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An Arabidopsis Peptide Transporter Is a Member of a New Class of Membrane Transport Proteins

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An Arabidopsis peptide transport gene was cloned from an Arabidopsis cDNA library by functionally complementing a yeast peptide transport mutant. The Arabidopsis plant peptide transporter (AtPTR2) allowed growth of yeast cells on dipeptides and tripeptides but not peptides four residues and higher. The plant peptide transporter also conferred sensitivity to a number of ethionine-containing, toxic peptides of chain length three or less and restored the ability to take up radiolabeled dileucine at levels similar to that of the wild type. Dileucine uptake was reduced by the addition of a variety of growth-promoting peptides. The sequence of a cDNA insert of 2.8 kb indicated an open reading frame encoding a 610-amino acid polypeptide (67.5 kD). Hydropathy analysis predicted a highly hydrophobic protein with a number of potential transmembrane segments. At the amino acid level, the Arabidopsis plant peptide transporter shows 24.6, 28.5, and 45.2% identity to the Arabidopsis nitrate-inducible nitrate transporter (CHL1), the rabbit small intestine oligopeptide transporter (PepT1), and the yeast peptide transporter (Ptr2p), respectively, but little identity to other proteins known to be involved in peptide transport. Root growth of Arabidopsis seedlings exposed to ethionine-containing toxic peptides was inhibited, and growth was restored by the addition of certain peptides shown to compete with dileucine uptake in yeast expressing the Arabidopsis transport gene. Consistent with the observed inhibition of root growth by toxic peptides, the peptide transporter is expressed in the roots of Arabidopsis seedlings. This study represents the characterization of a plant peptide transporter that is a member of a new class of related membrane transport proteins.

INTRODUCTION

Peptide transport systems mediating the uptake of intact peptides distinct from amino acid uptake are present in bacteria, fungi, plants, and mammalian tissues (for reviews, see Becker and Naider, 1980; Higgins and Payne, 1980; Matthews and Payne, 1980; Payne, 1980; Naider and Becker, 1987). Peptide transport is a specific biochemical process in which small peptides (≤6 amino acids) are transported across a membrane by energy-dependent, saturable carriers. A large number of genes that encode components of oligopeptide transport systems in bacteria have been cloned and sequenced, whereas only two eukaryotic peptide transport genes have been cloned and sequenced (Fei et al., 1994; Perry et al., 1994).

In the yeast system, peptides are transported with little amino acid side chain specificity but with strong stereospecificity for L-amino acids. At least three genes (*PTR1*, *PTR2*, and *PTR3*) are known to be involved in this process (Island et al., 1991). Yeast *PTR1* and *PTR2* mutants are completely peptide-transport deficient as defined by their resistance to toxic dipeptides and lack of uptake of radiolabeled peptide substrates (Island et al., 1991). *PTR2* encodes a 601-amino acid residue polypeptide (Ptr2p) with 12 putative hydrophobic segments, and it demonstrates all the previously identified characteristics of peptide transport in yeast (Perry et al., 1994). A data base search indicated the yeast peptide transporter may be the second protein discovered in a new class of membrane-bound transport proteins (Tsay et al., 1993; Perry et al., 1994).

The function of peptide transport is primarily for utilization of peptides as sources of nitrogen and carbon (Becker and Naider, 1980; Matthews and Payne, 1980; Payne, 1980; Adibi, 1987). However, a few reports have shown peptide transport to be involved in bacterial sporulation (Mathiopoulos et al., 1991; Perego et al., 1991), chemotaxis in bacteria (Manson et al., 1986), and recycling of bacterial cell wall peptides (Goodell and Higgins, 1987). In plants, peptide transport has been demonstrated in isolated scutella from germinating grains of barley, wheat, rice, and maize and postulated to be involved in supplying amino acids, stored in the form of peptides, from the endosperm to the germinating embryo (Higgins and Payne, 1978a; Sopanen et al., 1978; Salmenkallio and Sopanen, 1989). Peptide transport in germinating barley seeds has been demonstrated with nonhydrolyzable, nonphysiological peptide substrates, which were accumulated intact and against a concentration gradient (Burston et al., 1977; Higgins and Payne,

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1977a, 1977b; Sopanen et al., 1977). The transport system exhibits saturation kinetics and is inhibited by a range of metabolic inhibitors (Higgins and Payne, 1977b). A wide variety of peptides has also been shown to be transported intact into the barley embryo and subsequently hydrolyzed intracellularly (Higgins and Payne, 1978a, 1978c). The plant peptide transport system can transport both dipeptides and tripeptides (Sopanen et al., 1977; Higgins and Payne, 1978b). Two proteins of ~66 and 41 kD were initially identified by thiolaffinity labeling as components of the plant peptide transport system in barley grains (Payne and Walker-Smith, 1987). Subsequently, an additional or similar protein of 54 kD was identified by photoaffinity labeling (Hardy and Payne, 1991).

Here, we report the isolation and characterization of a plant peptide transport gene cloned by functional complementation of the yeast *ptr2* mutation. Comparison of DNA sequences indicated that the Arabidopsis nitrate transporter, and the yeast, Arabidopsis, and rabbit small intestine peptide transporters belong to a new family of eukaryotic transport proteins that are distinct from their prokaryotic counterparts.

RESULTS

Complementation of Peptide Uptake

Yeast 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. To isolate a plant gene that encodes a peptide transporter, strains PB1X-9B and PB1X-2AΔ were transformed with an Arabidopsis cDNA library, and the transformants were selected on minimal medium containing the dipeptides His-Leu and Lys-Leu. A total of 21 primary transformants were recovered (15 from PB1X-9B and six from PB1X-2AΔ). The plasmids isolated from the transformants were reintroduced back into their respective mutant strains and plated on dipeptide medium. Two transformants each from PB1X-9B (designated PTF3 and PTF4) and PB1X-2AΔ (designated DTF1 and DTF2) were able to restore both growth on peptides and peptide transport to the mutants, whereas the other 17 plasmids recovered from the primary screen did not restore growth on peptides when reintroduced into their respective mutants. NotI restriction digest of plasmids pDTF1 and pDTF2 showed a 2.0-kb insert, whereas plasmids pPTF3 and pPTF4 contained a 2.8-kb insert. PB1X-2AΔ transformed with pDTF1 or pDTF2 did not show sensitivity to toxic peptides, whereas PB1X-2AΔ transformed with pPTF3 or pPTF4 displayed sensitivity to most toxic peptides tested.

