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Selection of higher regenerative callus and change in isozyme pattern in rice (*Oryza sativa* L.)

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Summary. Calli were initiated from seedling roots in rice (*Oryza sativa* L. var. Tadukan) and subcultured at 45-day intervals on MS medium supplemented with 2 mg/l 2,4-D. Sectors of callus which differentiated shoot meristems (green spots) under the same 2,4-D concentration were selected from the calli subcultured 90 days after initiation. The selection was continued for about 2 years. Responses to 2,4-D between original and selected lines differed considerably, although differentiation was not generally seen in rice callus in the presence of 2 mg/l 2,4-D. After 180 days, calli of the selected line differentiated into numerous shoot-bud primordia and grew out new callus tissues under 2 mg/l 2,4-D concentration; the frequency of the differentiation exceeded 90%. On the other hand, no calli of non-selected line differentiated into shoot-buds under 2 mg/l 2,4-D, and the frequency of the shoot-bud was only about 50% under lower 2,4-D concentration (0.1 mg/l). The pattern and activity of peroxidase isozyme varied markedly between calli of the selected and non-selected lines. First, two strong peroxidase bands which show fast mobility and one intermediate peroxidase band with slow mobility were detected only in the calli of selected line. Secondly, changes in band pattern of proteins separated by SDS-PAGE were observed. In the calli of selected line, there was a loss of the polypeptide bands with molecular weight of 24 and 42 K in the selected calli, but they were clearly present in the unselected line. The appearance of new peroxidase isozyme bands and loss of polypeptide bands, change in response to auxin and increased ability for shoot bud differentiation are closely correlated to each other.

Key words: *Oryza sativa* L. – Regenerative callus line – Organogenesis – Peroxidase isozyme – SDS-PAGE

Introduction

Somaclonal variation arising through tissue culture has been demonstrated in a number of plant species (Larkin and Scowcroft 1981). Some variants resistant to amino acid or their analogues (Wakasa and Widholm 1987) and pathotoxin (Gengenbach and Green 1975) were obtained through cell selection and, thereafter, plant regeneration. Somaclonal variation useful for rice breeding has been also reported (Oono 1978; Sun et al. 1983).

High frequency of plant regeneration from callus cultures is one of the essential factors for application of tissue culture in crop improvement. Induction of somatic embryogenesis from rice callus tissues has been demonstrated (Heyser et al. 1983; Abe and Futsuhara 1985). These reports, however, showed that ability of embryoid formation and plant regeneration decreased during a long-term subculture. The decrease of plant regeneration in the prolonged subcultures is common in many species, and precise reasons for this are not known. Chromosome aberration in such cultures may affect plant regeneration (Ahloowalia 1982). However, at the same time, a mutation capable for efficient plant regeneration may take place. Selection of such a sector harbouring the regenerative potentiality would be useful for plant breeding as reported in *Pennisetum americanum* (Vasil and Vasil 1981), where selection and transfer of compact and white to pale-yellow callus to regeneration medium was favourable for the occurrence of somatic embryogenesis. The present investigation was carried out to select highly regenerative calli and to determine the changes in isozyme pattern during the selection.

Materials and methods

Callus establishment and subculture

Callus cultures from root segment of rice (*Oryza sativa* L. cv Tadukan) were established on MS basal medium (Murashige

and Skoog 1962) supplemented with 2 mg/l 2,4-D, 30 g/l sucrose, and 8 g/l agar. The calli were subcultured at 45-day intervals on the same medium. During transfer, the callus was divided into smaller pieces (30–50 mg) and five pieces were placed in 100-ml flasks containing 50 ml of medium. Ten flasks were provided for each subculture.

Selection, testing of regenerative ability and responses to 2,4-D

Three sectors, which differentiated into green spot on the medium containing 2 mg/l 2,4-D, were selected from callus tissues subcultured 90 days after initiation in the second culture passage. In the second selection, only one clone showed the regenerative trait and the clone was transferred. Selection was continuously conducted and the clone was subcultured on N6 medium (Chu et al. 1975), as well as on MS medium, for about 2 years. Six flasks containing 18 calli were prepared for the evaluation of the shoot-bud differentiation from the calli which responded to different 2,4-D levels in the media at the fifth culture passage. The ability to differentiate shoot-bud primordium from the selected calli was compared with that of the non-selected ones. The same experiment was carried out at the 15th culture passage, which was about 2 years after callus induction.

