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Non-proline-accumulating rice mutants resistant to hydroxy-L-proline

H. Hasegawa [1] and S. Mori [2]

[1] Radiation Center of Osaka Prefecture, 704 Shinke-cho, Sakai, Osaka 593, Japan
[2] Faculty of Agriculture, Kyoto Prefectural University, Shimogamo, Sakyo-ku, Kyoto 606, Japan

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Summary. Three rice (*Oryza sativa* L.) mutants resistant to hydroxy-L-proline (Hyp), HYP 101, HYP 202 and HYP 203, were selected from an ethylene imine mutagenized M_2 population of the original variety, 'Nipponbare', and their biochemical and genetical characteristics were investigated. The sensitivity of the mutants to Hyp could be clearly differentiated from that of the original variety when seeds were germinated and cultured with $10^{-4} \sim 10^{-3}$ M Hyp for 10 days. A difference in Hyp sensitivity was also observed among the HYP mutant lines, HYP 101 being the most resistant line. When free amino acids in seeds and 15-day-old seedlings were analyzed, the composition of the amino acids in the mutants was somewhat different from that found in the original variety. However, free proline accumulation was not detected in either the HYP mutants or the original variety. In each mutant line, HYP resistance was transmitted with a single recessive nuclear gene (*hpr*). These results suggest that the mechanism of Hyp resistance controlled by the recessive gene do not involve free proline accumulation.

Key words: Rice – Hydroxy-L-proline – Resistant mutant – Recessive gene – Free amino acids

Abbreviations: Hyp = hydroxy-L-proline, T-Pro = thioproline

Introduction

In recent years, some mutants, such as those carrying amino acid analog resistance and lysine plus threonine resistance, have been selected from a mutagenized population of several plants. Their mechanism of resistance has been shown to be due to an insensitivity to feedback inhibition of the pertinent amino acid biosynthetic pathway. Such mutants can be useful material, not only for the analysis of amino acid metabolism but also for breeding to improve nutritional quality, because the corresponding free amino acid accumulates in the mutants (Green and Phillips 1974; Brock and Langridge 1975; Miflin et al. 1983; Gengenbach 1984). With respect to proline analog-resistant mutants in crop plants, Kueh and Bright (1981, 1982) selected four barley mutants resistant to Hyp from a sodium azide mutagenized M_2 population and showed that the mutant leaves accumulated free proline.

In a previous paper (Hasegawa and Inoue 1983), it was reported that over 20 rice mutants resistant to Hyp were selected from a M_2 population mutagenized by several chemical mutagens and that ethylene imine was the most effective mutagen in inducing the resistant mutant. This paper describes further investigations on the characterization of Hyp resistant mutants, using the progenies of the mutants HYP 101, HYP 202 and HYP 203. The results demonstrate that free proline does not accumulate in the HYP mutant lines and that HYP resistance is controlled by a single recessive nuclear gene.

Materials and methods

Plant materials and chemicals

The progenies of three rice (*Oryza sativa* L.) mutants resistant to Hyp, HYP 101, HYP 202 and HYP 203, and their original variety, 'Nipponbare' (*Japonica* type), were used in the present study. The mutants were selected from a M_2 population mutagenized by 0.2% ethylene imine for 2 h (Hasegawa and Inoue 1983). HYP 101 was recognized as a homozygous line for HYP

resistance in the M_3 generation and HYP 202 and HYP 203 in the M_4. Further investigations on the characterization of the mutants were carried out with the M_4 and M_6 seeds.

Hyp and T-Pro were purchased from Wako Pure Chemicals (Japan) and Sigma Chemicals (USA), respectively.

Proline analog resistance

The M_6 seeds were germinated and cultured with 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3} or 10^{-2} M Hyp, or 10^{-4}, 10^{-3}, 5×10^{-3}, 10^{-2} and 5×10^{-2} M T-Pro (another proline analog) in a growth chamber at 25 ± 1 °C under natural daylight. The analog solutions were exchanged every two days. Seedling height was measured 10 days after treatments and taken as a parameter of the resistance. Each treatment was separately replicated three times using 30 seeds per treatment.

