PHYSIOLOGICAL AND MOLECULAR ANALYSIS OF A VALINE RESISTANT MUTANT IN *ARABIDOPSIS THALIANA*

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ABSTRACT

The passage of amino acids across membrane barriers in plant cells usually requires an amino acid transporter which links the transport to the proton motive force. In recent years many amino acid transporters have been characterized using various molecular and biochemical approaches. Another commonly used approach to identify genes, is to look for mutant phenotypes in T-DNA insertion mutagenized plants. A T-DNA tagged *Arabidopsis* seed population was screened with high concentrations of valine, and a putative valine transport mutant was isolated. Southern blotting displayed a single T-DNA insertion in the genome. Valine uptake deficiency of the mutant plant revealed by the whole seedling uptake assays using ^{14}C-labeled amino acids supports the hypothesis that the mutated gene is a valine transporter.

Key Words: Valine resistance, uptake deficiency, T-DNA mutagenesis.

ÖZET

Bitki hücrelerinde amino asitlerin membranlardan geçişi genellikle taşımayı proton taşıma gücüne bağlayan bir amino asit taşıyıcısı gerektirir. Son yıllarda çeşitli moleküler ve biyokimyasal yaklaşımlar kullanılarak bir çok amino asit taşıyıcısı karakterize edilmiştir. Genleri tanımlamak için yaygınca kullanılan bir başka yöntem ise T-DNA ile mutasyona uğratılmış tohumların arasında mutant fenotipler aramaktır. Bu çalışmada bir T-DNA mutantı tohum populasyonu yüksek valin ihtiva eden ortamda taramaya tabi tutularak bir valin taşıma eksikliği gösteren mutant bitki izole edildi. Southern Blot metoduyla yapılan analiz, mutant bitkinin genomuna sadece bir T-DNA molekülünün eklendiğini gösterdi. Radyoaktif karbonla (^{14}C) işaretlenmiş amino asitlerle yapılan 'tüm fide taşıma' deneyinin, mutant bitkinin dokularına valin alımının azalmış olduğunu göstermesi, mutasyona uğramış olan genin bir valin taşıyıcısı olduğu hipotezini güçlendirmektedir.

Anahtar Kelimeler: Valine dayanıklılık, taşıma eksikliği, T-DNA mutagenezi.

1. INTRODUCTION

1.1 Valine Resistance

A putative amino acid transport mutant was previously identified (1, 2). Unlike wild type seedlings that stop growing when germinated on media containing valine, this mutant plant is able to survive.

A unique feature about valine is that its biosynthesis is linked to that of leucine, and isoleucine. The pathways that synthesize valine, leucine and isoleucine all pass through the enzyme called acetolactate synthase or acetohydroxy acid synthase (AHAS). This enzyme is regulated by feedback inhibition by excess amounts of valine and leucine (3,4,5). Therefore, when either of these amino acids is in excess, AHAS stops the biosynthesis of all three branched amino acids (Figure 1), and the plant will die due to the starvation for the other amino acids (3). Based on this effect, valine resistant plants were screened in a T-DNA mutagenized population of *Arabidopsis*. In this screen, seeds were germinated on 1 mM valine, and resistant plants were selected based on their ability to grow.

