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A 5-methyltryptophan resistant rice mutant, MTR1, selected in tissue culture

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Summary. Cell lines resistant to tryptophan analogue 5-methyltryptophan (5MT) were selected in seed-derived calli of *Oryza sativa* L. var. 'Norin 8'. Plants were regenerated (R_1) from one selected callus line (MTR1). In three out of the six R_1 plants, 5MT resistance was inherited in the R_2 and R_3 generations as a dominant nuclear mutation. Segregation ratios in the progeny of heterozygous plants were 1 : 1. Morphological and fertility variation seen in some of the R_2 plants were not correlated with 5-methyltryptophan resistance. Resistance in the MTR1 callus was due to the accumulation of high levels of free tryptophan (87-fold) that was associated with an increase in free phenylalanine content (9-fold). The leaves of resistant plants also contained elevated levels of free tryptophan and phenylalanine.

Key words: 5-Methyltryptophan resistance – Plant tissue culture selection – Rice mutant – Tryptophan

Introduction

The use of plant tissue cultures for the selection of mutants resistant to various compounds can be a very powerful tool since many millions of cells can be placed under a uniform and controlled selection pressure. This method is especially advantageous when the mutation frequency is low or when selection would be difficult at the whole plant level. Selection for amino acid analogue resistance to improve nutritional quality is a worthwhile goal for plant breeders and is difficult to carry out at the whole plant level.

Several examples of selection for resistance to the tryptophan (trp) analogue 5-methyltryptophan (5MT) have been reported (Widholm 1972; Widholm 1978; Carlson and Widholm 1978; Widholm 1980; Ranch et al. 1983) and the resistance is, in most cases, due to the presence of a feedback altered anthranilate synthase which allows the accumulation of free trp. Anthranilate synthase is the key feedback control enzyme in the trp biosynthetic branch of the shikimate pathway (reviewed by Gilchrist and Kosuge 1980).

For cellular selection to be useful for plant breeding, plants must be regenerated which can pass the selected trait to their progeny. An important question in all in vitro selection experiments is whether the trait selected at the cell level will be expressed in regenerated plants. In the case of 5MT resistance, tobacco plants regenerated from resistant cells did not express the altered form of anthranilate synthase (Widholm 1980; Brotherton et al. 1986) while *Datura innoxia* plants did (Ranch et al. 1983).

Rice callus was used by Schaeffer and Sharpe (1981) to select for resistance to the lysine analogue, aminoethylcysteine. In regenerated plants the lysine proportion of the seed protein was increased by 10% and the seed protein content was increased by 48% in the next generation.

In this report we describe the use of rice callus in selecting for 5MT resistance. We report plant regeneration from one line and show inheritance of the resistance trait to the seed progeny. Preliminary reports on this research have already been published (Wakasa and Widholm 1982; Wakasa 1985).

Materials and methods

Calli were initiated from hulled rice seeds (*Oryza sativa* L. var. 'Norin 8') that were surface sterilized in 70% ethanol for 1 min and subsequently in 7% Na hypochlorite for 60 min. For callus induction the seeds were placed on Murashige and

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; MS = Murashige and Skoog (1962) basal medium; 5MT = D,L-5-methyltryptophan; phe = phenylalanine; trp = tryptophan; tyr = tyrosine

Skoog (1962) basal medium (MS) with 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l α-naphthaleneacetic acid, 0.01 mg/l kinetin and 0.9% Difco agar (MXNK medium).

For selection of 5MT resistance, calli (50 days after initiation) were divided into 10 to 20 mg fresh weight pieces and placed on selection medium (MXNK plus 300 μM 5MT). Calli which grew on the selection medium were placed onto plant regeneration medium (MS basal medium with 0.01 mg/l indoleacetic acid, 6.75 mg/l benzyladenine and 0.9% Difco agar) and the plantlets which formed were grown further on the same medium without growth hormones. The regenerated plants were transplanted into an autoclaved mixture of vermiculite and soil (1 : 1) and grown in a culture room at 27° to 28 °C under a 12 h fluorescent light regime (ca. 100 $\mu Em^{-2} s^{-1}$). Plants which survived were transplanted into soil in pots in the greenhouse and grown to maturity.

