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Transformation and Expression of the PAT Gene in *Arabidopsis* Tryptophan Mutants

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Phosphoribosylanthranilate transferase (PAT) catalyzes the second step of the tryptophan biosynthetic pathway and is encoded by a single-copy gene that complements all the visible phenotypes of the tryptophan mutant (*trp*1-100) of *Arabidopsis*. The *trp*1-100 is blue fluorescent under UV light because it accumulates anthranilate. To obtain a plant with reduced PAT activity, PAT1 genes with several internal deletions in different promoter regions (pHS 101, pHS102, pHS104, pHS105, and pHS107) were introduced into *trp*1-100 via *Agrobacterium*. Then, homozygous T_3 plants were isolated and examined for blue fluorescence. Introduction of the PAT1 gene fusants results in the reversion of fluorescence phenotype except in the case of pHS105. These results prompted us to perform a parallel analysis of anthranilate synthase and PAT in terms of the genetic complementation. A plant line carrying pHS105 gene fusant dose not completely complement the blue fluorescence but it accumulates less anthranilate than *trp*1-100. The activity of PAT was reduced in the transgenic mutant as well. The plant carrying these constructs will add to the growing collection of molecular tools for the study of the indolic secondary metabolism.

Keywords: anthranilate, anthranilate synthase, *Arabidopsis thaliana*, PAT, transformation

The tryptophan biosynthetic pathway in higher plants is not only the source of an essential amino acid but also provides precursors for a variety of secondary metabolites, including plant growth regulator auxin (IAA) (Wright *et al.*, 1991; Normanly *et al.*, 1993), antimicrobial phytoalexins (Tsuji *et al.*, 1993), alkaloids (Cordell, 1974), and glucosinolates (Haughn *et al.*, 1991). Although we have a sophisticated understanding of the biosynthetic pathway in microorganisms, work on the genetics of tryptophan biosynthesis in higher plants has recently begun to receive close scrutiny (Rose and Last, 1994).

Mutants of *Arabidopsis* are now available at four steps in the tryptophan biosynthetic pathway. The *trp*1-100 is deficient in the enzyme phophoribosyl anthranilate transferase (PAT) (Last and Fink, 1988; Rose *et al.*, 1992). PAT catalyzes the second step of the tryptophan branch which catalyzes the conversion of anthranilate to 5-phosphoribosyl anthranilate (Fig. 1).

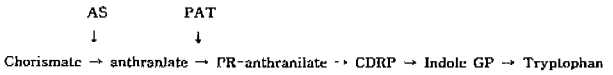

Fig. 1. The tryptophan biosynthetic pathway. The enzyme defective in *trp*1-100 mutants (PAT) is indicated and also anthranilate synthase. AS, anthranilate synthase; PAT, phosphoribosylanthranilate transferase: PR-anthranilate, 5-phosphoribosylanthranilate; CDRP, 1-(o-carboxyphenylamino)-1-deoxyribulose 5-phosphate; indole GP, indole-3-glycerol phosphate.

Decreased PAT activity results in the accumulation of anthranilate, which is blue fluorescent under UV light. The enzyme anthranilate synthase (AS) catalyzes the first step, converting chorismate to anthranilate. Even though available information indicates that AS plays a key role in the regulation of Trp biosynthesis, it was reported that the *trp*1-100 mutant strain has an AS activity equal to that of wild type *Arabidopsis* (Last & Fink, 1988).

PAT1 gene has been cloned in *A. thaliana* (Rose *et al.* 1992). The PAT1, as unique single-copy gene, is genetically linked to *trp*1-100 and complements

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the mutant phenotype. Since the enzyme plays an obligatory role in both tryptophan synthesis and indolic secondary metabolism, it is desirable to obtain mutants with patially reduced phosphoribosylanthranilate transferase (PAT) activity. Until now, no such mutants have been reported. However, this could be accomplished by introducing a PAT1 gene with partially deleted promoter sequences into a *trp*1-100 which is defective in the PAT activity. Since the blue fluorescent phenotype can be casily scored by measuring the amount of anthranilate compounds, the transformants with reduced PAT activity can be screened in this way. The plant carrying these characteristics will be a valuable molecular tool in the analysis of the indolic secondary metabolism.

In this study, we constructed several internal deletions in promoter region of the PAT1 gene and each construct with PAT1 gene was introduced into *Arabidopsis* tryptophan mutants (*trp*1-100). To find a plant carrying a reduced level of PAT activity, the amount of the precursor, anthranilate, was measured, and anthranilate synthase and PAT activity as well. Finally, various amounts of anthranilate will reflect the degree of genetic complementation by introduced PAT1 gene with partially deleted promoter sequences.