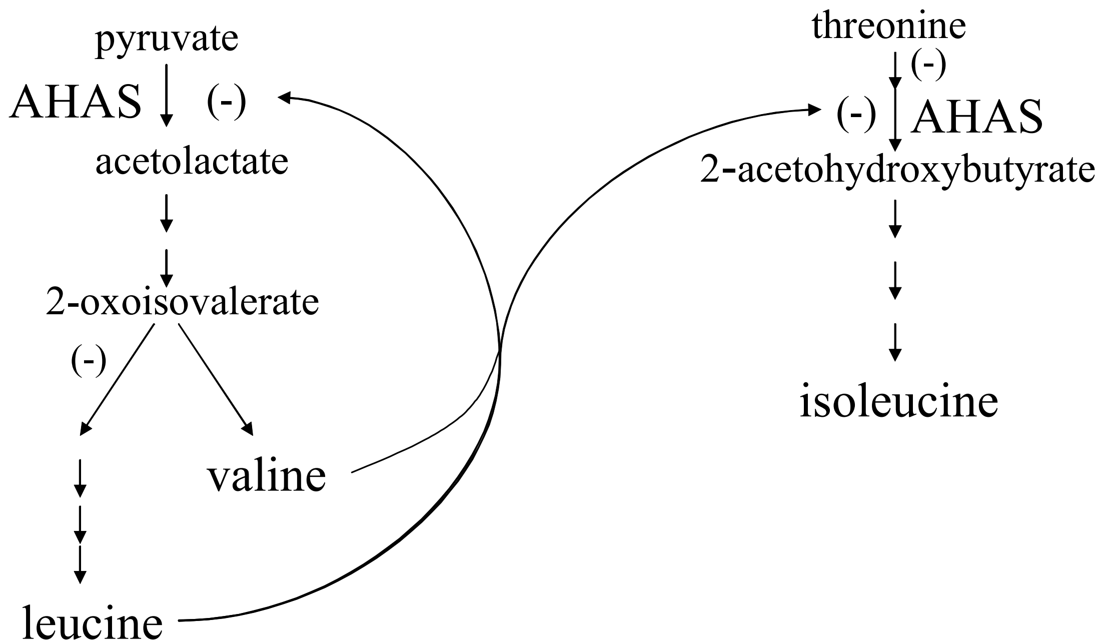

Figure 1. Regulation of AHAS through feedback inhibition of valine and leucine. Both valine and leucine alone can stop the biosynthesis of both amino acids, when in excess amounts. They bind to AHAS and block the first step in both leucine-valine pathway and in isoleucine pathway (adapted from Dey and Harborne, 1997).

Resistance to high concentrations of valine could be due to a mutation in AHAS that results in the loss of feedback inhibition of the enzyme. As an alternative explanation a mutated transporter could also account for the resistant phenotype. The mutant transporter could be at the plasma membrane (blocks uptake) or it could be located in the plastid membrane blocking uptake to the stroma where AHAS is located (6, 7). It must be pointed out, however, that the mutated gene does not necessarily have to be a transporter but it could be a common regulatory gene for most (if not all) the amino acid transporters. Azaserine, a toxic amino acid analog, has been reported to be transferred by a leucine favoring amino acid transporter in animal cells (8). Since azaserine is a purine synthesis inhibitor (9), it is lethal to plants if taken up. In earlier work (1) the valine resistant mutant was also resistant to azaserine. This observation favors the 'membrane transport mutant' hypothesis over 'a change in the target enzyme'. It is important to note that, many amino acid transporters with broad substrate specificity have been described in plants. Thus, it is now surprising this screen worked. This may suggest a key function and / or location of the putative transporter identified in this screen.

An explanation on the role of this putative amino acid transporter in nitrogen assimilate partitioning, and its unique role that allowed us to identify it using a mutant screen against high concentration of valine and against toxic amino acid analogues, would generate many useful insights toward understanding the physiology of plants. Taking the distinct role of amino acid transport in the plant's life into account, this research would provide a significant step forward in our understanding of amino acid transport as well as nitrogen assimilate partitioning in plants.

1.2 T-DNA Mutagenesis in *Arabidopsis*

Transferred DNA (T-DNA) is a part of the Ti plasmid of *Agrobacterium tumefaciens* that is transferred into the host plant genome during the infection by the bacteria. The purpose of this transfer, as far as the bacterium is concerned, is to program the host genome so as to produce nutrients and structure (i.e. crown gall) that are essential for the bacteria. The bacterium does this programming by means of the genes encoded in the T-DNA that is transferred to the host genome (4, 10). Once transferred into the plant cell, the T-DNA part of the plasmid integrates itself into the nuclear genome through a special machinery that involves the participation of various proteins (11,12,13,14).

Feldmann and Marks (15) generated a T-DNA tagged *Arabidopsis* seed library for mutant screening and made it available for distribution (16) through the Ohio State University *Arabidopsis* Biological Resource Center (ABRC), and through Nottingham *Arabidopsis* Stock Center (NASC) Department of Life Sciences. Since *Arabidopsis* introns are small and because there is very little intergenic material (17) in its genome, with a large enough population of T-DNA transformed lines, it is possible to saturate the genome with mutations in practically every gene. A study (18) reporting the random distribution of T-DNA integrations into *Arabidopsis* genome supports this hypothesis. More recent work (19) has indeed reported the near saturation of the gene space.