Plant morphological observations and chromosome number determinations were carried out with plants grown in pots outside.

Chromosome pairing was studied in pollen mother cells fixed in ethanol : acetic acid (3 : 1) containing 0.5% ferric chloride and stained with aceto-carmine.

In order to test inheritance of 5MT resistance, calli were initiated from seeds as described above or from roots of seedlings germinated on MS medium lacking hormones. The root pieces used to obtain callus were placed on MS medium with 2 mg/l 2,4-D while the seedlings were transplanted to soil to obtain seed. Calli from individual seedlings were first multiplied then tested for resistance by placing 10 mg pieces on the above medium containing different 5MT concentrations. To score resistance phenotype fresh weights of six to ten pieces were averaged after a 21 day incubation.

Sensitivity of seedlings to 5MT was tested directly by germinating surface sterilized husked seeds on MS medium without hormones but with 0.3 or 1 mM 5MT. Plants were examined after a 14 day incubation.

Free amino acids were extracted by homogenizing a 2 g fresh weight callus or a 1 g fresh weight sample of leaves with a glass-teflon homogenizer in 10 ml chloroform : methanol : water (5 : 12 : 3). Following centrifugation at 12,000×g for 5 min, the pellet was homogenized again in 10 ml chloroform : methanol : water, centrifuged and the supernatants combined with 5 ml chloroform and 7.5 ml water. The mixture was centrifuged as above to separate the phases and the top phase was removed and dried under vacuum at 45 °C. The residue was dissolved in 2 ml 0.15 M Li citrate buffer (pH 2.2) and analyzed in a Beckman 119CL amino acid analyzer using physiological fluid methodology.

Results and discussion

Callus selection

When 1,000 rice calli pieces, each between 10 to 20 mg fresh weight, were incubated on medium containing the normally inhibitory concentration of 5MT (300 μM), three pieces showed growth after 21 days and one after 42 days. Only the latter callus, line MTR1, formed plants when transferred to the regeneration medium. Six of these plants set seed in the greenhouse. In this paper the regenerated plants are denoted R_1 and their selfed progeny R_2, etc. The six plants which produced seed are numbered 2 through 7.

Inheritance of 5MT resistance in R_2

In order to establish the level of 5MT that is suitable to distinguish resistant and sensitive progeny, calli were initiated from individual R_2 seeds or roots and grown in the presence of different 5MT concentrations (Table 1). Significant differences in the response of individual R_2 progeny were detected with 30 and 100 μM 5MT which completely inhibited the sensitive control calli. Calli from three R_2 seedlings (2, 3 and 5) grew at these 5MT concentrations while the calli from the other three R_2 seedlings (4, 6 and 7) and the two wild type controls did not. Table 2 contains a summary of the

Table 2. Inheritance of 5MT resistance in R_2 as established by testing inhibition of callus growth

R_1 plant origin	No. of calli	
	r	s
2	9	5
3	7	12
4	0	17
5	4	9
6	0	13
7	0	14
'Norin 8' control	0	9

Table 1. Test of R_2 calli for resistance to 5MT initiated from individual R_2 rice seeds or roots. The fresh weights of 6 to 10 calli pieces (10 mg each when inoculated) were measured after 21 days of incubation and the means with standard deviations are listed

R_1 plant origin	Sensitivity rating of the calli	Fresh wt (mg) of calli with different 5MT concentrations (μM)					
		0	10	30	100	300	1,000
2	r	51.5± 9.8	56.2±18.9	85.6±24.7	36.0±14.1	22.7±23.3	5.0±1.4
3	s	34.8± 9.1	27.7±12.9	12.6± 2.2	9.0± 2.2	6.5± 2.0	9.0±2.8
	r	31.3±11.0	37.8± 9.2	32.6±19.1	31.8± 9.0	15.3±14.9	8.1±3.6
4	s	31.0± 5.8	50.6±17.6	6.6± 2.7	5.6± 1.0	6.8± 2.0	5.5±1.2
5	s	22.0± 7.9	13.6± 4.7	6.8± 2.5	6.8± 1.6	8.1± 2.3	3.8±1.6
	r	84.0±26.0	105.8±46.1	106.5±46.4	100.6±43.0	4.8± 4.1	3.5±1.2
6	s	43.6±22.4	16.6± 5.2	12.6±11.6	9.0± 2.8	6.6± 2.1	7.3±3.9
7	s	59.3±12.8	22.1± 8.4	8.8± 1.8	7.6± 2.0	5.6± 1.2	7.0±2.6
'Norin 8' control	s	23.6± 8.3	25.3±19.7	7.5± 2.2	4.8± 0.9	4.5± 0.8	5.5±1.8
'Norin 8' control	s	35.5± 9.5	14.6± 3.7	19.3±20.5	8.1± 2.4	8.0± 1.5	6.3±2.2