MATERIALS AND METHODS

Plant Lines and Culture Conditions

A mutant line of *Arabidopsis*, homozygous for *trp* 1-100, was grown on an agar medium or soilless mix in a growth chamber at 22°C, 3000 LUX with a 16 hrs photoperiod.

Construction of Plasmids

A genomic clone (pAR189) containing the PAT1 gene of *A. thaliana* was kindly provided by Dr. Alan Rose (UC Davis). Plasmids containing the PAT1 gene with deleted promoter were subcloned into the binary vector pEND4K (Klee *et al.*, 1985). pHS101, pHS 102, pHS104, and pHS105 contain a series of internal promoter deletions from -30 to -846. pHS107 contains a 7.9 kb-KpnI fragment including the full sequence of PAT1 promoter (Fig. 2).

Plant Transformation

Each of these plasmids was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. *Agrobacterium* strains containing gene fusion constructs were used to cocultivate root explants of *trp*1-100 mutant and kanamycin-resistant plants were regenerated as described (Last *et al.*, 1991). All independently transformed plants (T_1) were regenerated for each gene fusion. The T_1 plants were self-pollinated, and the resultant T_2 seeds were germinated on PNS medium containing 50 mg of kanamycin sulfate/L. Homozygous plants were tested by scoring the blue fluorescence and kanamycin resistant phenotype of the T_3 progeny from self-pollination of T_2 plants.

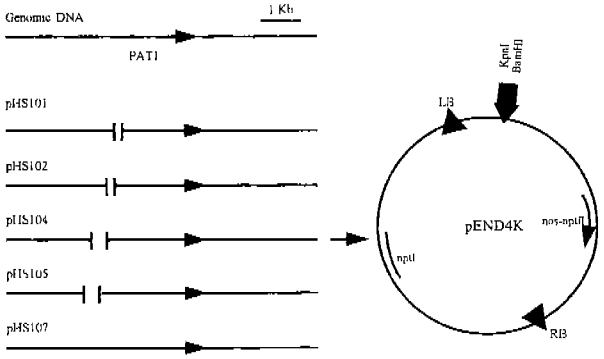

Fig. 2. A series of internal deletion mutants of the PAT1 promoter were constructed and subcloned into Kpn1 site of pEND4K. (pHS101, deleted from -30 to -184; pHS102, deleted from -120 to -350; pHS104, deleted from -235 to -606; pHS105, deleted from -442 to -846; pHS107, contained PAT promoter)

Measurement of enzyme activity and anthranilate content

The homozygous progenies (T_3) were analyzed in parallel for anthranilate content and the activities of AS and PAT in relation to the genetic complementation by the introduced PAT1 gene. Samples of 10-day-old seedlings were pooled and homogenized in equal volume of Tris-Cl Buffer (pH 7.5) and the extract was used for enzyme assay. AS and PAT activities were measured as described by Last and Fink (1988). Briefly, anthranilate synthase activity was measured in an 1 ml reaction buffer containing 50 µl plant extract, 0.1 µM chorismic acid, 20 µM glutamine, 2.0 µM $MgCl_2$, and 25 µM Tris-Cl (pH 8.0). The anthranilate produced was extracted into 3 ml of ethyl acetate and then quantified with a Perkin-Elmer fluorescense spectrophotometer (excitation 340 nm, emission 40 nm). PAT was assayed by monitoring the PRPP-dependent disappearance of anthranilate. The reaction buffer contained

