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Selection and characterization of a rice mutant resistant to 5-methyltryptophan

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Summary. A rice plant resistant to 5-methyltryptophan (5MT) was selected from mutagenized M3 seeds (*Oryza sativa* L. var. Sasanishiki) originating from panicles treated with ethylene imine (0.2%) 2 h after flowering. When germinated on 5MT-containing medium, the seeds (M4) from selfed plants segregated with a 3 resistant:1 sensitive ratio, indicating that the plant was heterozygous for a resistance gene and that the resistance was dominant. The resistance was also expressed in callus derived from seeds. Analysis of the free amino acids in seeds, seedlings, and calli showed that homozygous resistant plants (*TR1*) contained higher levels of total free amino acids than sensitive plants. In particular the levels of tryptophan, phenylalanine, and histidine were, respectively, 8.5, 5.4, and 4.9 times higher than those in the sensitive plants.

Key words: Ethylene imine – Dominant gene – Free amino acids – 5-methyltryptophan resistance – Rice mutant

Introduction

The mutants resistant to amino acid analogs are useful not only for studying amino acid biosynthesis in higher plants (Miflin et al. 1983), but also for improving the nutritional quality of the major cereal grains (Brock and Langridge 1975).

Free amino acids accumulate in the mutant tissues (Widholm 1972). Several resistant mutants to amino acid analogs have been selected from cell cultures (Schaeffer and Sharpe 1981; Ranch et al. 1983; Negrutiu et al. 1984; Wakasa and Widholm 1987) as well as from mutagenized seeds (Hasegawa and Mori 1986) and embryos (Bright et al. 1979). Tryptophan analog-resistant mutants were selected in *Datura* (Ranch et al. 1983) and in rice (Wakasa and Widholm 1987), and they accumulate free tryptophan in callus and leaf.

In this paper, we describe the induction, selection, and characterization of a 5-methyltryptophan (5MT) resistant mutant. The results demonstrate that the mutant accumulates free amino acids in seeds and that the resistance is controlled by a single, dominant nuclear gene.

Materials and methods

Mutagenic treatment

The rice (*Oryza sativa* L. var. Sasanishiki) grown in pots was used in all experiments. Three to four hundred panicles in different developmental stages, at 2 h before flowering, and at 2 h, 5 days, 10 days, and 15 days after flowering, were immersed in a 0.2% ethylene imine (EI) solution for 24 h at about 25 °C, and then washed immediately in running tap water. The plants were cultivated in a greenhouse at 25°–30 °C. M_1 seeds were harvested approx. 50 days after treatment.

Frequency of chlorophyll mutations

The M_1 seeds obtained from each EI treatment were sown on the seed beds and grown in the greenhouse at 25°–30 °C. After 10 days, the number of chlorophyll mutants was recorded.

Selection of 5MT-resistant mutants

M_3 seeds from the plants treated with EI at 2 h after flowering were incubated in water for 48 h and grown at about 25 °C in a greenhouse in nutrient solution (Satake and Koike 1984) containing 25 ppm 5MT. After 10 days of culture, surviving seedlings were selected, transplanted to seed beds, and kept in the greenhouse for ca. 30 days. The surviving plants were transplanted into soil in pots and grown in a plant growth cabinet 12 h photoperiod, with day and night temperatures of 25° and 18 °C, respectively.

Test for 5MT resistance

The M_4 seeds from 5MT-resistant plants (*TR1*, *TR2*) were sterilized in 2% sodium hypochlorite for 15 min, and washed three times in sterile distilled water. They were then cultured on agar (0.8%) containing 25 ppm 5MT. The length of plantlets was measured after 10 days at 25 °C under fluorescent light (3,000 lx). To test 5MT resistance in the callus induced from M_4 seeds it was cultured on MS (Murashige and Skoog 1962) medium containg 2 ppm 2.4-dichlorophenoxyacetic acid (2.4-D) and 25 ppm 5MT. The cultures were incubated under continuous light (3,000 lx) at 25 °C. The rate of callus formation was investigated after 30 days. The level of 5MT resistance of the callus was tested on small pieces of callus (about 0.5 mg fresh weight) transferred to MS medium containing a range of 5MT concentrations and cultured under the same conditions described above. Fresh weight of callus was measured after 30 days incubation.