An α-^{32}P-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 or pDTF2 plasmid DNA, indicating that the plasmids isolated from the two different sets of clones did not contain identical inserts. Because the DTF plasmids were not able to restore sensitivity to toxic peptides and pPTF3 demonstrated a vector band twice the expected vector size, these cDNAs were not characterized further. DNA gel blot hybridization to EcoRV-digested Arabidopsis genomic DNA using the 2.8-kb NotI insert of pPTF4 showed one strong hybridization signal around 5.4-kb plus two weaker hybridization bands under high-stringency conditions (data not shown). The 2.8-kb insert from pPTF4 was selected for sequencing and further analysis.

Sequence Analysis of pPTF4

DNA sequence analysis of the insert isolated from pPTF4 showed an 1830-bp open reading frame within the 2799-bp insert. The open reading frame encoded a polypeptide of 610 amino acids (67,510 D). Hydropathy analysis (Kyte and Doolittle, 1982) showed the predicted protein of Arabidopsis, AtPTR2, to be highly hydrophobic with a number of hydrophobic segments that are candidates for transmembrane domains (Figure 1). A precise model of transmembrane domain topology will require further biochemical evidence. A search of the protein sequence data base using the NCBI BLAST algorithm (Altschul et al., 1990) revealed homologies to the yeast peptide transporter Ptr2p (probability high score 654, 52% identity, 68% similarity), the rabbit small intestinal peptide transporter PepT1 (probability high score 140, 29% identity, 53% similarity), and the Arabidopsis nitrate transporter CHL1 (probability high score 82, 36% identity, 47% similarity). All other probability scores were less than 50.

Sequence Comparison Analysis

Protein sequences AtPTR2, Ptr2p, PepT1, and CHL1 were aligned with the Genetics Computer Group (GCG; University of Wisconsin, Madison) program Pileup, and a consensus

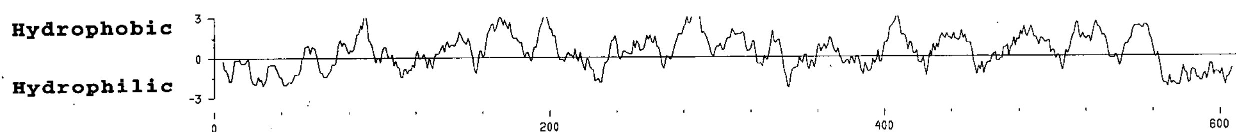

Figure 1. Hydropathy Plot of AtPTR2.

A plot of the hydrophilic and hydrophobic moments as predicted by Kyte and Doolittle (1982) for the entire AtPTR2 amino acid sequence is presented. Positive numbers indicate hydrophobicity.

sequence was generated with the GCG program Pretty from the pairwise alignment generated with Pileup (Figure 2). The consensus sequence was most conserved in the first and last third of the proteins with a 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.

Disc Assay of Ethionine-Containing Peptides

It was shown previously that the peptide transport mutant PB1X-9B is resistant to peptides containing the toxic amino acid analog ethionine (Eth) (Island et al., 1991), whereas the peptide transport–proficient strain PB1X-2A (*his⁻ leu⁻ lys⁻ PTR2⁺*) was sensitive to dipeptides and tripeptides containing this amino acid analog. Transformation of PB1X-9B with pPTF4 harboring the plant peptide transport gene restored sensitivity to the toxic dipeptides alanyl-ethionine (Ala-Eth), Leu-Eth, leucine-*m*-fluorophenylalanine (Leu-f-Phe), Lys-Ala-Eth, and Lys-Leu-Eth (Table 1). None of the strains tested was sensitive to Lys-Leu-Ala-Eth, Lys-Leu-Leu-Ala-Eth, Lys-Leu-Leu-Eth, or Lys-Leu-Leu-Leu-Eth. Both the yeast and plant peptide transporters expressed in yeast showed similar patterns of sensitivity to toxic peptides.

Peptide Utilization in pPTF4 Transformants

A number of peptides varying in both composition and length were examined for their ability to support growth of the PB1X-9B transformants (Table 2). The mutant PB1X-9B showed no growth on any of the peptides, whereas PB1X-9B transformed with the yeast peptide transport gene showed growth on all peptides except tri-Lys, tetra-Lys, poly-Lys, and Met-Met-Leu. Yeast transformants containing *AtPTR2* showed a similar pattern to that containing *PTR2*, except that these transformants were able to utilize Met-Met-Leu. The peptide transport–proficient yeast strain PB1X-2A does not utilize Met-Met-Leu, indicating that the uptake of this peptide is a result of specificity conferred by the *AtPTR2* gene (Table 2). None of the strains was able to utilize peptides longer than three residues. The results indicated that *AtPTR2* confers transport of both dipeptides and tripeptides with a specificity similar to that of *PTR2*, with the exception of one peptide tested (Met-Met-Leu).