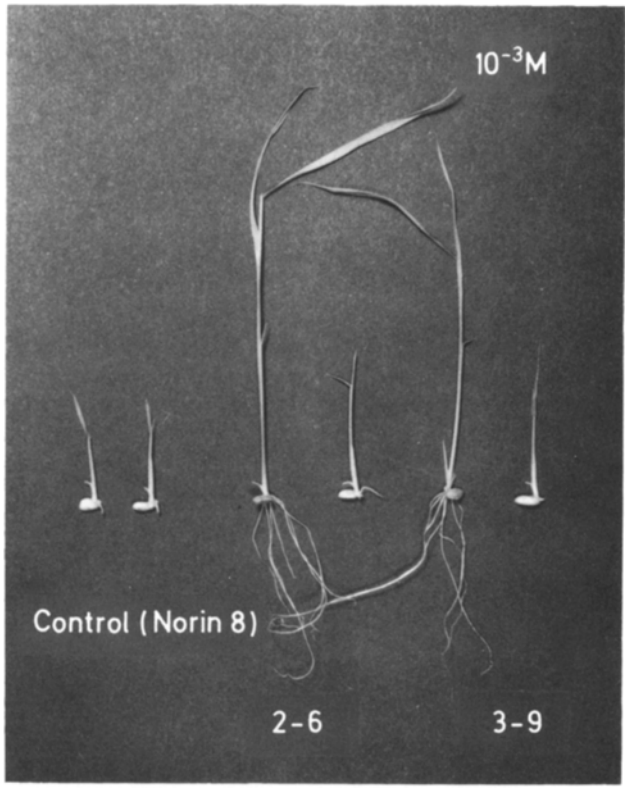

Fig. 1. Effect of 1 mM 5MT on the growth of control ('Norin 8') and segregating R_3 populations (2–6 and 3–9) after 14 days. One seedling each of R_3 progeny from plants 2–6 and 3–9 shows either resistance and sensitivity to 5MT

data on the inheritance of 5MT resistance in R_2. R_1 plants No. 2, 3 and 5 produced progeny upon selfing which segregated for 5MT resistance. Accordingly, the R_1 plants were heterozygous for 5MT resistance that behaved as a dominant trait. The number of seedlings tested was too low to establish a segregation ratio. No resistance was detected in calli originating from progeny of plants 4, 6 or 7.

Inheritance of 5MT resistance in R_3

Plants were grown to maturity from several of the R_2 seeds, the root calli of which were tested for 5MT resistance. Seed obtained by selfing these plants was tested for 5MT resistance by germination in the presence of 300 μM 5MT or 1 mM 5MT under aseptic conditions rather than by the callus assay used to score R_2. These 5MT concentrations severely inhibited the growth of the wild type and some but not all of the R_3 seedlings (Fig. 1, Table 3). When previous testing had shown the R_2 plant calli to be 5MT sensitive then all of the R_3 seedlings produced from the plant were scored as sensitive (Table 3) while if the R_2 calli were 5MT resistant then some of the R_3 seedlings were also resistant. The segregation of resistance and sensitivity in the R_3 regeneration best fits a 1 : 1 ratio (Table 3).

