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Free proline contents in two different groups of rice mutants resistant to hydroxy-L-proline

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Summary. In four rice (*Oryza sativa* L.) mutants resistant to hydroxy-L-proline (Hyp), HYP101, HYP203, HYP205 and HYP210, and in their original variety, Nipponbare, free proline and Hyp contents in the seeds and in the 14-day-old seedlings have been determined. The four mutants can be divided into two groups: HYP101 and HYP203 are classified as to recessive gene and the levels of free proline are similar to that of the original variety; the second group includes mutants HYP205 and HYP210 where the Hyp resistance is transmitted heterozygously and, both in the seeds and in the seedlings, a remarkable increase in free proline content is observed. In particular, free proline contents in the seeds of HYP205 and HYP210 are, respectively, 24 and 12 times that of the original variety. Hyp is detected only in the seedlings cultured with Hyp solution. In the Hyp resistant seedlings of HYP205 and HYP210, Hyp contents are twice that of the original variety and less than half in the seedlings of HYP101 and HYP203. Hyp resistance and differential proline levels are also evident in the callus initiated from the mutants. This suggests that the Hyp resistant mutants are good genetic markers both in planta and in vitro. The Hyp mutants are also discussed with regard to stress resistance.

Key words: *Oryza sativa* L. – Hydroxy-L-proline resistance – Proline accumulation – Callus – Genetic marker

Introduction

In higher plants, proline is credited with playing a role in resistance to stresses such as salt, drought and frost (Stewart and Larher 1980; Aspinall and Paleg 1981; Pandey and Ganapathy 1985; Van Swaaij et al. 1986, 1987; Duncan and Widholm 1987). In fact, free proline accumulation is frequently observed in plants grown under such conditions. The proline biosynthetic pathway in higher plants is not, however, well-characterized and the mechanism of proline accumulation under stress is still unclear (Miflin et al. 1983).

Mutants resistant to proline analog are thought to be a useful tool for studying proline biosynthesis and stress resistance in higher plants (Miflin et al. 1983). In rice more than 20 mutants resistant to hydroxy-L-proline (Hyp), a proline analog, have been selected (Hasegawa and Inoue 1983). Three of these do not accumulate free proline either in the seeds or in the seedlings, and their Hyp resistance is controlled by a single recessive nuclear gene (Hasegawa et al. 1985). Furthermore, Mori et al. (1985, 1986) demonstrated that the Hyp resistance was also expressed in the callus.

In this paper the progenies of several Hyp-resistant mutants were re-examined and analysis of free proline and Hyp in the seeds and in young seedlings was carried out. A new type of Hyp-resistant mutant, a proline-accumulating one, was identified. Hyp resistance and free proline accumulation in the callus initiated from the mutants were also investigated.

Materials and methods

Plant materials

The seeds of M_7 progenies of four rice (*Oryza sativa* L.) mutants resistant to Hyp, namely HYP101, HYP203, HYP205 and HYP210, and their original variety, Nipponbare (*Japonica* type), were used. The isolation of the mutants was previously described (Hasegawa and Inoue 1983). Hyp resistance of HYP101 and HYP203 is controlled by a single recessive gene designated as *hpr* (Hasegawa et al. 1985), while Hyp resistance

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of HYP205 and HYP210 is transmitted heterozygously (Mori et al. 1986). The seeds of HYP205 and HYP210 used in this experiment were obtained from the M_6 plants which had shown Hyp-resistance at the young seedling stage.

Effect of Hyp on seedling growth

The seeds were germinated and cultured in the presence of 10^{-3} *M* Hyp (purchased from Wako Pure Chemicals, Japan) in a biotron, Type LH-200-RD (Nippon Medical and Chemical Instruments, Japan), at 25±1 °C under fluorescent light (6,000 lux, 16 h photoperiod). Nutrient solutions were exchanged every 2 days. Seedling height on the 7th day after treatment was measured to monitor Hyp-resistance. In each treatment, 120 seeds were used.