Radiolabeled Dileucine Uptake in PB1X-9B

pPTF4 transformed into the yeast peptide transport mutant PB1X-9B restored ^{3}H-dileucine uptake to wild-type levels (Figure 3). Uptake of the radiolabeled substrate could be inhibited with 100-fold cold dileucine, whereas 100-fold cold leucine had no effect on the uptake rate of radiolabeled dileucine (data not shown), indicating peptide uptake is not via an amino acid

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   AtPTR2  ......MSSI EEQITKSDSD FIISEDQSYL SKEKKADGSA TINQADEQSS   44
    Ptr2p  MLNHPSQGSD DAQDEK.QGD FPVIEEE... .KTQAVTLKD SYVTDDVANS   45
    PepT1  .......... .......... .......... .......... ..........
     CHL1  .......... .......... .......... .......... ..........
Consensus  --------S- --Q--K---D F---E----- -K-------- -----D---S

   AtPTR2  TDELQKSMST GVLVNGDLYP SPTEEELATL PSVCG.TIPW KAFIIIIVEL   93
    Ptr2p  TERYNLSPSP ....EDEDFE GPTEEEMQTL RHVGG.KIPM RCWLIAIVEL   90
    PepT1  .......... .......... ......MGMS KSLSCFGYPL SIFFIVVNEF   24
     CHL1  .MSLPETKSD DILLDAWDFQ GRPADRSKTG GWASAA.... ...MILCIEA   42
Consensus  T--L--S-S- --L----DF- GPTEEEM-TL -SVSG--IP- --F-I-IVEL

   AtPTR2  CERFAYYGLT VPFQNYMQFG PKDATPGALN LGETGADGLS NFFTFWCYVT  143
    Ptr2p  SERFSYYGLS APFQNYMEYG PNDSPKGVLS LNSQGATGLS YFFQFWCYVT  140
    PepT1  CERFSYYGMR ALLILYF... .....RNFIG WDDNLSTVIY HTFVALCYLT   66
     CHL1  VERLTTLGIG VNLVTYL... .....TGTMH LGNATAANTV TNFLGTSFML   84
Consensus  CERFSYYGL- -P--NYM--G P-D---G-L- LG--GATGLS -FF-FWCY-T

   AtPTR2  PVGAALIADQ FLGRYNTIVC SAVIYFIGIL ILTCTAI... ...PSVIDAG  187
    Ptr2p  PVFGGYVADT FWGKYNTICC GTAIYIAGIF ILFITSI... ...PSVGNRD  184
    PepT1  PILGALIADA WLGKFKTIVW LSIVYTIGQA VTSLSSV... ...NELTDNN  110
     CHL1  CLLGGFIADT FLGRYLTIAI FAAIQATGVS ILTLSTIIPG LRPPRCNPTT  134
Consensus  PVLG-LIADT FLG-YNTIVC -A-IY-IGI- ILTL-SI--- ---PSV-D--

   AtPTR2  KS........ .MGGFVVSLI IIGLGTGGIK SNVSPLMAEQ LPKIPPYVKT  228
    Ptr2p  SA........ .IGGFIAAII LIGIATGMIK ANLSVLIADQ LPKRKPSIKV  225
    PepT1  HDGTPDSLPV HVAVCMIGLL LIALGTGGIK PCVSAFGGDQ FE........  152
     CHL1  SSHCEQASGI QLTVLYLALY LTALGTGGVK ASVSGFGSDQ FDETEP....  180
Consensus  SS-------- --G-F--ALI LI-LGTGGIK ANVS--GADQ --K--P--K-

   AtPTR2  KKNGSKVIVD PVVTTSRAYM IFYWTINVGS LSVLATTSL. ........ES  269
    Ptr2p  LKSGERVIVD SNITLQNVFM FFYFMINVGS LSLMATTEL. ........EY  266
    PepT1  .........E GQEQRNRFFS IFYLAINAGS LLSTIITPMV RVQQCGIHVK  193
     CHL1  .........K ERSKMTYFFN RFFFCINVGS L..LAVTVLV YVQD......  213
Consensus  -K-G--VIVD ---T--RFFM IFYF-INVGS LS-LA-T-LV -VQ-----E-

   AtPTR2  TKGFVYAYLL PLCVFVIPLI ILAVSKTAF. .TSTLLPPVP SLFVLVKCSS  317
    Ptr2p  HKGFWAAYLL PFCFFWIAVV TLIFGKKQY. ....IQRPIG D...KVIAKS  308
    PepT1  QACYPLAFGI PAILMAVSLI VFIIGSGMY. ..KKFKPQGN ILSKVVKCIC  240
     CHL1  DVGRKWGYGI CAFAIVLALS VFLAGTNRYR FKKLIGSPMT QVAAVIVAAW  263
Consensus  -KGF--AY-- P-C-FVIALI V-I-GK--Y- --K---PP-- -L--VV---S

   AtPTR2  LLLKTNLISK KL......N. HLALLLLERY VKDQWDDLF. ..........  349
    Ptr2p  FKVCWILTKN KF......DF NAAKPSVHPE KNYPWNDKF. ..........  341
    PepT1  FAIKNRFRHR SK......QF PKRAHWLDW. AKEKYDERL. ..........  272
     CHL1  RNRKLELPAD PSYLYDVDDI IAAEGSMKGK QKLPHTEQFR SLDKAAIRDQ  313
Consensus  F--K--L--- K-------DF -AA--SL--- -K-PWD--F- ----------

   AtPTR2  .......... .........I DELKRALRAC KTFLFYPIYW VCYGQMTNNL  380
    Ptr2p  .......... .........V DEIKRALAAC KVFIFYPIYW TQYGTMISSF  372
    PepT1  .......... .........I AQIKMVTRVL FLYIPLPMFW ALFDQQGSRW  303
     CHL1  EAGVTSNVFN KWTLSTLTDV EEVKQIVRML PIWATCILFW TVHAQLTTLS  363
Consensus  ---------- ---------- DEIKR-LRA- K-FIFYP--W T-YGQMTS--

   AtPTR2  ISQA....GQ MQTGNVSNDL FQAFDSIALI IFIPICDNII YPLLRK...Y  423
    Ptr2p  ITQA....SM MELHGIPNDF LQAFDSIALI IFIPIFEKFV YPFIRR...Y  415
    PepT1  TLQATTMSGR IGILEIQPDQ MQTVNTILII ILVPIMDAVV YPLIAK...C  350
     CHL1  VAQSETLDRS IGSFEIPPAS MAVFYVGGLL LTTAVYDRVA IRLCKKLFNY  413
Consensus  I-QA-T--G- -G--EIP-D- MQAFDSIALI IFIPI-D-VV YPLIRK---Y