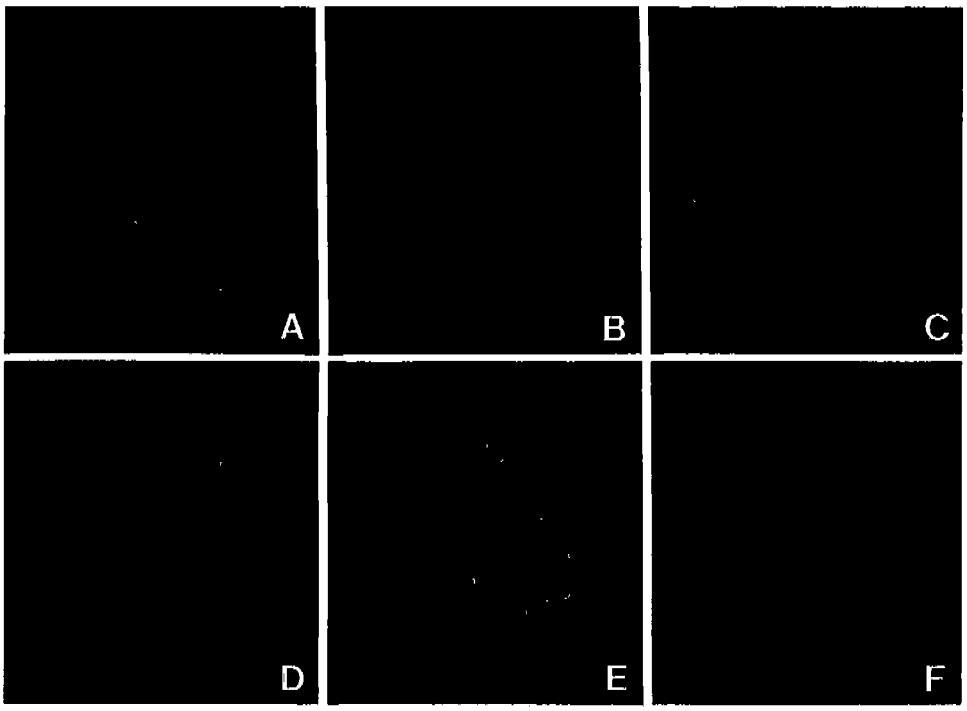

Fig. 3. Photographs of blue fluorescenced cotyledons of the transformants carrying deleted promoter sequences. (A, *trp*1-100; B, pHS101; C, pHS102; D, pHS104; E, pHS105; F, pHS107)

8 nM of anthranilate, 100 mM of PRPP, 2 μM of $MgCl_2$, 25 μM of Tris-Cl (pH 8.0) and 50 μl of plant extract in 1.0 ml.

RESULTS

Deletion analysis of the regulatory region of the PAT1 gene

Internal deletion mutants of the PAT1 promoter region were constructed. DNA fragments containing the regulatory elements were digested with the suitable restriction enzymes to generate a serial deletion of the promoter region (Fig. 2). pHS101 is a 7.8 kb fragment from pAR189 containing PAT1 promoter deletion, -30 to -184. pHS102 is 7.7kb fragment containing deletion, -120 to -350 and pHS104 is 7.6kb deleted from -235 to -606 and pHS105 containing promoter deletion, -442 to -846. pHS107 is a 7.9kb fragment containing PAT1 promoter.

Introduction of the PAT gene with partially deleted promoter regions into *trp*1-100 mutant

Deleted promoter followed by the PAT1 gene was introduced into a tryptophan mutant (*trp*1-100). Transfer of the gene fusants to *trp*1 plants allowed us to analyze promoter activity in transgenic plants.

Agrobacteria carrying a recombinated plasmid DNA was cocultivated with the root explants of *trp*1-100 mutant. Selection was carried on the callus induction medium containing kanamycin (25 mg/l), 2 days after the cocultivation. Regenerated shoots on the selection media were acclimated in a green house. Each transformed plant was self-pollinated to maintain the genetic line and finally homozygous T_3 seeds were germinated on solid media containing kanamycin (50 mg/l).

Complementation of blue fluorescence in *trp*1-100 mutant

Complementation assay for the blue fluorescence allowed examination of the effects of promoter mutations in the transgenic progenies. Germinated seedlings of T_3 were examined under UV light (Fig. 3). Table.1 shows the results of the complementation assay for the blue fluorescence in the homozygous T_3 plants. As control, *trp*1-100 shows blue fluorescence. For pHS101, pHS102, pHS104, and pHS107, the seedlings were completely complemented even though a few seedlings were segregated so that they re-

Table 1. Summary of complementation results from T_3 seeds.

Plasmid introduced	Kanamycin resistant	Kanamycin sensitive	Fluorescent	Non-Fluorescent	Complementation (%)
*trp*1-100	0	100	100	0	0
C24	0	100	0	100	0
pHS101	93	7	5	95	100
pHS102	98	2	4	96	98
pHS104	95	5	3	97	100
pHS105	81	19	97	3	0
pHS107	65	35	37	63	97

*The number of analyzed seeds was 100. Each T_3 line was derived from an individual T_2 line after regeneration of primary transformant (T_1) by self pollination.

Table 2. Quantity of anthranilate and the activity of anthranilate synthase and phosphoribosyl anthranilate transferase in a series of transformant carrying deleted promoter sequences.