The transport mutant 2607 was isolated (1) by screening the Feldmann tagged seed library for resistance against 1 mM valine. The T-DNA construct in Feldmann lines (15) and the restriction map of endonucleases used for Southern analysis, are shown in Figure 2.

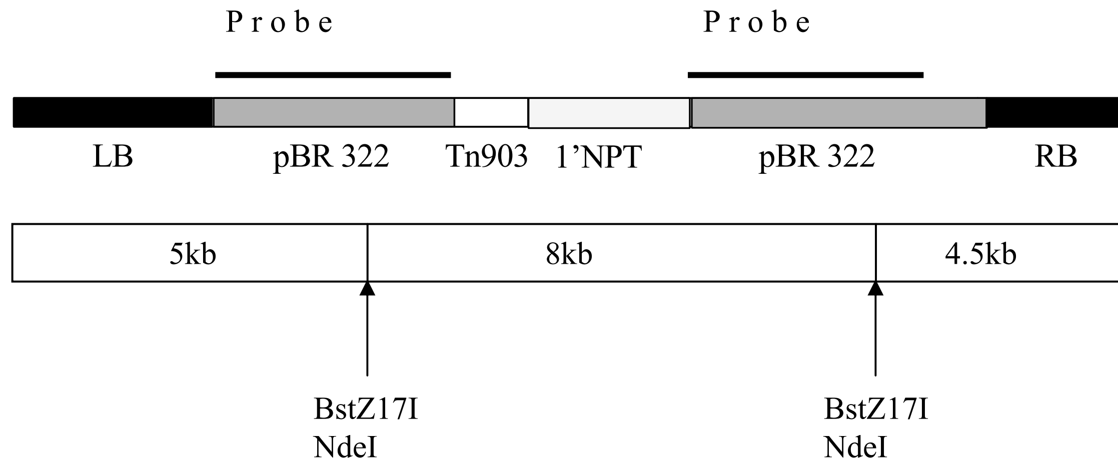

Figure 2. The structure and the restriction map of the T-DNA construct used to generate 2607. BstZ17I and NdeI (New England Biolabs) cut T-DNA about 50 nucleotides apart from one another and hence they practically generate same size fragments. When double digestion products are probed as indicated, there will be 3 bands for a single insertion, 5 bands for a double insertion, 7 bands for 3 insertions, and so on. This approach can only detect the number of insertion sites which would very helpful to relate the phenotype to the insertion, but not the number T-DNA cassettes inserted side by side such as head to head or head to tail. The probe is a 3.5 kb fragment of pBR322.

2. MATERIALS AND METHODS

2.1 *Arabidopsis* Growth Conditions

Arabidopsis thaliana ecotype Wassilewskija (WS), and the T-DNA tagged *Arabidopsis* seed library (15, 20) was acquired from the *Arabidopsis* Biological Resource Center (ABRC) at The Ohio State University. *Arabidopsis* seeds were sterilized and grown based on Sundaresan et al.(21). The seeds were soaked in 95% alcohol for 10 min, 20% Clorox 0.1% tween 20 for 5 min, washed with sterile water for 2 min twice and added 0.1% agar (top agar). The sterilized seeds were stratified 1-3 days and then transferred into plates that were made with 4.5 $g{\bullet}L^{-1}$ Murashige and Skoog (MS) salts (Invitrogen Corp., Carlsbad, CA, USA), 1% sucrose (Sigma-Aldrich, St. Louis, MO, USA), and 4.5 $g{\bullet}L^{-1}$ Agargel (Sigma-Aldrich, St. Louis, MO, USA) or 6 $g{\bullet}L^{-1}$ Bacto™ Agar (BD Biosciences, Boston, MA, USA). 1 mL from a 50 $mg{\bullet}mL^{-1}$ sterile kanamycin stock solution (50 $\mu g{\bullet}mL^{-1}$ final concentration), and valine to desired final concentration were added after media were cooled down to 55 ^{o}C, when needed. Plates with stratified seeds were grown in a controlled growth chamber that was set to 21 ^{o}C and 10 h 150 μE $m^{-2}{\bullet}s^{-1}$ light cycle. Seedlings were transferred into well-watered Sunshine Mix soil (Wetsel Seed Co., Harrisonburg, VA, USA) when needed, and they were grown under controlled growth chambers that were set to 21 ^{o}C and 8 h 200 $\mu E m^{-2}{\bullet}s^{-1}$ light cycle.