   AtPTR2  NIPFKPILRI TLGFMFATAS MIYAAVLQ.. .......... ..........  451
    Ptr2p  .TPLKPITKI FFGFMFGSFA MTWAAVLQ.. .......... ..........  442
    PepT1  GLNFTSLKKM TIGMFLASMA FVAAAILQVE IDKTLPVFPK ANEVQIKVLN  400
     CHL1  PHGLRPLQRI GLGLFFGSMA MAVAALVELK RLRTAHAHG. ..........  452
Consensus  --P-KP---I TLGF-F-SMA M--AAVLQ-- ---T------ ----------

   AtPTR2  .......... .......... .......... .......... ..........
    Ptr2p  .......... .......... .......... .......... ..........
    PepT1  VGSENMIISL PGQTVTLNQM SQTNEFMTFN EDTLTSINIT SGSQVTMITP  450
     CHL1  .......... .......... .......... .......... ..........
Consensus  ---------- ---------- ---------- ---------- ----------

   AtPTR2  .......... .......... .......... .......... ..........
    Ptr2p  .......... .......... .......... .......... ..........
    PepT1  SLEAGQRHTL LVWAPNNYRV VNDGLTQKSD KGENGIRFVN TYSQPINVTM  500
     CHL1  .......... .......... .......... .......... ..........
Consensus  ---------- ---------- ---------- ---------- ----------

   AtPTR2  .AKIYQRGPC YANF...... .......... .......... ..........  464
    Ptr2p  .SFVYKAGPW YNEP...... .......... .......... ..........  455
    PepT1  SGKVYEHIAS YNASEYQFFT SGVKGFTVSS AGISEQCRRD FESPYLEFGS  550
     CHL1  .......... .......... .......... .......... ..........
Consensus  --KVY--GP- YN-------- ---------- ---------- ----------

   AtPTR2  .......... ........TD TCVSNDISVW IQIPAYVLIA FSEIFASITG  496
    Ptr2p  .......... ........LG HNTPNHVHVC WQIPAYVLIS FSEIFASITG  487
    PepT1  AYTYLITSQA TGCPQVTEFE DIPPNTMNMA WQIPQYFLIT SGEVVFSITG  600
     CHL1  .......... .........P TVKTLPLGFY LLIPQYLIVG IGEALIYTGQ  483
Consensus  ---------- ---------- T--PN---V- WQIP-Y-LI- F-EIFASITG

   AtPTR2  LEFAFTKAPP SMKSIITALF LFTNAFGAIL SICISSTAV. ..........  535
    Ptr2p  LEYAYSKAPA SMKSFIMSIF LLTNAFGSAI GCALSPVTV. ..........  526
    PepT1  LEFSYSQAPS NMKSVLQAGW LLTVAVGNII VLIVAGAGQ. ..........  639
     CHL1  LDFFLRECPK GMKGMSTGLL LSTLALGFFF SSVLVTIVEK FTGKAHPWIA  533
Consensus  LEFAYSKAP- SMKS-ITALF LLTNAFG-I- S---S---V- ----------

   AtPTR2  ....NPKLTW MYTGIAVTAF IAGIMFWVCF HHYDAMEDEQ NQLEFKRNDA  581
    Ptr2p  ....DPKFTW LFTGLAVACF ISGCLFWLCF RKYNDTEEEM NAMDYEEEDE  572
    PepT1  ....INKQWA EYILFAALLL VVCVIFAIMA RFYTYVNPAE IEAQFE.EDE  684
     CHL1  DDLNKGRLYN FYWLVAVLVA LNFLIFLVFS KWYVYKEKRL AEVGIELDDE  583
Consensus  -----PKL-W -YT--AVL-F I-G--FWVCF R-Y-Y-E-E- NE--FE-EDE

   AtPTR2  LTKKDVEKEV HDSYSMADES QYNLEKANC                        610
    Ptr2p  FDLNPISAPK ANDIEILEPM ESLRSTTKY                        601
    PepT1  KKKNPEKNDL YPSLAPVSQT QM                               707
     CHL1  PSIPMGH                                                590
Consensus  --KNP----- --S------- Q---------
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Figure 2. Alignment of AtPTR2, Ptr2p, PepT1, and CHL1 Protein Sequences and the Derived Consensus Sequence.

Identities are shown in the consensus sequence where two or more identical amino acids are considered a consensus. Dots indicate gaps in the amino acid sequences used to optimally align the sequences. Dashes indicate the lack of consensus among the amino acid sequences.

Table 1. Effect of Toxic Peptides on Transport Proficient, Mutant, and Transformed Yeast Strains[a]

	Diameter (mm) of Zone of Inhibition			
Toxic Peptide	PB1X-2A	PB1X-9B	PB1X-9B(pJP9)	PB1X-9B(pPTF4)
Eth	39.0 ± 1.0	44.5 ± 3.5	46.5 ± 1.5	46.5 ± 0.5
f-Phe	24.0 ± 2.0[b]	14.0 ± 1.0	20.0 ± 0.0	19.5 ± 0.5
Leu-Eth	38.0 ± 1.0	0	47.0 ± 1.0	45.0 ± 1.0
Ala-Eth	35.5 ± 0.0	0	42.5 ± 2.5	35.0 ± 1.0
Leu-f-Phe	31.0 ± 1.0[c]	0	20.5 ± 3.0[c]	37.0 ± 1.0
Lys-Ala-Eth	31.0 ± 0.0	0	36.0 ± 1.5	37.5 ± 1.5[b]
Lys-Leu-Ala-Eth	0	0	0	0
Lys-Leu-Leu-Ala-Eth	0	0	0	0
Lys-Leu-Eth	29.0 ± 1.0	0	36.0 ± 0.5	35.0 ± 1.0
Lys-Leu-Leu-Eth	0	0	0	0
Lys-Leu-Leu-Leu-Eth	0	0	0	0

[a] Values are given as mean ± SE of the mean of at least two independent experiments.
[b] Broad border around halo.
[c] Hazy background in halo.

transporter. Uptake rates conferred by the plant peptide transporter were consistent with uptake rates of PB1X-9B transformed with the yeast peptide transporter (Perry et al., 1994). Competition with uptake of ^{3}H-dileucine similar to that observed by Leu-Leu was seen for Ala-Ala, Ala-Ala-Ala, Ala-Met, Met-Met-Leu, Met-Met, and Leu-Phe in PB1X-9B transformed with pPTF4.