Free amino acid analysis

Free amino acids in the seeds (hull-less seeds) and in the 15-day-old seedlings, which were cultured in a growth chamber at 25 ± 1 °C under natural daylight, were analysed in the M_6 generation. One gram of seeds or 0.5 g of seedlings were extracted by 80% boiling ethanol as described by Shiomi and Hori (1973) and the levels of 17 amino acids were determined with an auto amino acid analyzer, ATTO MLC-703 (Japan). The amino acid analysis were repeated there times with separately extracted samples.

Genetical analysis of HYP mutants

In the M_4 generation, HYP 101 and 'Nipponbare' were crossed reciprocally. Both F_1 and F_2 seeds were germinated and cultured with 10^{-3} M Hyp. The crosses, HYP 202 (♀)×'Nipponbare' (♂) and 'Nipponbare' (♀)×HYP 203 (♂) were also carried out in the M_4 generation. Hyp resistance was evaluated by measuring the seedling height as described above.

Results

Proline analog resistance

There was marked difference in seedling height 10 days after treatment with Hyp at concentrations of $10^{-4}\sim10^{-3}$ M between three HYP lines and 'Nipponbare' (Fig. 1). A typical plant response to 10^{-3} M Hyp is shown in Fig. 2. At concentrations of 5×10^{-3} M or higher, however, the seedling growth of both HYP lines and 'Nipponbare' was severely inhibited. A difference in Hyp resistance was also observed among the HYP lines. The concentrations of Hyp causing a 50% reduction in seedling height (calculated from Fig. 1) were 2.6×10^{-3}, 1.9×10^{-3}, 1.9×10^{-3} and 2.6×10^{-4} for HYP 101, HYP 202, HYP 203 and 'Nipponbare', respectively, thus showing a ten-fold difference in resistance between HYP 101 and 'Nipponbare'. HYP 101 was the most resistant line to Hyp. The extent of Hyp resistance in HYP 202 was almost equal to that in HYP 203.

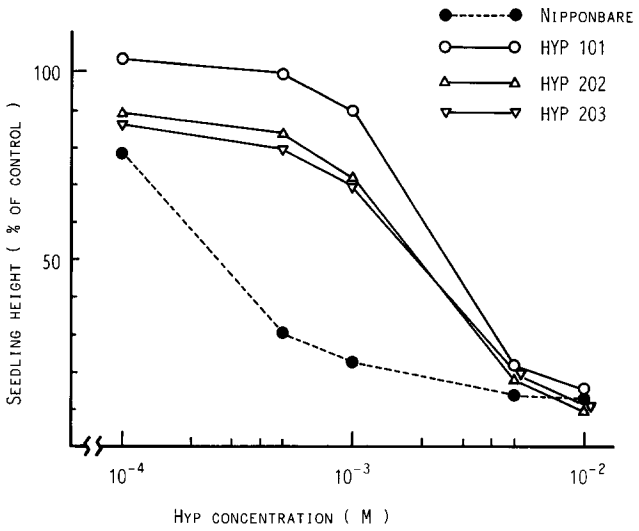

Fig. 1. Difference of Hyp sensitivity between HYP mutant lines and the original variety, 'Nipponbare'. Seedling height was measured 10 days after treatment

T-Pro at concentrations of 5×10^{-3} M or higher equally inhibited the seedling growth of HYP lines and 'Nipponbare' and no significant difference in T-Pro resistance was observed (Fig. 3). Chlorosis of seedlings was also observed at concentrations of 10^{-3} M or higher in both HYP lines and 'Nipponbare'.