Effect of Hyp on callus growth

For HYP101, HYP203, HYP210 and Nipponbare, small pieces of calli (about 0.3 mg fresh weight) which were initiated from the seed were cultured on the solid medium of Linsmaier and Skoog (1965), supplemented with 10^{-5} *M* 2,4-D containing 0, 10^{-4}, 10^{-3} and 10^{-2} *M* Hyp in an incubator, MIR-251 (SANYO Electric Tokki, Japan), at 27±1 °C in the dark. The growth of callus was evaluated by measuring the diameter of the 21st day after treatment. In each treatment, 45 calli were used.

Analysis of free proline and Hyp

Free proline and Hyp were evaluated in seeds (hull-less) and in 14-day-old seedlings, and calli were analyzed. The seedlings were cultured with distilled water or 10^{-3} *M* Hyp in a biotron, Type LH-200-RD (Nippon Medical and Chemical Instruments, Japan), at 25±1 °C under fluorescent light (6,000 lux, 16 h photoperiod). To obtain callus for amino acid analysis, seedlings were aseptically cultured with Hyp. From Hyp-resistant plants, as evaluated 10 days after treatment, calli were initiated from the roots and cultured on Linsmaier and Skoog medium (1965) in the presence of 10^{-5} *M* 2,4-D at 27±1 °C in the dark for 40 days. Approximately 1 g (fresh weight) of each sample was considered for analysis. The methods of extraction of free amino acids have been reported elsewhere (Hasegawa and Mori 1986; Hasegawa 1988). The levels of proline and Hyp were determined by absorbance at 440 nm with an amino acid auto analyzer (ATTO MLC-703, Japan).

Results

Hyp resistance in seedling

Figure 1 shows the distribution of seedling height in four mutant lines and Nipponbare 10 days after treatment. When the seedlings were cultured with 10^{-3} *M* Hyp, seedlings longer than 15 mm and with no visual damage of Hyp were classified as resistant. All seedlings of Nipponbare were sensitive, while all those of HYP101 and HYP203 were resistant. Seedlings of HYP205 and HYP210 included both types (Fig. 2). In this experiment, the ratio of resistant seedlings to sensitive seedlings was approximately 1:1 and 1:4 in HYP205 and HYP210, respectively.

Hyp resistance in callus

Resistance to Hyp was categorized according to five classes, based on the diameter of callus. Figure 3 shows that the callus growth was inhibited by Hyp at a concen-