2.2 Polymerase Chain Reactions and Sequencing

Tail-PCR was performed as described (22) in a DNA-Engine PTC-200 (MJ Research, Inc., Watertown, MA). The PCR protocols and nested primers were as described by Krysan et al.(23), and the arbitrary degenerate (AD) primers were as described by Liu and Whittier (22). The nested primers used in the first amplification were L1 (5'-GAT GCA CTC GAA ATC AGC CAA TTT TAG AC-3') and R1 (5'-TCC TTC AAT CGT TGC GGT TCT GTC AGT TC-3'); and in the second amplification were L2 (5'-GGA TGT GAA TTC AGT ACA TTA AAA ACG TC-3') and R2 (5'-GTC AGT TCC AAA CGT AAA ACG GCT TGT CC-3'). The AD primers used were AD1 (5'-NTC GA(G/C)T(A/T)T(G/C)G(A/T)GTT-3'), AD2 (5'-NGT CGA (G/C)(A/T)GANA(A/T)GAA-3'), AD3 (5'-(A/T)GTG NAG(A/T)ANCANAGA-3'). Two additional AD primers AD2a (5'-STT GNT AST NCT NTG C-3') and AD5 (5'-WCA GNT GWT NGT NCT G-3') (24) were also utilized when needed.

Nested primers for pTiC58 were pTiL1 (5'ACC TTC ACA TCC AGC ACA AGC ATA TCA-3'), pTiL2 (5'-ATC TTT GCG CAG CTC ATT CTT GAC CAA T-3'), pTiL3 (5'-TAG TTT TAT TGA TCA GCG GTT CCG CAA-3'), pTiR1 (5'-ACG AAA ATA TCC GAA CGC AGC AAG ATA T-3'), pTiR2 (5'-TTG AAG GCC AAA GCC TGG AAC TCA CTT T-3'), and pTiR3 (5'-TGT TTT TCG GAT GCC CGT TGA CGT ATT T-3'). Primers were designed using Primer3 software (25) on the web (http://www-genome.wi.mit.edu/cgi-bin/primer/ primer3_www.cgi), and ordered from Integrated DNA Technologies (Coralville, IA, USA).

Inverse polymerase chain reaction (I-PCR) and the primers were as reported (26). All standard polymerase chain reactions were done using standard molecular biology protocols (27) in a DNA-Engine PTC-200 (MJ Research, Inc., Watertown, MA).

For sequencing the TAIL-PCR products directly from the second or third amplification, the brightest bands were gel extracted using a gel extraction kit (Qiagen Inc., Valencia, CA, USA), and sequenced by the Keck Center at the University of Illinois at Urbana-Champaign.

2.3 Southern Blotting

Plant genomic DNA was isolated as described by Dellaporta et al. (28). Genomic DNA was digested with selected restriction enzymes, and separated on a 0.8% agarose gel in TAE buffer. After running approximately 3 h at 80 volts (constant), the gel was depurinated in 0.25 M HCl, denatured in 0.5 M NaOH / 1.5 M NaCl, and neutralized in 1 M Tris / 1.5 M NaCl (pH 8). Digestion products were transferred onto a Hybond N^+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in 10 X SSC (standard saline citrate, pH 7) for 16-24 h, cross linked to the membrane with a UV cross-linker (Stratagene, La Jolla, CA, USA). α-^{32}P-dCTP or was purchased from Perkin Elmer (Boston, MA, USA). ^{32}P-labeled probes were synthesized using a Mega Prime DNA labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Probes were cleaned up with Bio-Spin columns (Bio-Rad, Hercules, CA, USA), and probe specific activity was measured using a liquid scintillation analyzer (Packard Bioscience Company, Downers Grove, IL, USA). Pre-hybridization, hybridization and washing the membranes were done based on

Sambrook et al. (27). For hybridization 10^6 cpm•mL^{-1} per hybridization was used. Blots were washed twice in 50 mL 2 X SSC / 0.1% SDS for 15 min each time at 42 ^oC, and twice in 50 mL 0.1 X SSC / 0.1% SDS for 15 min each time at 62 ^oC. The blots were then exposed to an X-ray film for 1-7 days in an X-ray cassette at -80 ^oC freezer.