Analysis of free amino acids

Analysis of free amino acids was carried out using M_5 seeds from a homozygous resistant plant. The seedlings and callus were cultured in nutrient solutions with or without 25 ppm 5MT for 14 days at 25 °C. One gram of ground seeds (hull-less), 1 g of seedlings, and 2 g of fresh weight callus were homogenized and extracted twice with a mixture of 10 ml chloroform: methanol: water (5:12:3, v/v/v) (Wakasa and Widholm 1987). The extracts were subsequently dissolved in 2 ml 0.15 *M* lithium citrate buffer (pH 2.2) and analyzed with an automatic amino acid analyzer. SHIMADZU CTO-6A (Japan). Each experiment was repeated twice.

Results

Frequency of chlorophyll mutations in M_1 seedlings

In the treatment with EI at 2 h after flowering and 5 days after flowering, the frequency of chlorophyll mutants was 11.5% of 182 and 1.2% of 248 M_1 seeds sown, respectively. In the other three treatments, no chlorophyll mutants were observed.

Selection of 5MT-resistant seedlings

Twenty-four seedlings resistant to 5MT were screened from ca. 22,000 M_3 seeds originated from the treatment of EI at 2 h after flowering. These resistant seedlings grew in the nutrient solution containing 25 ppm 5MT, while control seedlings did not. Among the resistant seedlings, two seedlings developed to mature plants and produced seeds. The other seedlings died during cultivation or did not produce any seeds. Genetic analysis indicated that the two plants and their progenies behaved as 5MT-resistant mutants. The mutants were designated as *TR1* and *TR2*.

Inheritance of 5MT resistance

M_4 seeds obtained from the selfing of *TR1* and *TR2* heterozygous plants were tested for 5MT resistance with 25 ppm 5MT, which completely inhibited the growth of

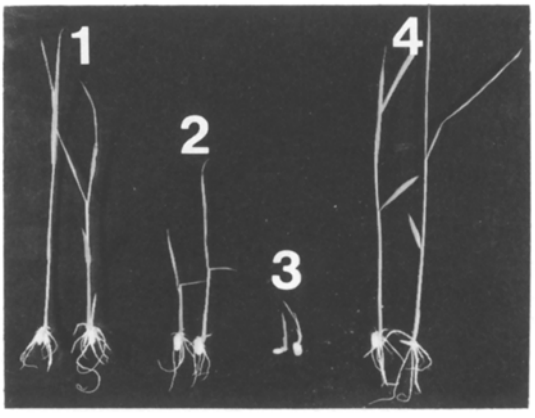

Fig. 1. Growth of progenies of *TR1* (1), *TR2* (2), and of the original variety, Sasanishiki (3, 4), in nutrient solution with 5MT or without 5MT. 1, 2, 3: with 25 ppm 5MT; 4: without 5MT

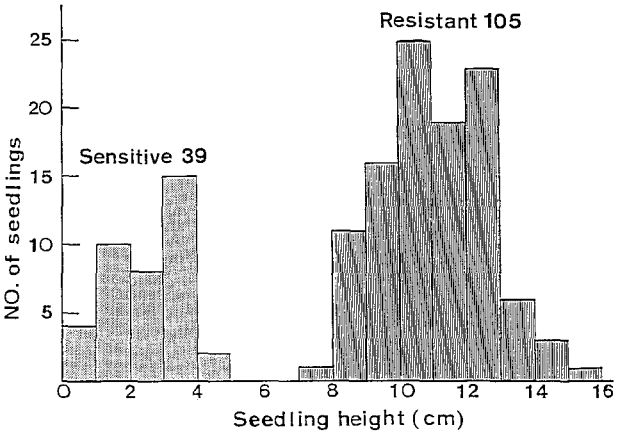

Fig. 2. Distribution of seedling height in the progeny of the heterozygous mutant plant *TR1* resistant to 5MT. Seedlings longer than 7 cm were classified as resistant. Seedling height was measured 10 days after culturing with 25 ppm 5MT