Analysis of free amino acids

Table 1 shows the levels of 17 free amino acids in both seeds and 15-day-old seedlings. There were some dif-

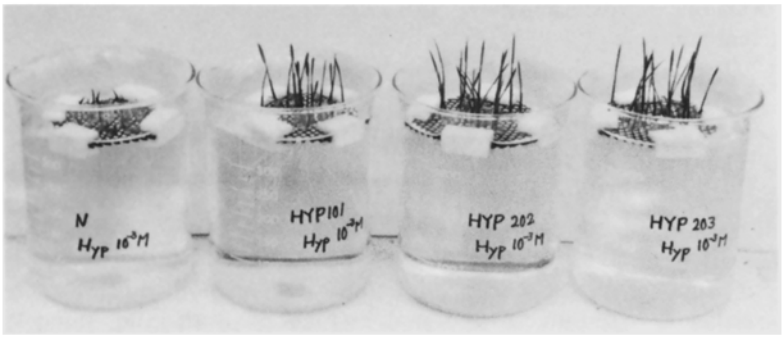

Fig. 2. Seedlings of HYP mutants and the original variety, 'Nipponbare', cultured with 10^{-3} M Hyp for 10 days. *Left to right:* 'Nipponbare', HYP 101, HYP 202 and HYP 203

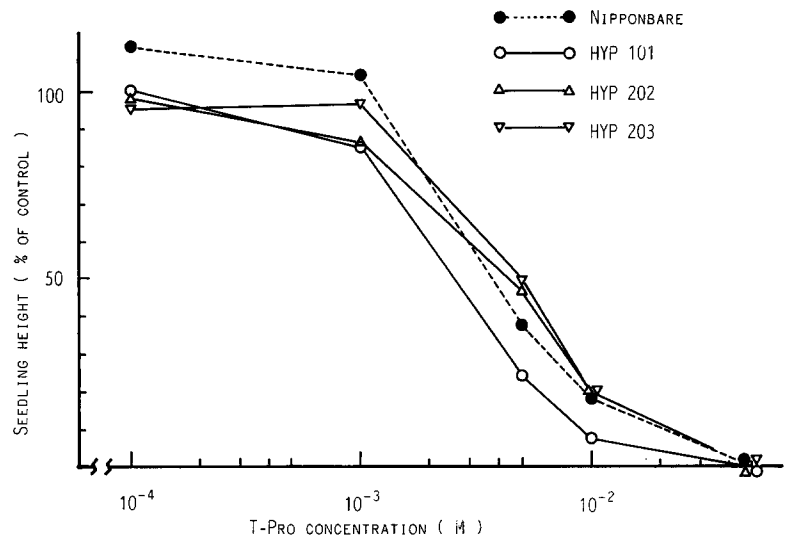

Fig. 3. Effect of T-Pro on seedling growth of the HYP mutant lines and the original variety, 'Nipponbare'. Seedling height was measured 10 days after treatment

Table 1. Free amino acid content of seed and 15-day-old seedlings of HYP mutants and 'Nipponbare'

Amino acid	'Nipponbare'		Amino acid content (nmol/g fresh weight)[a]					
	Seed	Seedlings	HYP 101		HYP 202		HYP 203	
			Seed	Seedling	Seed	Seedling	Seed	Seedling
Aspartate	393±46	603± 224	337±18	277± 69	325±28	346± 35	323±49	381± 22
Threonine	155± 2	4,183±1,022	243±17**	2,457±398	217±31	3,101±336	185±33	2,971±255
Serine	158±72	839± 157	282±40	1,660±544	237±22	2,400±623*	259±67	2,062±112**
Glutamate	767±90	1,534± 344	783±31	1,108±226	801±84	1,444±105	795±81	1,514±325
Proline	Tr	ND	Tr	ND	ND	ND	ND	ND
Glycine	107±18	780± 218	89± 7	614±123	106± 9	840± 83	91± 9	768±205
Alanine	288±25	2,264± 773	391±30*	1,824±278	336±15*	2,850± 96	363±54	2,195±384
Cystine	ND	ND	ND	ND	ND	ND	ND	ND
Valine	ND	647± 47	Tr	436±149	24±16	749± 13	ND	553± 34
Methionine	Tr	ND	ND	ND	ND	ND	ND	ND
Isoleucine	Tr	149± 89	Tr	172± 25	Tr	266± 40	Tr	221± 29
Leucine	Tr	146± 5	15± 0	138± 15	Tr	197± 46	15± 2	156± 12
Tyrosine	Tr	ND	58±19	ND	Tr	ND	Tr	ND
Phenylalanine	Tr	Tr	41±28	Tr	18± 1	Tr	Tr	ND
Lysine	30±11	134± 49	37± 3	111± 79	40± 1	137± 39	27± 5	161± 23
Histidine	101±15	573± 143	120± 7	282± 60*	113±15	279±100*	101± 7	187± 7**
Arginine	107±30	100± 26	190±62	77± 18	143±41	113[b]	146±37	130± 50