Analysis of peroxidase isozyme

Two samples were used from each strain. After excluding the green regions, 0.2 g calli of selected and non-selected lines was homogenized in 2.0 ml of sampling buffer containing 0.0625 *M* TRIS-HCl, 10% glycerol and 0.15% Triton-X 100, at 0 °C and pH 8.0. The homogenates were centrifuged at 14,000 × g for 20 min at 0 °C. The supernatant was used for peroxidase assay. For electrophoresis, 15 µl of protein extract was applied. Isozymes were separated by 1.0-mm thick polyacrylamide gel (8%–16% gradient) at 5 °C, and stained in a solution containing 0.03% diamino benzidine and 0.03% H_2O_2.

Protein analysis by SDS-PAGE

Protein for SDS-PAGE was extracted from calli using extraction buffer containing 0.0625 *M* TRIS-HCl (pH 6.8), 1% SDS, 2% mercaptoethanol and 10% gylcerol, after excluding the green tissues. One gram of the calli was homogenized in 7 ml of extraction buffer. The homogenates were centrifuged at 14,000 × g for 20 min. The supernatants were boiled for 3 min and again centrifuged at 14,000 × g for 5 min. SDS-PAGE was carried out according to the procedure of Laemmli (1970) with a slight modification of the slab gel (Gradient polyacrylamide, 8%–16%, 1 mm thick), containing 0.1% SDS. Fifteen microliters of the extract was applied to each lane. Standard proteins (Pharmacia LMW Calibration Kits) for identifying molecular weight were also used. The electrophoresis was performed at room temperature with a constant voltage of 80 V. After electrophoresis, the gels were stained with 0.25% (w/v) Coomassie brilliant blue R-250 in 45% ethanol:10% acetic acid. The gels were de-stained in 25% ethanol:8% acetic acid. All experiments of peroxidase and protein analyses were carried out in at least two replicates.

Results

A preliminary experiment of callus cultures of the rice variety showed that a few of the shoot-buds could initiate in relatively high 2,4-D concentration (1–2 mg/l) as well as in low 2,4-D levels. In this experiment, three green sectors which contained the shoot-bud primordium emerged from 50 calli at the late period of the second subculture passage under a high concentration of 2,4-D (2 mg/l). The green sectors were dissected from the calli and transferred separately into the new media. At the third subculture passage, only one clone initiated many shoot-buds and two clones did not, and the regenerative clone was exclusively transferred. Continuous selection was conducted at every transfer to the similar medium. Morphological differences were obvious between the selected and the non-selected calli, especially at the late period of culture. Calli of the non-selected line were watery and yellowish, while calli of the selected line were greenish, with many shoot-bud primordia scattering on the calus surface. Responses to 2,4-D were different between the two callus lines (Table 1).

Table 1. Response of rice callus to 2,4-D between non-selected and selected lines at 5th culture passage

2,4-D (mg/l)	No. and percent of calli showing shoot or GS initiation[a]			
	Non-selected line		Selected line	
	No.	%	No.	%
2.0	0	0	13	71
1.0	6	33	12	67
0.5	7	39	15[b]	74
0.1	9	50	17	94

[a] In each case, 18 calli were examined
[b] In each case, 20 calli were examined
GS, Green shoot-bud primordium

At a high 2,4-D concentration (2 mg/l), 72% of the selected calli differentiated into shoot-bud primordia on the callus surface, whereas the non-selected did not, and the callus mass only grew on the similar culture medium. At low 2,4-D concentration (0.1 mg/l), the non-selected calli differentiated the shoot primordia about 50% less than did the selected ones. On the other hand, the seleced calli differentiated at a rate of more than 90%. The high ability of shoot-bud differentiation was maintained for 2 years in the selected lines, whereas the non-selected ones lost the ability after long-term subcultures.

After 2 years, N6 medium with a high 2,4-D concentration (2 mg/l) was more favourable for callus growth (data not shown) and differentiation of the shoot-but primordia than MS medium, and calli on N6 medium were vigorous and a fleshy pale-yellow, although the frequency of shoot initiation was higher on MS medium than on N6 medium. In 2,4-D free MS medium, the rates of the primordium differentiation and shoot initiation were 94% and 83%, respectively, in contrast to the frequency of 83% and 55% on N6 medium (Table 2). Multiple shoot-buds were frequently formed from the calli of selected lines (Fig. 1 a), and proliferated as microtellering