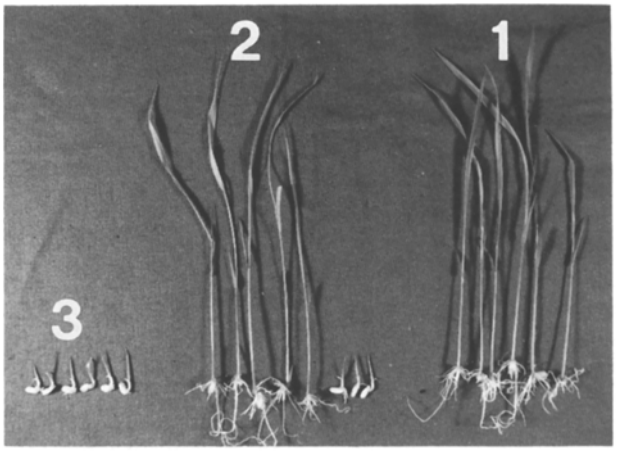

Fig. 3. Presence versus absence of segregation of homozygous and heterozygous progenies derived from the original plant of the *TR1* mutant. Seedlings of the homozygote (1) and the heterozygote (2) *TR1* progenies and of the original variety, Sasanishiki (3) were cultured with 5MT 25 ppm for 10 days

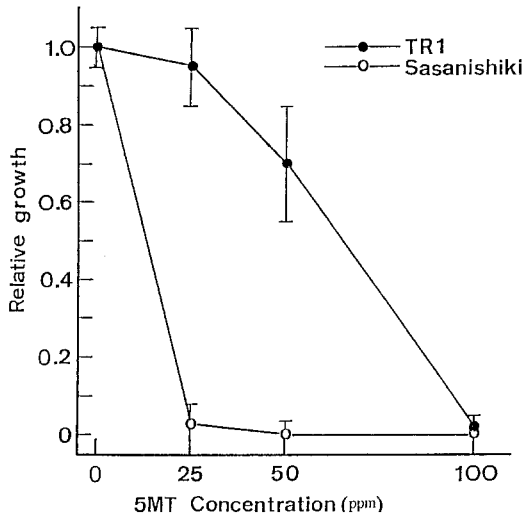

Fig. 4. Effect of 5MT concentration on callus fresh weight of *TR1* as compared to Sasanishiki. The fresh weight was determined after 1 month of incubation

Table 1. Callus formation in the M_4 progeny of the original heterozygous *TR1* plant on MS medium containing 25 ppm 5MT. The callus formation was recorded after 30 days

Phenotype	No. of M_4 seeds transferred to 5MT medium	No. of seeds forming the callus	No. of seeds not forming the callus	Percent of callus formation (%)
TR1	50	36	14	72
Sasanishiki (Control)	10	0	10	0

the control plants (Fig. 1). The segregation ratio of resistant to sensitive seedlings in progeny of *TR1* was approximately 3:1 ($X^2=0.33$, $0.5<P<0.75$) (Fig. 2). The *TR2* segregations were more complex (this mutant will not be described here). *TR1* seedlings were generally larger and had better root growth than *TR2*. For further genetical analysis of *TR1*, M_5 seeds were obtained from all selfed M_4 plants showing resistance, and their resistance was tested with 5MT-containing medium. Of 71 progenies examined, 22 plants produced all resistant seedlings, and 49 plants had both resistant and sensitive seedlings (Fig. 3). The ratio of homozygote to heterozygote in resistant plants of the M_4 generation was 1:2 ($X^2=0.175$, $0.5<P<0.75$).

Callus formation in the segregating M_4 progeny of *TR1* on the medium containing 25 ppm 5MT is shown in Table 1. The percentage observed was similar to the segregation ratio recorded for the seedlings. *TR1* callus grew at 50 ppm 5MT, whereas sensitive callus from the original variety did not grow even at 25 ppm (Fig. 4).