Plasmid introduced	Anthranilate content (μg/g.FW)	Activity of Anthranilate synthase	Activity of PAT
*trp*1-100	1.87	28.4±4.5	< 1
C24	0.04	20.6±1.6	16 ±1.4
pHS101	0.01	16.1±1.7	17.5±2.1
pHS102	0.03	24.2±2.2	14±1.3
pHS104	0.02	19.1±1.3	13.5±1.2
pHS105	1.21	30.2±2.7	3±1.7
pHS107	0.02	21.4±0.9	19.5±2.0

*Specific activities were in μM of anthranilate per fresh weight(g). All numbers were the average of valucs from three independent experiments.

vealed blue fluorescent. However, seedlings for pHS 105 still have fluorescence.

Deletion effect on the PAT activity in homozygous T_3 plants

PAT1 gene expression was initially examined by quantitative assay of anthranilate in extracts from pooled tissues of the homozygous T_3 plants (Table 2). No significant increase above the background fluorescence in extracts from wild type (C24) was detected in plants containing deletion mutants from pHS101, pHS102, and pHS104. However, a reduced level of anthranilate was observed for the -401 to -805 PAT1 deletion mutants (pHS105). The activities of anthranilate synthase were revealed consistently in the non-fluorescensed. However, it was higher in plants carrying promoter deletions from pHS105 and lower in the case of pHS107. The PAT activity in the plants carrying pHS107 was higher than that of wild type and all of the transgenic plants showed higher PAT activity than that of *trp*1-100 mutant.

DISCUSSION

Direct enzyme assays on crude extracts of wild type and mutant plants indicate that the *trp*1-100 homozygotes contained very low level of the anthranilate phosphoribosyl (PR)-transferase activity. The defect in activity causes the accumulation of anthranilate in the plant which exhibits a striking blue fluorescence under either longwave or shortwave ultraviolet light (Rose *et al.*, 1992). The plants grow at a normal rate and look similar to the wild type. Since *trp*1-100 mutation is genetically linked to PAT1 gene, PAT1 complements phenotype of *trp*1-100 mutant. Here, we report partially complemented *trp*1-100 transformants into which the PAT1 gene with partially deleted promoter sequence was introduced.

Our results demonstrate that only distal promoter regions of the PAT1 gene are required for correct expression. The results of progressive deletion of the promoter regions suggest that the expression of the PAT1 gene is achieved without main proximal region of the promoter to complement the mutant phenotype. Therefore, no evidence was obtained for positive regulatory elements that modulate PAT1 promoter activity in the transformants. Because these putative regulatory elements did not display in the transgenic analysis, further studies are required to confirm their properties and precise locations within the PAT1 promoter.

Anthranilate synthase activity remained invariably in those transgenic lines even though available information indicates that AS plays a key role in regulation of Trp biosynthesis (Li and Last, 1996). This result is consistent with the accumulation of steady state level of AS subunit which did not change in response to the growth of plants on 50 μM Trp in *A. thaliana* (Niogi and Fink, 1992). It suggests that accumulation of Trp biosynthetic enzymes is not repressed by the end product. The activity of phosphoribosylanthranilate transferase was reduced in the transgenic mutant carrying PAT1 gene from pHS105 while other transgenic lines showed some activities. Therefore, the transgenic plants, carrying PAT1 from pHS105, had partially recovered PAT activity so that they have limited precursor pool for tryptophan and IAA biosynthesis.

Although the pathway for the biosynthesis of IAA from tryptophan has been determined (Bialek *et al.*,

1992), the exact sequence of reactions should be clarified in plants (Nonhebel *et al.*, 1993). Mutants blocked at the later stages of Trp biosynthesis results in increasing indolic precursor pools. Increased rates of precursor formation at IAA and/or tryptophan biosynthesis would allow the development of the IAA mutant (Normanly *et al.*, 1993). This class of putative IAA mutants has been found in several plants (Tam *et al.*, 1995). In contrast, the suppression of precursor biosynthesis would allow the development of a mutant in which the IAA biosynthesis is partly reduced. The *trp*1-1 tryptophan mutants are consistent with a defect in IAA synthesis but, show too severe growth retardation (Rose *et al.*, 1992). The transgenic line for pHS105 has limited the precursor pools for IAA biosynthesis by blocking the early steps of the Trp biosynthesis pathway. Therefore, we expect that the PAT1 gene, in conjunction with the *trp*1-100 mutants, could be used as a tool for studying of IAA biosynthesis. The availability of tryptopan mutant in *A. thaliana* would permit the elucidation of IAA biosynthetic pathway and provide a genetic basis for the study of hormone action.

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