*,** Significantly different from 'Nipponbare' at 5% and 1% level, respectively
[a] Average of 3 replications, ±SD, Tr: trace, ND: not detected, [b] Not replicated

ferences in amino acid composition between HYP mutant lines and 'Nipponbare'. Seeds of HYP 101 had a significantly higher content of threonine and alanine than those of 'Nipponbare'. A significant increase in alanine was also observed in the seeds of HYP 202. The seedlings of 'Nipponbare' contained higher levels of aspartate, threonine and histidine than those of the HYP mutant lines. On the other hand, serine content in HYP 202 and HYP 203 was significantly higher than that of 'Nipponbare'. Considerable amounts of tyrosine and phenylalanine were detected only in the seeds of HYP 101. However, free proline was detected in neither HYP lines nor 'Nipponbare' except in the seeds of HYP 101 and 'Nipponbare', where a trace amount of free proline was detected but its exact content was not determined.

Genetical analysis of the HYP mutant

In various combinations of crosses, the growth of all the F_1 seedlings was inhibited by 10^{-3} M Hyp. In the F_2 generation of the HYP 101 (♀)×'Nipponbare' (♂) cross, 34 out of 120 seedlings were resistant and 86 were sensitive to Hyp (Fig. 4a). In the reciprocal cross, 24 out of 120 F_2 seedlings were resistant and 96 were sensitive to the analog (Fig. 4b). Both ratios fitted to