If resistance is controlled by a dominant nuclear mutation, as concluded from segregation in R_2, selfed seeds of resistant R_2 plants would be expected to

Table 3. Inheritance of 5MT resistance in R_3 produced by selfing R_2 plants. Resistance of seedlings was established by growth inhibition data on 300 μM 5MT or 1 mM 5MT

R_2 plant origin	Chromosome no. (2n)	Phenotype of callus from R_2 plant	No. of seedlings			Significance test to 1:1 segregation in χ^2 test ($P > 0.05$)
			Resistant	Sensitive	ng[b]	
2-1	24	r	46	31	11	NS
2-2	24	r	40	39	8	NS
2-3	–	r	47	36	5	NS
2-4	24	r	37	26	3	NS
2-6	24	r	45	40	10	NS
			33	30	2[a]	NS
3-2	–	r	67	99	11	NS
3-4	24	r	38	38	10	NS
3-6	–	s	0	95	5	
3-7	24	r	88	87	7	NS
3-9	24	r	41	37	2	NS
4-8	24	s	0	99	0	
5-1	24	r	58	62	11	NS
			39	37	10[a]	NS
5-3	24	s	0	99	0	
5-5	24	r	111	109	9	NS
6-7	–	s	0	95	0	
7-7	–	s	0	92	0	
Control-1	24	s	0	95	1	
Control-2	24	s	0	62	4[a]	

[a] On 1 mM 5MT
[b] No. of seeds which did not germinate

Table 4. Free amino acids in callus initiated from R_2 rice seeds or roots. Numbers given are nmol/g fresh wt

Amino acid	5MT sensitive (R_2 plant of origin)								
	4-7	4-1	3-3	7-5	5-4	5-3	6-6	'Norin 8' control	Mean
ASP	233	282	285	253	180	222	178	259	236
THR	132	93	107	204	138	117	180	115	136
SER	440	503	485	1,125	844	740	393	612	643
ASN	231	243	106	267	30	375	314	262	228
GLU	746	1,103	906	2,092	1,660	1,426	394	1,320	1,206
GLN	205	381	455	919	751	220	182	475	448
PRO	23	0	40	315	53	0	105	38	72
GLY	146	186	251	402	390	178	208	221	248
ALA	277	360	860	1,626	1,440	243	274	427	688
VAL	155	150	175	251	335	167	176	174	198
CYS	158	114	129	526	268	118	156	143	202
MET	15	36	38	65	52	36	59	39	42
ILE	58	56	54	103	126	62	58	61	72
LEU	51	51	87	151	137	45	32	82	80
TYR	36	40	50	52	44	37	81	48	48
PHE	166	162	173	104	130	157	140	153	148
TRP	3.1	0	0	0	0	0	0	0	0.4
LYS	127	109	225	227	247	290	286	240	219
HIS	80	55	74	58	94	68	138	71	80
ARG	158	127	218	204	171	467	421	257	253
Total	3,440	4,051	4,718	8,944	7,090	4,968	3,775	4,997	5,248

Amino acid	5MT resistant (R_2 plant of origin)					Amino acid ratio of resistant to sensitive
	5-5	3-4	3-2	2-4	Mean	
ASP	268	286	252	352	290	1.2
THR	149	154	88	136	132	1.0
SER	671	645	421	660	599	0.9
ASN	437	103	118	206	216	0.9
GLU	1,320	1,310	879	1,197	1,176	1.0
GLN	228	950	306	519	501	1.1
PRO	0	104	0	33	34	0.5
GLY	181	370	174	287	253	1.0
ALA	334	1,488	348	586	689	1.0
VAL	225	290	183	273	243	1.2
CYS	119	148	112	140	130	0.6
MET	14	26	37	25	26	0.6
ILE	70	98	59	73	75	1.0
LEU	52	176	74	132	108	1.4
TYR	65	70	59	101	74	1.5
PHE	1,715	903	896	1,432	1,236	8.4
TRP	34.5	60.5	14.3	30.0	34.8	87
LYS	315	207	204	399	281	1.3
HIS	139	109	79	148	119	1.5
ARG	412	154	210	414	298	1.2
Total	6,748	7,652	4,513	7,143	6,514	1.2

segregate 3 : 1 if the R_2 plant was a heterozygote. In the progeny of a homozygote no segregation should be expected. It is apparent that none of the 11 resistant R_2 plants was homozygous for the resistance trait since segregation was seen in the R_3 progeny (Table 3). The possibility exists that homozygous resistance may be lethal. However, if the resistance is controlled by a single dominant gene and if the homozygous dominants are then eliminated due to lethality then the heterozygous to homozygous recessive ratio should be 2 : 1 and not 1 : 1 as seen here. The possibility also exists that pollen carrying the 5MT resistance is not capable

of fertilization so that the trait is only transmitted through heterozygous females which would produce a 1 : 1 ratio of sensitive to resistant progeny. It is also possible that the female gametes carrying 5MT resistance could be inviable. Further genetic studies involving a large number of plants should resolve these questions.