2.4 Amino Acid Uptake from Whole Seedling

Amino acid uptake assay of whole seedlings was done based on a modified version of a published protocol (29). The ^{14}C-labeled amino acids were purchased from Perkin Elmer (Boston, MA, USA). One week old *Arabidopsis thaliana* seedlings grown on agar plates were separated from agar and placed into the transport solution that contained 200µM amino acid of interest and 0.4µCi of its ^{14}C-labeled solution. Seedlings were taken out of the solution at desired time points, washed with cold water, placed into a scintillation vial and counted for radioactivity using a scintillation counter.

3. RESULTS

3.1 Isolating T-DNA Mutants Resistant to High Valine Concentration

Since the original screening was done with 4900 individual seeds (1), a lower level of valine concentration (1 mM) was used to isolate maximum number of potential valine resistant mutants. This concentration, however, is not enough to unequivocally differentiate between valine resistant and sensitive seedlings of *Arabidopsis* (2). Therefore seeds of 2607 that are always resistant to 5 mM valine were isolated (
Figure 3).

3.2 Determining the Number of Insertion Sites in the Genome

There are many approaches to obtain a genomic sequence flanking T-DNA. Some of the standard techniques include genomic library construction, plasmid rescue and inverse polymerase chain reaction (14, 16, 26, 30). More recent techniques include Thermal Asymmetric Interlaced PCR (TAIL-PCR) (22) and various standard or inverse PCR based methods that involve pretreatment of restriction digestion products (31,32,33). Unfortunately none of these methods are universally successful for all plants (26, 31, 34). Most of the problems in T-DNA insertion mutants arise from aberrant or multiple insertions of the T-DNA vector such as head to head, head to tail, tail to tail, or one into another. In addition, many "untagged" mutations are caused by unsuccessful attempts of T-DNA to insert into chromosomal DNA that leaves short insertions or deletions.

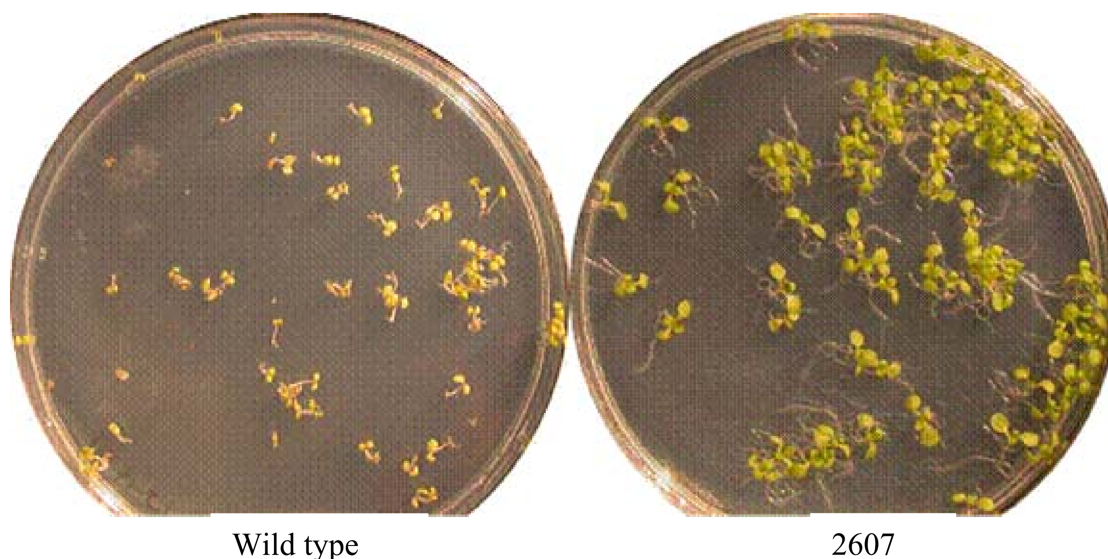

Figure 3. Growth of wild type and 2607 seedlings on 5 mM valine containing MS plates.

Wild type seedlings can not grow on MS media containing 5 mM valine, while T-DNA insertion mutant 2607 is resistant. The seedlings were 11 days old when pictured. 2607 can continue growing and set seeds with no detected phenotype.

