; Thurmann and Street, 1962), modified di- or tripeptide phytotoxins (e.g., tabtoxin and phaseolotoxin, Walton, 1990; Willis et al., 1991), and the natural dialanine-based herbicide, bialaphos (Damm et al., 1993). Consistent with this idea, phaseolotoxin has been

shown to be transported via the oligopeptide permease (Opp) system in *E. coli* and *S. typhimurium* (Staskawicz and Panopoulos, 1980).

Peptide transport in plants has been characterized only in barley grains and broad bean leaf tissues. In barley seeds, this process is involved in retrieval of the peptides resulting from the hydrolysis of seed proteins by the embryo (Higgins and Payne, 1977; Sopanen et al, 1977). Jamai et al. (1994) reported that a peptide transporter also exists in mature broad bean leaves and it may be responsible for the exchange of nitrogenous compounds (Jamai et al., 1994). The high level expression of *AtPTR2-B* in all *Arabidopsis* organs implies that *AtPTR2-B* is of central importance with respect to plant growth and development.

To assess the *in vivo* role of the *AtPTR2-B* protein, *Arabidopsis* plants were transformed with an antisense or sense construct of the *AtPTR2-B* cDNA under the control of the CaMV 35S promoter. In several transformants, reduced amounts of *AtPTR2-B* mRNA were found in some tissues. The reduction in *AtPTR2-B* expression led to marked effects on physiological phenotypes such as seed development, flowering, and lateral root and root hair formation.

As described previously, peptide transport may play a nutritional role in seed development (Higgins and Payne, 1980). Thus, we have examined seeds at different stages of development under the stereo microscope. Differences in seed development were readily apparent between the wildtype and transgenic plants. The wildtype plants produced normal

and healthy seeds, while the antisense and BS2 transgenic plants generated a large proportion of aborted seeds. Closer examination indicated that before or at the heart stage of embryo development, no differences in seed shape and color were observed in transgenic siliques. However, after the heart or torpedo stage, the difference was apparent in the transgenic siliques. Some seeds contained green and intact embryos that were easily distinguished from other parts of seeds, whereas the rest of seed appeared empty. After entering the mature cotyledon stage of development, the normal and aborted seeds could be easily distinguished. The normal seeds appeared healthy like those in the wildtype siliques, while the other seeds were much smaller. These observations strongly suggest that the arrested seed development occurred after the heart or torpedo stage of embryo development. This result is consistent with our speculation that the peptide transporter may be involved in the import of nitrogen into the embryo which occurs during the maturation stage of seed development (Patton et al., 1990). The reduction in the level of the AtPTR2-B transporter as a result of antisense expression might affect the import of the nitrogenous nutrients into seeds, thus arresting normal seed development.

Our previous studies showed that toxic peptides, such as Ala-Eth and Leu-Eth, effectively inhibited root growth and this inhibition could not be reversed by addition of amino acids (Steiner et al., 1994), suggesting that the plant roots must have a peptide transport system responsible for this uptake. To determine if this uptake is mediated by the AtPTR2-B transporter, root inhibition assays were conducted using the transgenic plants. The results showed that the transgenic plants remained sensitive to toxic peptides even when the

minimal concentration for inhibition was applied (data not shown). These results could be interpreted to suggest that toxic peptide uptake by roots may be not mediated by the *AtPTR2-B* transporter. However, it was found that the endogenous level of *AtPTR2-B* mRNA was little affected in the antisense transgenic lines. Therefore, the transporter mediating toxic peptide uptake in roots remains to be identified. As described previously, most of transgenic shoots failed to producing roots when grown in the root-inducing medium. This might be due to the improper condition for rooting. However, it didn't rule out the possibility that reduced expression of *AtPTR2-B* resulted in failure to rooting since the survived transgenic plants didn't show the reduction in endogenous *AtPTR2-B* mRNA levels. Although transgene expression did not affect endogenous *AtPTR2-B* mRNA levels in roots, transgenic plants produced significantly more lateral roots and root hairs along the length of the primary roots than the wildtype plants. It is thought that the lateral roots and root hairs play a role in the absorption of water and nutrients by increasing the root surface area (Bowman, 1994). Thus, this phenotypic change in the transgenic roots could be viewed as an adaptive response to a shortage of nutrients due to the reduced transport of nitrogenous sources in the upper parts of the plant as a result of decreased *AtPTR2-B* expression.

The initiation of flowering is a complex process that is regulated by a combination of developmental programs and responses to enviromental signals (Lang, 1965; Vince-Prue, 1975). The transition to flowering in *Arabidopsis* is promoted by long-day photoperiods and vernalization, and also affected by growth temperature, nutrient availability, and light quality (Bowman, 1994). Analysis of various mutants affecting flowering indicates that the transition

to flowering may be promoted by more than one pathway (Koornneef et al., 1991). Surprisingly, our transgenic plants exhibited a typical late flowering phenotype followed by strong leaf growth. This change correlated with the reduced *AtPTR2-B* expression. The delayed flowering seen in the transgenic plants may be simply due to the nutrient defect caused by reduced peptide transport activity. However, we can't exclude the possibility that *AtPTR2-B*-mediated transport may involve a pathway that regulates the transition to flowering. Further experiments need to be conducted to explore this phenomenon. For example, the change of photoperiod to test if late-flowering transgenic plants restore the wildtype phenotypes.

Taken together, the reduced *AtPTR2-B* expression in *Arabidopsis thaliana* resulted in significant effects on plant growth and development, suggesting that *AtPTR2-B* plays a general role in plant nutrition. To further elucidate the role of *AtPTR2-B* in plants, more experiments need to be conducted, such as localization of the *AtPTR2-B* transporter in plants. These results will provide important information regarding the physiological function of *AtPTR2-B*. For example, if *AtPTR2-B* was found to be localized in the plasma membrane of the vascular system (phloem or xylem), that would strongly suggest that *AtPTR2-B* may be involved in distributing peptide-like compounds throughout the plant and may balance the import and/or export of these compounds among the different organs of the plant.

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