Plant morphology and chromosome numbers

The 42 R_2 plants which had been tested for 5MT resistance as calli (Table 2) were grown in pots and 39 of them were observed for morphological changes. One 5MT sensitive plant (5-7) originating from plant 5 was approximately 20 cm shorter than the wild type (105 cm) and produced no seed due to sterility. The chromosome pairing during meiosis in this plant (5-7) was abnormal due to the presence of 11 bivalent and two or three univalent small chromosomes. One resistant plant (3-9) showed less tillering than the control and another resistant plant (5-6) showed low fertility since the seed number was approximately 2 to 4% of the control. Another sensitive plant (4-3) was 10 cm taller than the control. The other 35 plants showed normal morphology and fertility. Of the 27 plants showing chromosome numbers and pairing in the pollen mother cells, only plant 5-7 showed abnormality. Nine resistant plants which were found to have a normal diploid chromosome number ($2n = 24$) showed a 1 : 1 segregation (Table 3). These results indicate that 5MT resistance and 1 : 1 segregation were not associated with aneuploidy or gross chromosome abnormalities.

Amino acid analysis

Callus from several R_2 seedlings was studied to determine the mechanism of resistance to 5MT. Since past work has shown that 5MT resistance is usually due to trp overproduction caused by an alteration in the feedback control properties of the trp biosynthetic control enzyme, anthranilate synthase (Widholm 1972), free amino acids were measured in the callus cultures. Calli originating from four resistant plants and seven sensitive plants were analyzed and the mean levels of free tryptophan and phenylalanine in the resistant calli were respectively 87× and 8.4× higher than in the sensitive lines (Table 4). The trp values in all the sensitive lines except one were zero so the increase in trp noted in the 5MT resistant calli is clear but the calculated fold increase is not necessarily quantitative. The other free amino acid levels were not very different in the resistant and sensitive calli. Total free amino acid content was increased in the resistant calli by approximately 20%.

Analysis of free amino acids in leaves of plants grown from seed showed that plants carrying 5MT resistance also contained higher levels of free trp and phe than sensitive control plants. The leaves of resistant plants 2-4-2 and 5-1r(1) contained 54 and 70 nmol free trp/g fresh weight while the control, 'Norin 8', contained 2.8. The respective resistant plants contained 494 and 105 nmol free phe as compared to 69 nmol phe for 'Norin 8' per g leaf. No other free amino acid showed over a two-fold difference. Thus, the 5MT resistance which is expressed in root callus is also correlated with increased free trp and phe levels in the plant leaves.

While there was a large increase in free trp in the 5MT resistant cells and plants there was also an increase in free phe. This phe increase could be due to the stimulation of the phe and tyr feedback control enzyme chorismate mutase by trp (reviewed by Gilchrist and Kosuge 1980). In studies with several 5MT resistant tobacco, carrot (Hauptmann and Widholm, unpublished; Widholm 1978), *Datura innoxia* (Ranch et al. 1983) and Asparagus (Curtiss and Widholm, unpublished), some cases of several fold phe and/or tyr increases were noted in addition to trp, which was always increased. Corn callus selected as 5MT resistant was very similar to rice since only free trp and phe were increased and not tyr (Miao, Duncan and Widholm, unpublished). Recently Jacobsen et al. (1985) reported that two potato cell lines selected for 5MT resistance had only very small increases in free trp but had from two to 13-times the normal levels of free phe and tyr. The lines showed a low level of resistance to 5MT and cross resistance to phe and tyr analogues.

These studies show that it is possible to use in vitro selection to obtain mutant plants with increased levels of certain free amino acids. However, if homozygous plants cannot be obtained the trait will not be useful commercially.

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