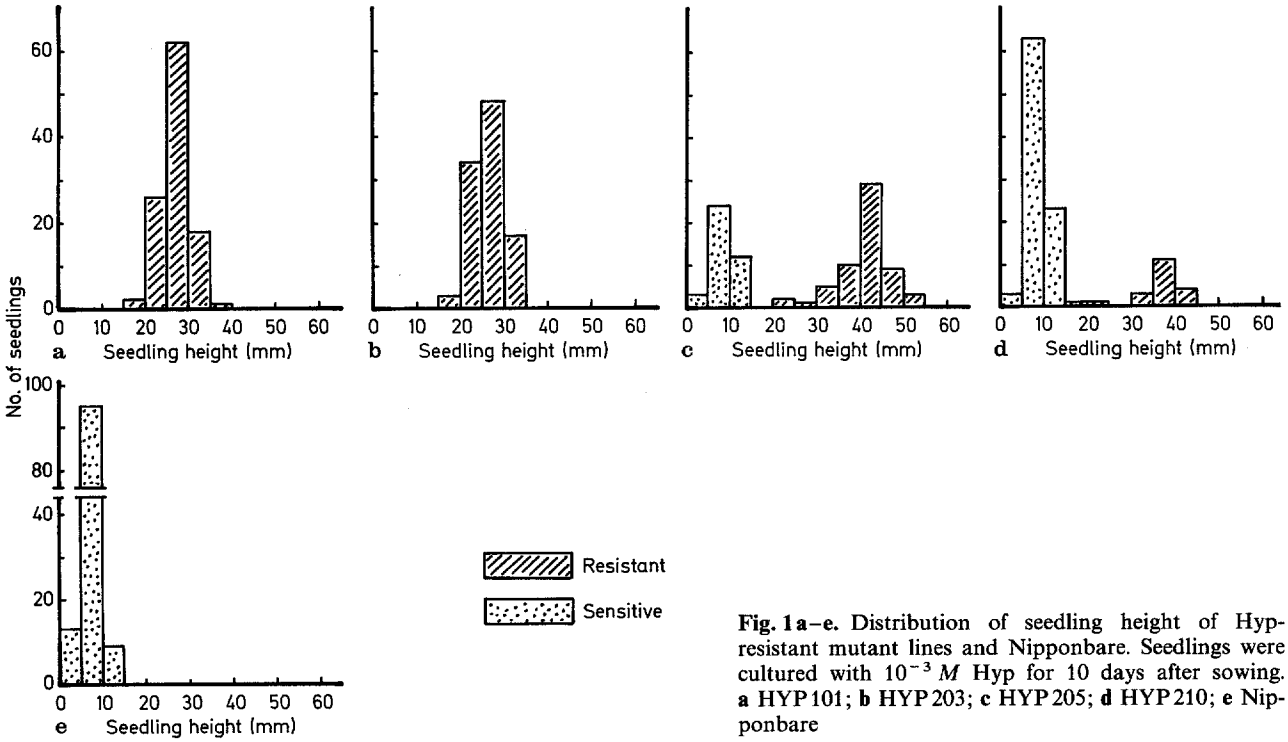

Fig. 1a–e. Distribution of seedling height of Hyp-resistant mutant lines and Nipponbare. Seedlings were cultured with 10^{-3} *M* Hyp for 10 days after sowing. **a** HYP101; **b** HYP203; **c** HYP205; **d** HYP210; **e** Nipponbare

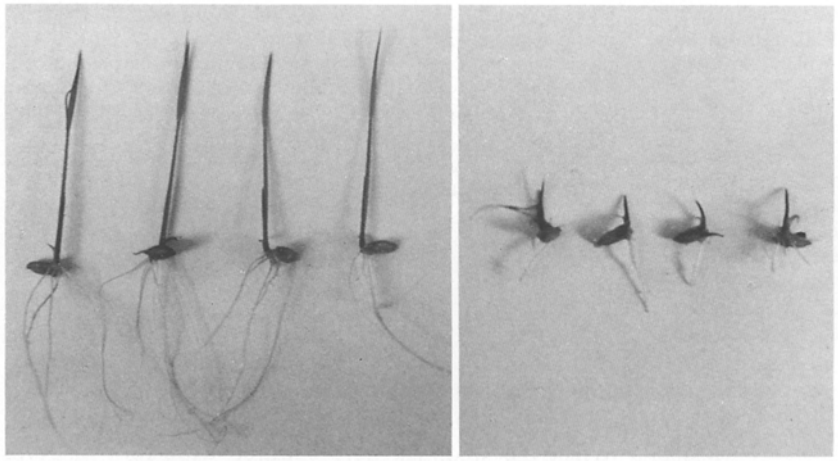

Fig. 2. Segregation of resistant (left) and sensitive (right) seedlings in HYP210. Seedlings were cultured with 10^{-3} *M* Hyp for 10 days after sowing