Germination Assay

Because it was not possible to isolate intact scutella from seeds as small as Arabidopsis (<1 mm), such as was done with barley grains, 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. In every case, the seeds were able to germinate and grow a root that had clearly penetrated the seed coat. The roots were able to grow 1 to 3 mm before growth was inhibited (data not shown). The addition of competitor peptides Ala-Met and Leu-Met to Ala-Eth and Leu-Eth, respectively, reversed the inhibition of root growth that resulted in the growth of seedlings comparable to the no-peptide controls. Germinating seeds in the presence of oxalysine and oxalysine-containing peptides, whose mechanism of inhibition is different from that of ethionine, showed growth patterns identical to seeds germinated with ethionine and ethionine-containing peptides (data not shown). Adding the amino acids leucine, alanine, and methionine as individual competitors for Leu-Eth or Ala-Eth had no effect on the inhibition of the toxic peptides. The fact that the seeds are able to germinate and initiate root growth in the presence of the toxic peptide suggests that expression of the peptide transporter occurs after germination is complete in Arabidopsis. The ability of nontoxic peptides to compete and reverse this growth inhibition indicates that a peptide transport system is present and functioning early in Arabidopsis root development.

Root Assay

To further assess whether Arabidopsis roots are sensitive to toxic peptides, 4-day-old seedlings were exposed to peptides

Table 2. Peptide Growth Assay

	Yeast Strain/Transformant			
Peptides	PB1X-2A[a]	PB1X-9B[b]	PB1X-9B(pJP9)[c]	PB1X-9B(pPTF4)[d]
Lys-Leu	+[e]	–	+	+
His-Leu	+	–	+	+
His-Lys	+	–	+	+
Lys-Lys	+	–	+	+
Lys-Lys-Lys	–	–	–	–
Lys-Lys-Lys-Lys	–	–	–	–
Poly-Lys	–	–	–	–
Leu-Leu	+	–	+	+
Leu-Leu-Leu	+	–	+	+
Ala-Leu	+	–	+	+
Ala-Ala-Leu	+	–	+	+
Ala-Lys	+	–	+	+
Lys-Ala-Ala	+	–	+	+
Met-Met-Leu	–	–	–	+
Gly-Leu-Gly-Leu	–	–	–	–
Thr-Pro-Arg-Lys	–	–	–	–
Tyr-Gly-Gly-Phe-Leu	–	–	–	–

[a] (*his⁻ leu⁻ lys⁻ PTR2⁺*).
[b] (*his⁻ leu⁻ lys⁻ ptr2⁻*).
[c] (*PTR2⁺*).
[d] (*AtPTR2⁺*).
[e] –, no growth; +, growth.

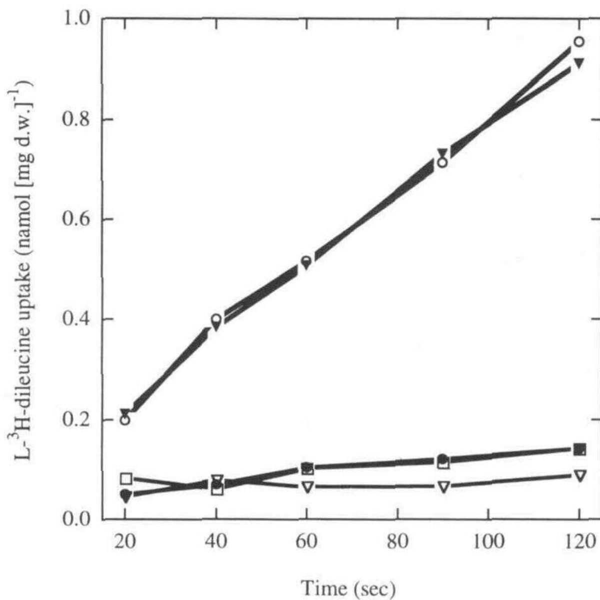

Figure 3. Radiolabeled Dileucine Transport by AtPTR2.

Uptake of L-^{3}H-dileucine (30 μM, specific activity 10 mCi/mmol) by yeast strains S288C, PB1X-9B, and PB1X-9B transformed with pPTF4 is expressed as nanomoles per milligram dry weight of tissue. The competitor is 100-fold cold dileucine. (○), PB1X-2A; (●), PB1X-2A plus dileucine; (▽), PB1X-9B; (▼), PB1X-9B(pPTF4); (□), PB1X-9B(pPTF4) plus dileucine. Standard error for data from three independent experiments was ≤9% of the mean.

containing toxic amino acids. In this assay, toxicity is equivalent to growth inhibition. Figures 4A and 4B show the inhibitory effects of Leu-Eth and Ala-Eth 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 (Figures 4D and 4E). Met-Met was able to reverse the toxicity of both Leu-Eth and Ala-Eth, although less effectively (data not shown). 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.

Expression of AtPTR2 in Arabidopsis Plants

RNA gel blot hybridization of the 2.8-kb insert from pPTF4 to total RNA from Arabidopsis seedlings failed to detect a signal, suggesting that *AtPTR2* is expressed at a very low level only in specific tissues or is highly regulated. Therefore, the presence of the *AtPTR2* mRNA was measured after first amplifying the transcript using a reverse transcriptase–polymerase chain reaction (RT–PCR). Figure 5 shows that the expression of *AtPTR2* occurs in the roots of young Arabidopsis seedlings and is consistent with the results of the germination and root assays. The probe also hybridized to a second band in both total root RNA and genomic DNA, indicating a possible second

