Characterization of Anthranilate Synthetase and Tryptophan Synthase in a 5-Methyltryptophan Resistant Mutant (MR 1) of *Zea mays* L.

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Summary

Anthranilate synthetase (AS) and tryptophan synthase (TS) enzymes were analyzed for characterization of 5-methyltryptophan (5MT) resistant mutant plants (MR 1) in maize. There were no significant differences in the level of AS activity between MR 1 and control seedlings when grown on MS medium without 5MT. However, the level of AS was 2 times higher in MR 1 seedlings grown on MS medium with 25 ppm 5MT than in control seedlings. The activity of AS from control seedlings was 50 % inhibited by 5 ppm tryptophan added to extract, while the enzyme from MR 1 seedlings was 50 % inhibited by 4 times higher concentrations (20 ppm) of tryptophan. The level of TS activity in MR 1 was 4 times higher than that in control seedlings when cultured on MS medium. For a more detailed study on TS, the gene encoding the tryptophan synthase B subunit (TSB) was isolated from maize and sequenced. The gene showed high homology to TSB isolated from other organisms. Northern hybridization analysis indicated that the gene expressed in MR 1 seedlings grown on MS medium at a higher level than in control seedlings. From the results, it was found that MR 1 plants showed differences in the character of AS, as reported previously in cell lines resistant to 5MT, and also notable diversity regarding TS activity when compared to control plants.

Key Words : *Zea mays.*, anthranilate synthetase, 5-methyltryptophan resistance, mutant, tryptophan synthase.

Introduction

In higher plants, it has been reported that L-tryptophan biosynthesis is controlled by feedback inhibition of anthranilate synthetase (AS), which catalyzes the first reaction branching from the aromatic amino acid pathway toward the biosynthesis of L-tryptophan, by the end product L-tryptophan, and tryptophan synthase (TS), which catalyzes the last step of the L-tryptophan biosynthesis pathway, is unaffected by L-tryptophan (Widholm 1974). It has been found that activity of anthranilate synthetase extracted from tobacco and carrot mutant cells selected for 5-methyltryptophan (5MT) resistance is not inhibited by 5MT and L-tryptophan as much as that from unselected cells. Therefore, the resistant mutants to 5MT accumulated much higher levels

of free L-tryptophan than the sensitive control cells (Widholm 1972, Lee and Kameya 1989). In most cases, the resistance of the cell lines was due to an altered anthranilate synthetase enzyme, which was less sensitive to inhibition by 5MT and also tryptophan. However, the studies of tryptophan biosynthetic enzymes in whole mutant plants resistant to 5MT have not been done because plant regeneration from the cell lines cultured for long period is not easy.

We have reported previously the selection of a 5MT resistant mutant plant (MR 1) in *Zea mays*, which is not inhibited by 50 ppm 5MT and accumulates higher levels of free tryptophan in seeds. We have also reported that these characters are controlled by a single dominant nuclear gene (Kang and Kameya 1993). Therefore, MR 1 is expected to be useful not only for studying amino acid biosynthesis but also for improving the nutritional quality of maize.

We describe here the activity of AS and TS enzymes extracted from MR 1 and control seedlings, and the feedback inhibition of AS by L-tryptophan. And we also report the sequence of the gene encoding tryptophan synthase B subunit (TSB) isolated from maize, which shows higher expression in MR 1 seedlings than that in control seedlings.

Materials and Methods

Plant materials

5-methyltryptophan resistant plants (MR 1) induced by EMS (ethylmethane sulfonate) treatment (Kang and Kameya 1993) and control plants (inbred line, Danggin) were used in this experiment. Seedlings were grown on MS agar (0.8 %) medium (Murashige and Skoog 1962) with or without 25 ppm 5-methyltryptophan at 25°C under continuous fluorescent light (4 W/m). Fourteen days old seedlings were harvested just prior to the extraction of the enzymes and total RNA.

Feeding experiments

Seeds of MR 1 and control plants were incubated in sterile water overnight, and then planted on the MS agar medium containing various concentrations of L-tryptophan. 14 days after the germination, seedlings were harvested, and their dry weight was measured.

Enzyme extraction

About 20 g of plant materials were homogenized in about 50 ml cold acetone with polytron-blender for 2 min..