Attempts to obtain genomic sequence flanking T-DNA vector in 2607 primarily using TAIL-PCR, have not yielded any useful result. Sequences obtained after second amplification of primary TAIL-PCR products which were obtained using a T-DNA nested primer and an arbitrary degenerate primer (22), did not yield any vector-genome junction sequence. Instead they yielded T-DNA left border sequence which suggested the head to head insertion, and pTiC58 (35) sequence which suggested the insertion of the T-DNA harboring Ti plasmid. Based on Ti plasmid sequence finding, three nested primers (for 3 rounds of TAIL-PCR) were designed to amplify a junction region between Ti plasmid and the genome. This approach also did not yield any junction sequence after getting the sequences of the candidate bands of third TAIL-PCR amplifications.

Researchers encountering difficulties with getting a quick answer with PCR, usually perform a Southern blotting analysis to determine the number of insertions in the genome (14, 26). Utilizing this approach a Southern blotting was performed and revealed a single insertion site in the genome (Figure 4).

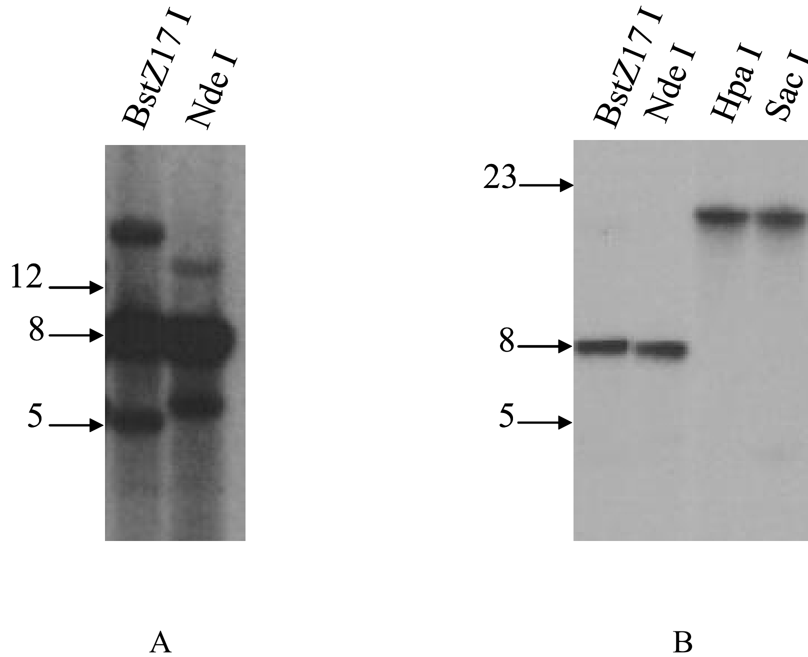

Figure 4. Southern autoradiographs of 2607 genomic DNA digestion. A. 40 µg DNA / lane. B. 10 µg DNA / lane. In both panels, the probe was a 3.5 kb EcoRI-SalI fragment of pBR322. BstZ17I and NdeI cut about 50 bp apart from one another in T-DNA, and hence their products are similar size for internal T-DNA fragments. Since both enzymes generated only 2 different sized fragments and 1 common fragment, this result suggests 1 insertion in the genome. The fact that the common internal T-DNA band had much higher intensity than the border bands, suggest the existence of multiple T-DNAs in single site. The reason the border bands are not visible in the second panel (B), is because of the insufficient amount of digested genomic DNA transferred to the blot. HpaI and SacI have no sites in T-DNA and hence they also suggest a single insertion site in the genome.

3.3 Transport Activity Measured by Whole Plant Dipping

Since the screening was done in the seedlings stage, and hence the primary contact to the valine containing medium was the roots, it was reasoned that the gene knocked out could be seedling and / or root specific. Therefore transport activity of the one week old mutant seedlings was tested along with the same condition wild type seedlings as the control. Wild type and mutant *Arabidopsis* seedlings were first germinated until the selection against 5mM valine starts, and then used for uptake assay. The results displayed that while there was no significant difference of alanine uptake between the wild type and the 2607 seedlings (Figure 5), valine uptake in the 2607 seedlings was reduced (Figure 6). These results strongly suggested either a valine transporter gene or a mechanism facilitating valine uptake is knocked out by the T-DNA insertion mutagenesis.