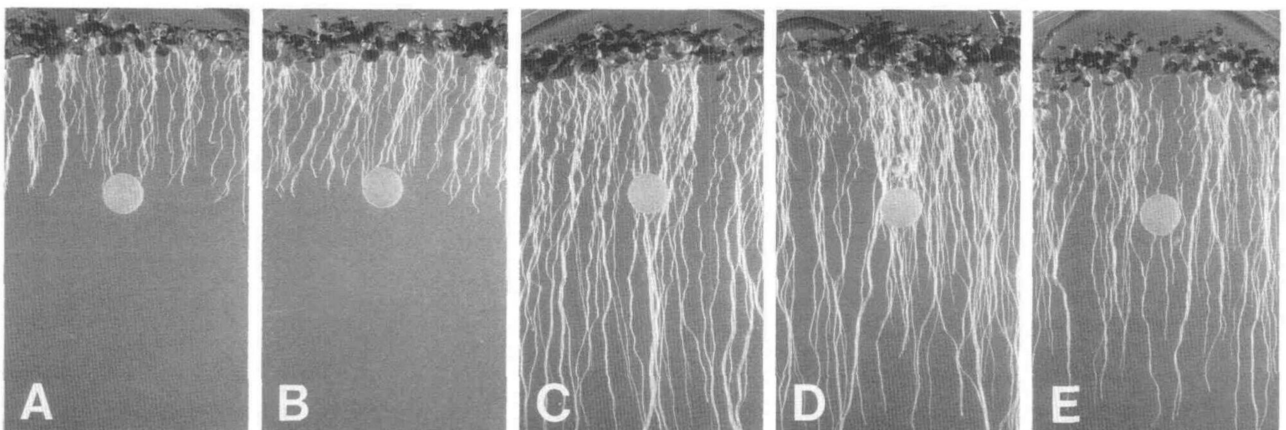

Figure 4. Inhibition of Arabidopsis Seedling Root Growth by Ethionine-Containing Peptides.

Arabidopsis seeds were sown on solid Arabidopsis growth media (see Methods). After 4 days growth, filter discs were placed on the agar surface, and 5 μL of an aqueous solution of dipeptides was applied to the discs. Roots are shown 6 days after the addition of peptides.

(A) Leu-Eth (0.19 μM).

(B) Ala-Eth (0.19 μM).

(C) Control (H_2O).

(D) Leu-Eth (0.19 μM) plus Leu-Met (4.00 μM).

(E) Ala-Eth (0.19 μM) plus Ala-Met (4.00 μM).

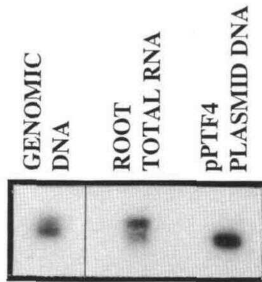

Figure 5. Expression of *AtPTR2*.

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

gene that cross-hybridizes with the 3′ specific *AtPTR2* probe. Because the probe is hybridizing to PCR-generated products, it is not possible to determine a size for the mRNA transcript.

DISCUSSION

As a physiological process, peptide uptake 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 recently 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). The method allows rapid identification of plant genes functionally homologous to yeast genes in well-characterized yeast mutants. Characterization of the plant peptide transport system reveals striking similarities to that of yeast on the physiological as well as the sequence level.

In general, the yeast peptide transport mutant (*ptr2*) transformed with the Arabidopsis peptide transport cDNA (*AtPTR2*) is able to grow on dipeptide media and shows sensitivity and specificity to toxic peptides similar to the yeast peptide transport gene (*PTR2*). *AtPTR2* expressed in the yeast *ptr2* mutant endows the cell with the ability to take up radiolabeled dileucine to a level comparable to that of the wild type.

The yeast peptide transport mutant transformed with *AtPTR2* is not sensitive to any toxic amino acid–containing peptide with a chain length of four residues or longer. This finding is identical to that observed with the yeast peptide transport gene. The specificity conferred by *AtPTR2* is most likely a result of a chain length limitation because the tripeptides Lys-Ala-Eth and Lys-Leu-Eth were toxic to both the wild type and transformants, whereas the longer peptides of similar composition were not toxic to the cells (Table 1). 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 PB1X-9B transformed with either *AtPTR2* 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 systems take up and utilize 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 3 and Results). This also supports earlier work on germinating barley seeds, which showed a strong bias toward transport of peptides composed in part or entirely 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 (see Results).

Nontoxic peptides tested for their ability to compete with the ethionine-containing peptides showed differences in their ability to compete in the root versus the radiolabeled uptake assays. Ala-Ala and Ala-Ala-Ala did not reverse the growth inhibition of Ala-Eth or Leu-Eth in root assays; this result was unexpected considering that these peptides competed very effectively with radiolabeled dileucine in the uptake assay. However, this may reflect a difference in expression of *AtPTR2* and its functional product in its native genetic background versus that in yeast. There may also be considerable differences in levels of competitor necessary to achieve strong reversal of the toxicity in the seedling assay versus the radiolabeled uptake assay as a result of the different time courses of the assays. Moreover, this difference in competitor specificity may indicate that other factors or components are present in roots versus yeast; these may determine to a greater degree the specificity of which peptides will be transported in plants. Surprisingly, methionine did not reverse the growth inhibition of ethionine or ethionine-containing peptides. The effect of ethionine in this in vivo assay may be very potent to 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.

Arabidopsis seedlings germinated under defined growth conditions expressed the AtPTR2 peptide transporter in the roots

(Figure 5). In addition, a second hybridization signal, which showed cross-hybridization to a 3′ specific probe, was present and may be an additional, related gene, whose presence would be consistent with previous reports identifying at least two and possibly three proteins associated with peptide transport in barley grains (Payne and Walker-Smith, 1987; Hardy and Payne, 1991).

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 4). This inhibition did not appear to affect other parts of the plant because the leaves looked comparable to the unaffected controls. Seeds germinated in the presence of peptides containing ethionine and oxalysine as well as ethionine and oxalysine themselves also demonstrated sensitivity to these toxic compounds. The seeds were able to germinate and grow a root 1 to 3 mm in length after which root growth was inhibited. In addition, no *AtPTR2* message could be detected in germinating 2-day-old imbibed seeds (data not shown). This suggests that the inhibitory effect of these toxic peptides occurs after germination of the seed and growth of the root primordia. It also indicates that roots are unable to transport peptides immediately upon imbibition and germination; transport occurs later, possibly after the emergence of the root primordia. Thus, inhibition by the toxic peptides may be developmentally dependent and occur after the radicle emerges from the seed, RNA synthesis begins, and the peptide transport system is expressed.