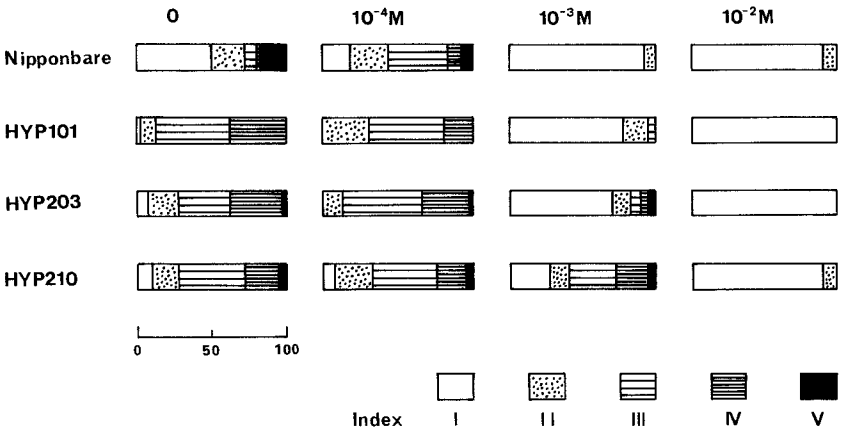

Fig. 3. Hyp sensitivity of calli derived from Hyp-resistant mutants and Nipponbare. Callus growth was followed by measuring the length in diameter (mm). I < 1; II 1 ~ 3; III 3 ~ 5; IV 5 ~ 6; V ≧ 6

Table 1. Free proline contents (nmol/g fresh weight) in seeds and 14-day-old seedlings of Hyp-resistant mutant lines and Nipponbare

	Free proline content		
	Seeds	Seedlings	
		Hyp 0 *M*	Hyp 10^{-3} *M*
Nipponbare	88± 4	244± 44	220± 45
HYP101	184± 7	206± 4	180± 70
HYP203	104± 4	278± 40	312± 92
HYP205	2,132±137	1,555± 63	317± 32 (S) 868±178 (R)
HYP210	1,085± 62	925±232	290± 66 (S) 899 (R)*

(S) and (R) Hyp-sensitive and -resistant segregants of the heterozygous lines
* Not replicated

tration of 10^{-3} *M* or higher. The difference in Hyp resistance among the lines used in this experiment was detected in the presence of 10^{-3} *M* Hyp. The callus from HYP210 was apparently the most resistant to Hyp. The frequency of well-grown calli in HYP101 and HYP203 was lower than that in HYP210.

Free amino acid analysis

As shown in Table 1, free proline contents in the seeds of HYP205 and HYP210 were about 24 and 12 times of that of Nipponbare, respectively, while a two-fold increase was observed in the seeds of HYP101. No remarkable change in proline content was observed in the seeds of HYP203. Increase in free proline content was also observed in the 14-day-old seedlings of HYP205 and HYP210 cultured on distilled water. When the seedlings were cultured in the presence of 10^{-3} *M* Hyp, differences in free proline content between sensitive seedlings and resistant seedlings of HYP205 and HYP210 were also found. In both lines, the level of free proline content in the resistant seedlings was about three times higher than that of the sensitive ones. However, proline content in the seedlings cultured in the presence of Hyp was lower than that in the seedlings cultured on distilled water. HYP101, HYP203 and Nipponbare did not show a remarkable difference in free proline content of the seedlings cultured with Hyp or without Hyp.

Proline content in the callus is shown in Table 2. The level of free proline content was higher than in those of the seeds and the seedlings, except for the callus from Hyp sensitive seedlings of HYP210. Remarkable increase

Table 2. Free proline contents (nmol/g fresh weight) in calli of Hyp-resistant mutant lines and in Nipponbare

Nipponbare	365
HYP101	655
HYP203	501
HYP210 (sensitive line)	141
HYP210 (resistant line)	8,085

Table 3. Free hydroxy-L-proline content (μmol/g fresh weight) in seeds and 14-day-old seedlings of Hyp-resistant mutant lines and in Nipponbare

	Seeds	Seedlings	
		Hyp 0 *M*	Hyp 10^{-3} *M*
Nipponbare	nd	nd	55.5 ± 26.9
HYP101	nd	nd	23.4 ± 2.1
HYP203	nd	nd	23.9 ± 7.2
HYP205	nd	nd	62.4 ± 5.8 (S)
			122.3 ± 19.0 (R)
HYP210	nd	nd	42.5 ± 13.2 (S)
			109.1 (R)

(S) and (R) Hyp-sensitive and -resistant segregants of the heterozygous lines
nd, not detected

in proline content (8,085 nmol/g fresh weight, about 22-fold increase) was observed in the callus derived from the resistant seedlings of HYP210, while proline content in the callus from the sensitive ones was less than that of Nipponbare. Minor differences were noted for the proline content in the callus of HYP101, HYP203 and Nipponbare.