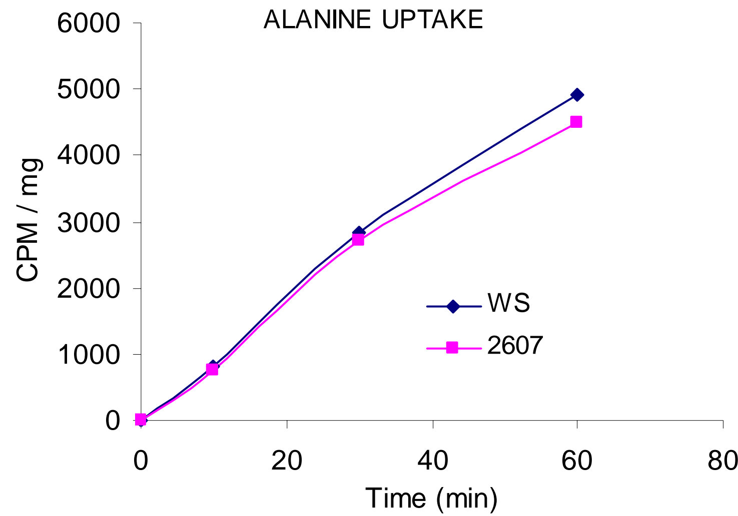

Figure 5. Uptake of ^{14}C-labeled alanine in wild type and T-DNA mutated plants (2607).

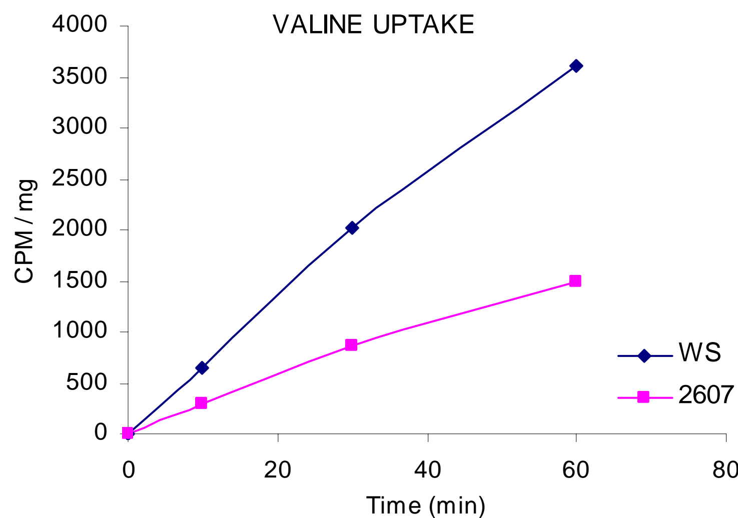

Figure 6. Uptake of ^{14}C-labeled valine in wild type and T-DNA mutated plants (2607).

4. DISCUSSION AND CONCLUSION

In this study, a valine transport mutant was investigated. Many amino acid transporters reported to date display broad specificity toward most amino acids. This means if an amino acid transporter is knocked out in a plant, its function could be replaced by

many other transporters. Therefore, a visible phenotype is very rare in plants that have a mutated single amino acid transporter gene (2). Research using multiple amino acid transporter knockouts, however, is yet to be explored.

The T-DNA mutant 2607 has been proposed as a valine transport mutant based on its ability to survive high concentrations of valine and azaserine (1). The reduced valine uptake of the mutant plant (Figure 6) also supported the "valine transporter" hypothesis. Given what we know today about the large number of plant amino acid transporters, it is very interesting that high concentrations of valine cannot kill the plant when (possibly) a single putative amino acid transporter gene is knocked out, or due to a single T-DNA insertion.

Resistance to high concentrations of valine could be due to a mutation at the biosynthetic enzyme acetolactate synthase (7), or due to inability of plant to acquire valine. Since many transporters have been reported to transport valine to various extents, the mutated transporter should be located at the membrane of a sub-cellular organelle if the transporter hypothesis is correct. In this case the putative gene must have a key function in amino acid partitioning, since it appears to have a vital role in valine distribution in plant.

The T-DNA cassette in 2607 appears to have inserted into a single location (Figure 3.2) but as multiple copies. Since many TAIL-PCR attempts with nested primers from left and right borders failed to amplify a junction sequence, the T-DNA borders might also be deleted partially or completely. Probably due to same reason, previous attempts to obtain a flanking sequence also failed (1). There are numerous alternatives untried (31,32,33), however, and it is still possible to find out the mutated region in the genome, which will further reveal the exact relationship of the valine resistance to a single gene. Accordingly, further work to clone the gene and to analyze its biochemical properties is being performed.

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