Free amino acid analysis

Analysis of free amino acids in *TR1* homozygous seeds, seedlings, and calli increased approximately two- to fourfold compared to sensitive control plants (Table 2). The levels of single free amino acids differed in seeds, seedlings, and calli and were individually affected by the mutant *TR1*. In the seeds tryptophan, phenylalanine, and histidine were, respectively, 8.0, 5.4, and 4.9 times higher in *TR1* than in the sensitive plants. However, calli and seedlings of *TR1* had lower levels of tryptophan than the

Table 2. Free amino acid contents in *TR1* homozygous seeds, seedlings, and calli compared to those of the original variety Sasanishiki. Numbers given are in nmol/g fresh weight

Amino acid	Sasanishiki			*TR1*			Ratio of resistant to senstive		
	Seeds	Seedlings	Calli	Seeds	Seedlings	Calli	Seeds	Seedlings	Calli
ASP	81	938	901	127	2,525	847	1.5	2.6	0.9
THR	54	1,303	165	79	2,866	1,120	1.4	2.2	6.8
SER	198	1,077	1,037	236	2,409	4,349	1.2	2.2	4.1
ASN	932	28,222	47	1,436	75,493	886	1.5	2.6	18.5
GLU	179	2,398	2,854	352	3,628	4,569	1.9	1.5	1.6
PRO	156	574	317	446	1,465	7,783	2.8	2.5	24.5
GLY	79	191	168	224	304	1,605	2.8	1.5	9.5
ALA	496	1,102	151	1,592	2,992	1,824	3.2	2.7	12.0
VAL	60	1,271	126	74	1,829	493	1.2	1.4	3.9
MET	10	47	52	25	131	133	2.4	2.7	2.6
ILE	15	714	56	20	891	192	1.3	1.2	3.4
LEU	18	301	71	26	437	282	1.4	1.5	3.9
TYR	33	166	87	66	146	71	2.0	0.9	0.8
PHE	15	536	80	85	639	107	5.4	1.2	1.3
TRY	25	210	18	204	128	17	8.0	0.6	0.9
HIS	17	649	100	85	661	151	4.9	1.0	1.5
ARG	68	1,097	14	150	2,015	72	2.2	1.8	5.1
Total	2,436	40,796	6,244	5,227	98,559	24,501	2.1	2.4	3.9

control variety. The levels of asparagine, proline, and alanine in *TR1* calli were, respectively, 18.5, 24.5, and 12.0 times higher than in Sasanishiki. *TR1* seedlings also had higher levels of threonine, asparagin, proline, and alanine.

Discussion

From an M_3 population obtained by EI treatment of panicles, a mutant, *TR1*, resistant to 5MT was selected. The 5MT resistance was inheritable and was expressed in the callus as well as in the seedlings. The segregation of resistance and sensitivity in progenies of *TR1* best fit a 3:1 ratio, allowing us to conclude that 5MT resistance is controlled in rice by a single, dominant nuclear gene.

The 5MT resistance of *TR1* calli indicates that the *TR1* mutation can generate useful materials for the study of somatic hybridization (see also Lee and Kameya 1989).

Resistance to 5MT in plant tissue cultures and regenerated plants from resistant lines is associated with increased amounts of free tryptophan (Ranch et al. 1983; Wakasa and Widholm 1987). However, up to now no report has shown an increase of this amino acid in seeds of 5MT-resistant plants, although a few reports have shown an increase of lysine in rice resistant to S-2-aminoethyl-L-cysteine (Schaeffer 1981), threonine in barley resistant to lysine plus threonine (Bright et al. 1982), and proline in rice resistant to hydroxy-L-proline (Mori et al. 1989).

The present work is the first example of selection of amino acid analog resistant plants, which are controlled by a single, dominant nuclear gene and which produce seeds containing higher levels of free amino acids than the sensitive plants. The homozygous resistant mutants obtained can be important in studies of amino acid biosynthesis and in improving the nutritional quality of rice.

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