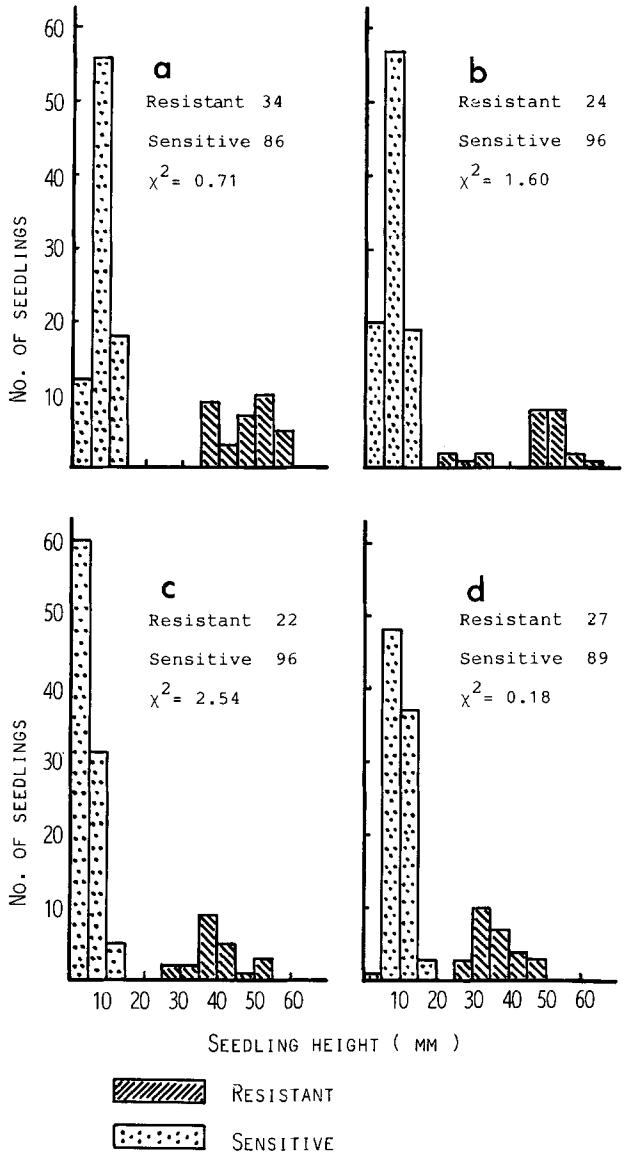

Fig. 4a–d. Distribution of seedling height of F_2 progenies derived from four crosses. Seedlings longer than 20 mm were classified as a resistant. Seedling height was measured 10 days after culturing with 10^{-3} M Hyp. **a** HYP 101 (♀)×'Nipponbare' (♂); **b** 'Nipponbare' (♀)×HYP 101 (♂); **c** HYP 202 (♀)×'Nipponbare' (♂); **d** 'Nipponbare' (♀)×HYP 203 (♂)

1:3 ratio ($0.3 < P < 0.5$ and $0.2 < P < 0.3$ from HYP 101×'Nipponbare' and 'Nipponbare'×HYP 101, respectively). As shown in Figs. 4c and d, the 1:3 ratio was also detected in the F_2 seedlings of the HYP 202× 'Nipponbare' and 'Nipponbare'×HYP 203 crosses. These results suggest that Hyp resistance in HYP 101 as well as HYP 202 and HYP 203 is inherited as a single recessive nuclear gene. These genetic results for Hyp resistance in HYP 202 and HYP 203 was also confirmed in a separate experiment (Mori et al. 1985a).

Discussion

Of 24 rice mutants resistant to Hyp (Hasegawa and Inoue 1983), three mutant lines have been established as a homozygous line. The difference in the degree of Hyp resistance was observed not only between HYP mutant lines and the original variety but also among the HYP lines. HYP 101 was more resistant to Hyp than HYP 202 and HYP 203, but the degree of resistance in HYP 202 was almost equal to that in HYP 203. The result that T-Pro resistance could not be detected in each HYP line was consistent with the observation of barley mutant resistant to Hyp (Kueh and Bright 1981). Differences in some morphological traits between HYP lines and the original variety have also been reported (Hasegawa et al. 1985).

The present experiment demonstrated two important characteristics of each HYP mutant: (1) free proline accumulated neither in the seeds nor in the seedlings; (2) Hyp resistance was inherited as a single recessive nuclear gene (*hpr*). These characteristics were different from those of the barley mutants resistant to Hyp in which the Hyp resistance was inherited as a semi-dominant gene and associated with approximately a 6-fold higher free proline content in the leaves than in those of the original variety (Kueh and Bright 1981, 1982). On the other hand, James and Jacobs (1976) reported that the recessive gene-controlled and p-fluorophenylalanine (a phenylalanine analog)-resistant mutant in *Arabidopsis thaliana* oversynthesized neither phenylalanine nor tyrosine. Bright et al. (1979a, b) demonstrated that S-2-aminoethyl-L-cysteine (a lysine analog) resistance of the barley mutant selected from a mutagenized M_2 population was also inherited as a single recessive gene and that the mutants did not accumulate free lysine either in seeds or in leaves. It thus appears that the lack of proline accumulation in the HYP mutants observed in the present study might be related to the fact that Hyp resistance was controlled by a recessive gene. Although we have not conducted a biochemical study for the possible mechanisms for Hyp resistance without proline accumulation, the decreased capacity of the mutants to take up the analog might be considered (Negrutiu et al. 1978; Bright et al. 1979a). With respect to proline accumulation, an additional mutation affecting proline metabolism is necessary for inducing accumulation (Hermann et al. 1972).

Recently, Mori et al. (1985b) showed that Hyp resistance was efficiently maintained in the calli initiated from the seeds of the three HYP mutants. This fact suggests that the Hyp resistance of the mutants used in the present study could be used as a genetic marker both in the cultured cell system and in the whole plant system.

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