A considerable amount of free Hyp was detected only in the seedlings which were cultured in the presence of Hyp (Table 3). Hyp contents in HYP101 and HYP203 were about half of that in Nipponbare. On the other hand, in HYP205 and HYP210, Hyp content of the resistant seedlings was about twice that of Nipponbare. No significant differences in Hyp content between the sensitive seedlings of HYP205 and HYP210 and Nipponbare were found.

Discussion

Hasegawa and Mori (1986) reported that the Hyp-resistant mutants HYP101 and HYP203 did not accumulate free proline; moreover, their Hyp resistance was transmitted as a single recessive trait. In the present experiment, two additional mutants, HYP205 and HYP210, were characterized and identified as different from HYP101 and HYP203. In fact, HYP205 and HYP210 accumulate free proline 24 and 12 times more than the original variety, respectively. Proline contents in the seedlings of HYP205 and HYP210 cultured on distilled water were similar to those found in the seeds.

HYP205 and HYP210 also show their resistance in the heterozygous state. The gene for Hyp resistance has not yet been conclusively identified. The segregation ratio of resistant seedlings to sensitive ones fits in most cases 1:1, but in rare cases it decreases lower than 1:1, as in the case of HYP210 (Fig. 1 d). No resistant seedlings have developed from the progeny of sensitive ones (data not shown). Similar cases were reported for lysine plus threonine resistance in barley (Bright et al. 1982) and 5-methyltryptophan resistance in rice (Wakasa and Widholm 1987). As for the Hyp resistance, a semi-dominant gene was also identified in barley (Kueh and Bright 1981, 1982). It is also known that a dominant or a semi-dominant mutant resistant to amino acid analog accumulates the corresponding free amino acid (Kueh and Bright 1981, 1982; Negrutiu et al. 1984), while a recessive gene-controlled mutant does not accumulate it (James and Jacobs 1976; Bright et al. 1979a, b; Hasegawa and Mori 1986). The present result are consistent with these findings.

Clear differences in Hyp content were found between non-proline-accumulating mutants and proline-accumulating: HYP101 and HYP203 had contents of Hyp less than half of that of Nipponbare, while the resistant seedlings of HYP205 and HYP210 accumulated twice the amount of Hyp compared to the original variety. The decreased Hyp uptake in HYP101 and HYP203 was similar to that of a recessive gene-controlled barley mutant resistant to S-2-aminoethyl-L-cysteine (a lysine analog) which did not accumulate free lysine (Bright et al. 1979a).

The characteristics of all the Hyp-resistant mutants considered in this study were expressed in the callus as well as in the seedling. Free proline accumulation was observed in the callus derived from Hyp-resistant seedlings of HYP210, but not so clearly in the callus from HYP101 and HYP203 or in the sensitive seedlings of HYP210. Altogether our results indicate that both groups of Hyp-resistant mutants are useful as markers in studies at the level of whole plants and of cultured cells.

Finally, it has been reported that in higher plants, free proline-accumulating cell lines resistant to a proline analog show salt or freezing tolerance (Ricardi et al. 1983; Van Swaaij et al. 1986, 1987). The lack of salt- or drought-resistance in barley mutant resistant to Hyp (Kueh and Bright 1981, 1982) was, on the contrary, attributed to its low content of free proline (Miflin et al. 1983). HYP205 and HYP210 could be of potential value for studies of stress-resistance in rice.

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