Crude extracts of enzyme were prepared according to the method reported by Widholm (1972 b) with the exception that initial extractions were done with 0.1 M Tris-HCl, pH 7.5, containing 0.1 M DTT, 0.1 mM disodium EDTA, 10 % glycerol, 20 mM L-glutamine and 4 mM MgSO₄ at 0 °C.

Anthranilate synthetase (AS) and tryptophan synthase (TS) assays

Enzyme activity of anthranilate synthetase was measured by the method reported previously (Widholm 1972 a) using 0.2 ml of extracts which had not been passed through a Sephadex G-25 column. Tryptophan synthetase was measured by the method described by Widholm (1971). The experiments were repeated three times.

RNA Isolation, cDNA Cloning, Screening and Sequence Analysis

Total RNA and poly(A⁺) RNA isolation from maize inbred line (IK), and subsequent cDNA library construction in λ gt 10 were essentially performed as described by Sano *et al.* (1991). Approximately 30000 recombinant plaques were screened with a maize TSB cDNA probe which was amplified by polymerase chain reaction (PCR) using synthetic primers. The forward primer, 5'-CACCGGGCGCTCACAAGA-3' and the reverse primer, 5'-ACGGTTTGGACATCCTTATC-3' correspond to the highly conserved amino acid sequence in the gene encoding tryptophan synthase B subunit (TSB) of various organisms. The probe was labeled by [³²P]-dCTP, and hybridization conditions were as described by Sano *et al.* (1991). Positive clones were subcloned into pUC 118 plasmid vector, and DNA sequences were determined with a model 373 A DNA sequencer using Taq Dye primer cycle sequencing kit (Applied Biosystems, Foster City, C. A., U. S. A.).

Assay of mRNA

All RNA samples were treated with RNase-free DNase (0.1 Units per microgram of RNA) for 15 min. at room temperature and then for 10 min. at 90 °C just prior to first strand cDNA synthesis. cDNA synthesis was primed by random hexamers, according to recommendations of the supplier (TAKARA technical manual). Synthesis reactions typically contained 10 ng of poly (A⁺) RNA in a volume of 20 μ l. The cDNA products were then serially diluted with TE buffer solution, and maize TSB gene specific cDNA was amplified simultaneously by PCR. Amplification condition was as follows : 30 cycles of 94 °C for 1 min., 55 °C for 2 min., 72 °C for 4 min. Aliquots of each reaction were then fractionated on 2 % agarose gels and Southern hybridization was conducted with maize TSB gene.

Northern hybridization

30 μ g of total RNA from MR 1 and control seedlings were denatured, fractionated on 1 % formaldehyde-agar-

ose gel, and blotted onto nylon membrane (Hybond N+, Amersham). Northern hybridization was performed as reported previously (Sano. *et al.* 1991)

Results

The effect of L-tryptophan on the growth of the 5MT resistant mutant (MR 1)

MR 1 and control seeds were cultured on MS agar medium containing L-tryptophan during a 14 days period following germination and harvested for dry weight measurements. Table 1 shows the effect of L-tryptophan on the growth of MR 1 and control seedlings. The dry weight in both MR 1 and control seedlings decreased gradually with increased amount of L-tryptophan. The decrease of MR 1 dry weight by L-tryptophan supplementation was lower than that of control. The MR 1 seedlings grown on medium containing 100 ppm and 1000 ppm L-tryptophan showed respectively 11.7 % and 42.1 % decreased dry weight, compared to those grown on medium without L-tryptophan. However, control seedlings grown on medium containing 100 ppm and 1000 ppm L-tryptophan showed respectively 23.2 % and 64.4 % decreased dry weight, compared to those grown on medium without L-tryptophan.

Enzyme assays

The AS activity was measured in MR 1 and control plants grown on MS agar medium with or without 5MT. As shown in Table 2, the activity of AS in MR 1 and control plants grown on the medium without 5MT showed no significant differences. However, the activity level observed in MR 1 plants grown on the medium with 5MT was 2 times higher than that of control plants. It was also detected that the activity of enzymes from MR 1 plants was 50 % inhibited when 20 ppm L-tryptophan was added to their extracts, while the enzyme from control plants was 50 % inhibited by 5 ppm

Table 1. The effect of L-tryptophan on the growth of MRI and control plants

Phenotype	Tryptophan concentration (ppm)	Dry weight ¹⁾ (mg/plants)	Inhibition percent
MR1	0	900±50	0
	100	798±72	11.7
	250	721±44	19.8
	500	660±47	26.5
	1000	520±24	42.1
Control	0	915±12	0
	100	702±65	23.2
	250	662±45	27.6
	500	469±27	48.7
	1000	325±29	64.4

¹⁾ The seeds were cultured on the MS agar (0.8 %) medium containing various concentrations of L-tryptophan. Dry weight of plant was measured after 14 days.