The Arabidopsis peptide transporter exhibits transport characteristics similar to that of the yeast peptide transporter, including transport of peptides with similar chain length and composition as well as inhibition of transport by various competing peptide substrates. Sequence comparison between these two proteins also shows considerable similarity at the amino acid level. Adding the CHL1 and the rabbit PepT1 sequences to the comparison produced a consensus sequence that shows similarity throughout the length of these proteins (Figure 2). As a group, AtPTR2, Ptr2p, PepT1, and CHL1 are distinct from other groups of proteins from bacteria, yeast, mammals, and plants: these 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 (H.-Y. Steiner and J.M. Becker, unpublished results).

The function(s) of peptide transport in plants in other than a nutritional role, as postulated for barley grains, is not known, although a wide range of peptides and peptide compounds are known to occur in plants (Higgins and Payne, 1980, 1982; Steffens, 1990). Identification of *AtPTR2* and other peptide transport genes can yield substantial information regarding the role that each of these peptide compounds might play in plants. In addition, the effect of toxic peptides on roots of Arabidopsis seedlings clearly indicates that peptide transport may have functions in plants other than delivery of peptides from the endosperm to the embryo. Isolation of a plant peptide transport gene will make it possible to exploit this system for delivery of toxic or growth-promoting substances to plants in a manner analogous to that postulated for human pathogens (Fickel and Gilvarg, 1973; Higgins, 1987; Becker and Naider, 1994). Indeed, a number of plant pathogens secrete toxins in the form of peptides (Walton, 1990), and it is conceivable that modifying the peptide transport system of important crop species could provide the necessary resistance to these pathogens.

METHODS

Bacteria and Yeast Strains

The *Escherichia coli* strain used was DH5α (*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*). The *Saccharomyces cerevisiae* strains used were S288C (*MATα SUC2 mal mel gal2, CUP1*), PB1X-9B (*MATa ura3-52 leu2-3,112 lys1-1 his4-38 ptr2-2*), PB1X-2A (*MATa ura3-52 leu2-3,112 lys1-1 his4-38 PTR2*), and PB1X-2AΔ (*MATa ura3-52 leu2-3,112 lys1-1 his4-38 PTR2::LEU2*). All yeast strains, except PB1X-2AΔ, were constructed using standard methods of yeast genetics (Sherman et al., 1986; Perry et al., 1994). PB1X-9B and PB1X-2A are segregants from sporulated diploids derived from a cross between mutagenized strain S288C and strain X2180-1A (Island et al., 1991). PB1X-2AΔ was generated from strain PB1X-2A using a one-step gene disruption/replacement strategy (Rothstein, 1983) in which a 653-bp region internal to the coding region was deleted and replaced by a 2.0-kb fragment containing the *LEU2* gene of yeast (Perry et al., 1994).

Media and Growth Conditions

Yeast strains S288C, PB1X-9B, PB1X-2A, and PB1X-2AΔ were routinely maintained on YEPD agar containing (w/v) 1% yeast extract, 2% bactopeptone (Difco Laboratories, Detroit, MI), 2% glucose, and 2% agar medium. Experiments with yeast were performed at 30°C in synthetic complete medium (SC) containing yeast nitrogen base (Difco) without amino acids and without ammonium sulfate, with 1 mg of allantoin per mL, 2% glucose, and amino acid supplements (Sherman et al., 1986). Amino acids were supplied at 0.15 mM unless indicated otherwise. Solid media contained 2% agar or 2% Noble agar in the case of disc assays. *E. coli* DH5α was routinely maintained on SOB medium (Sambrook et al., 1989); transformed DH5α strains were grown on SOB medium plus 100 μg/mL ampicillin at 37°C.

cDNA Library

An *Arabidopsis thaliana* (Landsberg *erecta* ecotype) cDNA library constructed in the yeast/*E. coli* expression vector pFL61 (Minet et al., 1992) was obtained from M. Minet and F. Lacroute (Centre National de la Recherche Scientifique, Gif sur Yvette, France). This library was made from mRNA obtained from complete young (stage two leaves) seedlings including roots. The cDNAs ligated to CACA adapters were inserted

into BstXI sites flanked by NotI sites in the vector. Expression in yeast is controlled by the constitutive phosphoglycerate kinase promoter.

Yeast Transformation and Selection

Yeast strains PB1X-9B and PB1X-2AΔ were transformed with the Arabidopsis cDNA library (Gietz et al., 1992), and transformants were selected on SC medium supplemented with L-leucine, L-lysine, and L-histidine (100 μM), washed from plates with sterile distilled H_2O, and replated on dipeptide medium (SC plus 80 μM His-Leu and 80 μM Lys-Leu in place of the required amino acids leucine, lysine, and histidine). 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 H_2O at 2×10^7 cells per mL and then spotted simultaneously (5 μL) on both SC minus uracil and dipeptide medium and scored for growth. Loss of the ability to grow on medium without uracil was always concomitant with loss of ability to grow on peptides as a source of required amino acids. Plasmid DNA was isolated and reintroduced into both PB1X-9B and PB1X-2AΔ. Only plasmids able to restore the growth of the mutants on dipeptide medium were further analyzed by restriction and DNA gel blot analyses.

DNA Gel Blot Analysis

High molecular weight genomic DNA was isolated from young seedlings using a cetyltrimethylammonium bromide nucleic acid extraction procedure (Rogers and Bendich, 1985). Genomic DNA (10 μg per lane) was completely digested with EcoRV and PstI at 37°C for 22 hr and subjected to electrophoresis in 1% (w/v) agarose. Following depurination (0.25 N HCl at 25°C for 30 min), denaturation (0.5 M sodium hydroxide and 1.5 M sodium chloride), and neutralization (1 M Tris-Cl and 1.5 M NaCl, pH 8.0) of DNA within the gel, genomic DNA was transferred to nitrocellulose membrane by vacuum blotting using 10 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) solution. The membranes were probed with a nick-translated, ^{32}P-labeled 2.8-kb NotI restriction fragment from pPTF4 consisting of the entire open reading frame of the Arabidopsis peptide transporter (*AtPTR2*) gene. Hybridization was at 42°C, and blots were washed twice at 65°C in 1 × SSC and 0.1% SDS for 30 min and twice in 0.1 × SSC and 0.1% SDS for 30 min and then exposed to Kodak X-OMAT AR film.