L-tryptophan. The activity of AS extracted from both plant materials was completely inhibited by 1,000 ppm L-tryptophan (Fig. 1). The results of TS assay are shown in Table 3. The activity of TS in MR 1 was four times higher than that in control plants.

Isolation of gene encoding tryptophan synthase B (TSB)

The maize inbred line (IK) leaf cDNA library was screened with selected fragments derived from PCR-amplified sequences. Fig. 2 shows amino acid homology of maize gene encoding tryptophan synthase B compared to the TSB genes from *Arabidopsis*. In terms of amino acid level, this gene has a high degree of similarity to the *Arabidopsis* gene and yeast gene; (ca 89 % and 63 % respectively), while the similarity to other organisms was in the range of 46 % -53 %.

TSB gene expression

To determine the expression of tryptophan synthase B gene, we assayed RNA isolated from seedling shoots,

seedling roots, tassels, silks and ears of control plants. As shown in Fig. 3, TSB specific RNAs were detected in all organs. In order to determine the level of expression of TSB gene in MR 1 and control plants, total RNA was directly isolated from MR 1 and control plants grown on MS medium with 25 ppm 5MT and on MS medium without 5MT, and all of them were used for Northern hybridization. The TSB gene showed higher levels of expression in the roots than in the shoots, and lower levels of expression in plants grown in presence of 5MT than in 5MT-free MS medium. It is also possible to note that the TSB gene in the roots of MR 1 plants grown in MS medium without 5MT showed higher levels of expression than in that of the control plants (Fig. 4).

Discussion

The resistant cell lines to 5-methyltryptophan accumulate higher levels of free tryptophan due to the altered feedback regulation of the tryptophan biosynthetic enzyme, anthranilate synthetase (Widholm, 1974). In the present study, we analyzed two tryptophan biosynthetic enzymes, anthranilate synthetase and tryptophan synthase in maize mutant plants (MR 1) resistant to 5-methyltryptophan which have increased amount of tryptophan (Kang and Kameya 1993). In assays of tryptophan biosynthetic enzyme activity, extracts from both MR 1 and control seedlings were able to convert chorismic acid to anthranilate acid and indole to tryptophan. In seedlings grown without 5MT, anthranilate synthetase activity of resistant mutant was similar to that of the control seedling but it was 2 times higher than that of control seedlings when grown with 5MT (Table 2). Moreover, the MR 1 plants had an anthranilate synthetase which was less sensitive to inhibition by L-tryptophan (Fig. 1) as pointed out in cultured cells by Widholm (1972). As a result of this relaxed feedback control, it is considered that free tryptophan levels increased in MR 1 as indicated in our previous report (Kang and Kameya 1993). The results of assay of tryptophan synthase showed that the enzyme activity of MR 1 was 4 times higher than that of the control (Table 3), which also may confirm the increment of free tryptophan levels in MR 1 mutants, although more continued studies are needed to understand the above mechanism.

In order to investigate the fundamental process for the biosynthesis of tryptophan, we isolated and sequenced TSB gene in maize. The isolated gene had a very high homology to that previously reported in *Arabidopsis* (Berlyn *et al.* 1989). And it was found that the TSB gene expression was higher in MR 1 than in controls, which has not been examined in cultured mutant cells (Widholm 1974).

The present report is the first characterization of AS and TS in whole plants resistant to 5MT, which may demonstrate that MR 1 plants help to establish an alternative mechanism for the biosynthesis of tryptophan.

Table 2. Activity of the anthranilate synthetase in maize seedlings at 14 days after germination

Genotype	Total tissue protein mg/g dry wt.	Anthranilate synthetase n mol/h×mg protein	
		Not 5MT treated	5MT treated
MR1	279.6	3.34±0.5	1.98±0.5
Control	264.1	3.16±0.5	0.85±0.5

Fig. 1. Feedback inhibition by L-tryptophan of anthranilate synthetase activity in extracts from MR 1 and control seedlings.

Table 3. Activity of tryptophan synthase in MR1 and control seedlings at 14 days after germination

Genotype	Total tissue protein mg/g dry wt.	Tryptophan synthase n mol/h mg protein
MR1	279.6	159.35±72
Control	264.1	40.59±92

		*		*	*	*	*	*	
M-TSB	QRPDSFGRFG	RFGGKYVPET	LMHALTELEN	AFHALATDDE	FQKRELDGIL	50			
A-TSB	<u>QRPDSFGRFG</u>	<u>RFGGKYVPET</u>	<u>LMHALSELES</u>	<u>AFVALATDDD</u>	<u>FORRELAGIL</u>				
	KDYVGRESPL	YFAERLTHEY	* * * *	YLKREDLNHT	GAHKINNAVA	100			
	<u>KDYVGRESPL</u>	<u>YFAERLTHEY</u>	<u>RRENGEGPLI</u>	<u>YLKREDLNHT</u>	<u>GAHKINNAVA</u>				
		*		*					
	QALLAKRLGK	QRIIAETGAG	QHG VATATVC	ARFGLEQIIY	MGGGAQDMER	150			
	<u>QALLAKRLGK</u>	<u>QRIIAETGAG</u>	<u>QHG VATATVC</u>	<u>ARFGLECIY</u>	<u>MGGGAODMER</u>				
		*							
	QALNVFRMRL	LGAEVRAVHS	GTATLKDATS	EAIRDWVTNV	ETTHYILGSV	200			
	<u>QALNVFRMRL</u>	<u>LGAEVRAVHS</u>	<u>GTATLKDATS</u>	<u>EAIRDWVTNV</u>	<u>ETTHYILGSV</u>				
		*	*	*	**				
	AGPHYPMMV	REFHKVIGKE	TRKQALERWG	MDPDVLVACV	GGGSNAMGLF	250			
	<u>AGPHYPMMV</u>	<u>RDEHAVIGKE</u>	<u>TRKQALEKWG</u>	<u>GKPDVLVACV</u>	<u>GGGSNAMGLF</u>				
		*	*	*	*				
	HEFVEDQDVR	LVGLEAAGHG	VDTDKHAATL	TKGQVGV LHG	SMSYLLQDDD	300			
	<u>HEFVNDTEVR</u>	<u>MTGVEAAGFG</u>	<u>VDTDKHAATL</u>	<u>TKGDVGV LHG</u>	<u>AMSYLLQDDD</u>				
	*								
	GQVIEPHSIS	AGLDYPGVGP	EHSFFKDMGR	AEYYSITDEE	ALEAFKRVSR	350			
	<u>GQVIEPHSIS</u>	<u>AGLDYPGVGP</u>	<u>EHSFFKDMGR</u>	<u>AEYYSITDEE</u>	<u>ALEAFKRVSR</u>				
			*	*	*				
	LEGIIPALET	SHALAYLEKL	CPTLPDGVRV	VLNCSGRGDK	DVHTAS	396			
	<u>LEGIIPALET</u>	<u>SHALAYLEKL</u>	<u>CPTLSDGIRV</u>	<u>VLNFSGRGDK</u>	<u>DVQTVA</u>				

Fig. 2. Alignment of the amino acid sequences of the tryptophan synthases B from *Arabidopsis* (A-TSB) and maize (M-TSB). Homologous or nonhomologous amino acids are indicated by underlines or stars, respectively.

Fig. 3. Detection of TSB-specific gene. Southern hybridization was conducted with maize TSB gene probe. A : 14 days old seedling shoots ; B : 14 days old seedling roots ; C : tassel ; D : silk ; E : ear.

Fig. 4. Northern hybridization analysis of TSB gene in maize. A 30 μ g sample of total RNA was fractionated by 1.2 % agarose gel electrophoresis, transferred to nylon membrane, hybridized with radioactivity labeled TSB cDNA probe. A : Control (no treatment) ; B : MR 1 (no treatment) ; C : Control (25 ppm 5 MT treatment) ; D : MR 1 (25 ppm 5 MT treatment).

tophan in higher plants.

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