DNA Sequencing and Analysis

Sequences on both strands were determined by the dideoxy chain termination method (Sanger et al., 1977) using Sequenase version 2.0 (United States Biochemicals). A NotI, 2.8-kb cDNA fragment was cloned into pBluescript II SK– (Stratagene). An ExoIII–mung bean deletion kit (Stratagene) was used to generate a deletion series of the fragment. Double-stranded template DNA was sequenced using T3 and M13 primers. Standard molecular techniques, unless otherwise stated, were performed according to Sambrook et al. (1989).

Nucleotide and amino acid sequence analysis was done with the Genetics Computer Group (GCG, Madison, WI) sequence analysis software package (Devereux et al., 1984), and data base searches were performed with the NCBI BLAST algorithm search program (Altschul et al., 1990). Multiple sequence alignments were constructed with the program Pileup in the sequence analysis software package of the GCG. A consensus sequence was generated by the GCG program Pretty using the output from Pileup. The consensus was generated at a minimum plurality of 2.00.

The following data bases were used in the searches and comparisons: GenBank, release 78.0; EMBL (modified), release 35.0; and Swiss-Prot, release 26.0.

Growth on Peptide Substrates

The yeast peptide transport mutant PB1X-9B and deletion strain PB1X-2AΔ transformed with Arabidopsis cDNAs were grown on various peptides to assess the specificity of the plant peptide transporter. In addition, untransformed PB1X-9B, PB1X-9B transformed with plasmid pJP9 encoding the yeast peptide transport gene (Perry et al., 1994), PB1X-2AΔ and PB1X-2A, and the *PTR2⁺* parental strain to the PB1X-2AΔ strain were also assayed. Each strain was grown overnight in SC medium with amino acids plus uracil (nontransformed) or minus uracil (transformed) plus amino acids to stationary phase and harvested by centrifugation, washed twice with sterile distilled H_2O, and resuspended at 5×10^6 cells per 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 supplemented with 80 μM of a specific peptide and auxotrophic requirements minus the amino acid components of the added peptide.

Disc Assays

Sensitivity to toxic amino acids and peptides was measured as described previously (Island et al., 1987). Briefly, a filter disc containing 0.38 μmol 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.

Transport Assays

Uptake of L-leucyl-L-^{3}H-leucine was performed as previously described (Island et al., 1987). Uptake is expressed as nanomoles per milligram dry weight. All competitors were supplied at 10-fold molar excess, except for dileucine, which was supplied at 100-fold excess.

Reverse Transcription–Polymerase Chain Reaction

Expression of *AtPTR2* was determined by reverse transcription–polymerase chain reaction (RT–PCR). Reverse transcription and PCR were performed as described previously (Sambrook et al., 1989). Total RNA (10 μg) from roots of 2-week-old Arabidopsis seedlings grown on Arabidopsis growth medium (Kranz and Kirchheim, 1987) under sterile conditions was pretreated with RNase-free DNase (Promega) and reverse transcribed with moloney murine leukemia virus 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) by Taq polymerase (Promega). The primers used are as follows: an upstream primer starting at base 1975, 5′-GCTTCCATGATTTACGCTGC-3′ (20mer) and a downstream primer starting at base 2528, 3′-GGCCATCCGCAA-

ATACG-5′ (17mer). These primers generate a 569-bp fragment of the *AtPTR2* cDNA. The amplified DNAs were electrophoresed and transferred to nitrocellulose filters. The filters were probed with an α-^{32}P-labeled, 720-bp BamHI-NotI DNA fragment from plasmid pPTF4. The blots were exposed to autoradiographic film at −80°C for 24 hr.

Germination Assay

Ten to 15 Arabidopsis seeds were placed on 2 mL of solid Arabidopsis growth medium per well in a 24-well plate. Each well was supplemented with 0.19 μmol of toxic amino acid analog or toxic peptide, which is five times the minimum inhibitory concentration (MIC, see below). Competitor peptides were added at 20-fold concentration. The plates were incubated at 30°C in the light for 5 days and then scored for germination.

Root Assay

Approximately 15 Arabidopsis seeds were placed in a row at one end of a 10-cm plate containing 20 mL of Arabidopsis growth medium. The seeds were incubated 3 to 5 days in the light with the plate set in an upright position with aluminum foil covering the bottom two-thirds of the plate. When the roots were ∼1 to 2 cm long, sterile filter paper discs were placed immediately in front of the root tips on the right and left side of the plate 2 cm from the edge. A line was drawn across the plate using the top of the discs as a guide marking zero growth. Five microliters of toxic amino acid analog, toxic peptide, or toxic peptide plus competitor was added to one disc while H_2O was placed on the other disc. The plates were incubated for an additional 3 to 5 days. The average length of the roots was measured from the line.

In both the germination and root assays, the minimum inhibitory concentrations of the toxic amino acid analog and toxic peptides were measured with decreasing concentrations of compound and assayed as described as above. The toxic amino acid analogs used in both assays were ethionine (Island et al., 1991; Perry et al., 1994), *m*-fluorophenylalanine (Kingsbury et al., 1983), and oxalysine (Basrai et al., 1992; Perry et al., 1994). The MIC for Ala-ethionine (Eth), Leu-Eth, and oxalysine (Oxalys)-Gly was 38 nmol for both seeds and seedlings, whereas ethionine and oxalysine inhibited root growth of germinated Arabidopsis seeds and root elongation of seedlings, at 3.8 nmol (data not shown). *m*-Fluorophenylalanine showed no inhibition in either assay.

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 as described previously (Naider et al., 1974).

Chemicals and Reagents

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

GenBank Accession Number

Sequence data has been submitted to GenBank as accession number U01171.

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