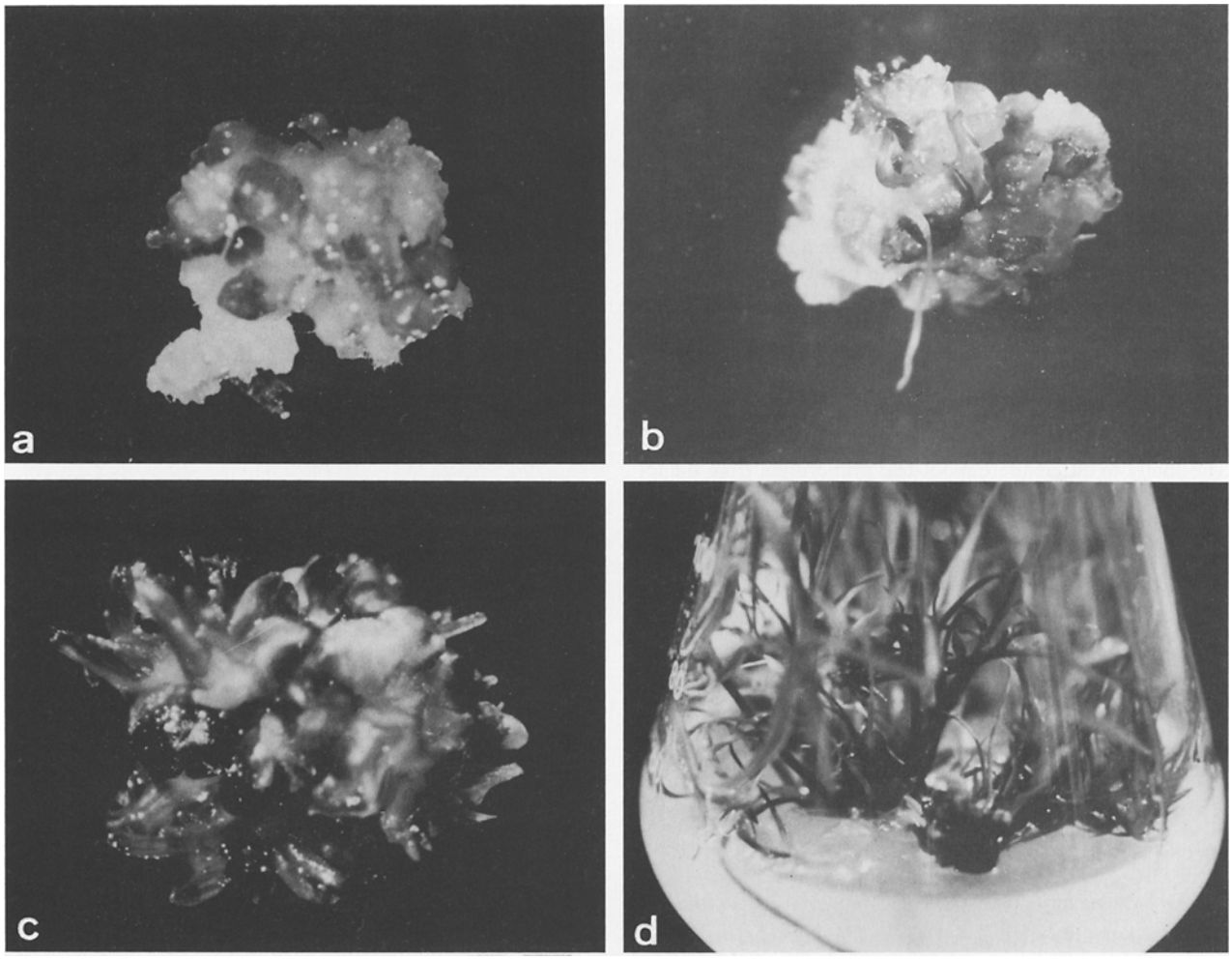

Fig. 1 a–d. Morphogenesis from calli of selected line in rice var. Tadukan. **a**: Callus with many green shoot-bud primordia in 2 mg/l 2,4-D (×4.5). **b**: A few shoot-buds with root differentiated from original callus (×3.0). **c**: Shoot-buds arising from the calli at the late period of 5th subculture (×4.5). **d**: Plantlets developed from multiple shoot-bud in 0.1 mg/l 2,4-D (×1.0)

(Fig. 1 c), They developed into plantlets (Fig. 1 d), but seldom had roots, which was characteristic of this selected line. The roots differentiated after transferring these shoot-buds into the medium supplemented with 0.1 mg/l of 2,4-D (Table 3). The pattern of differentiation was in contrast to that from non-selected calli, from which only one or two shoot-buds were differentiated even at the highest rate (Fig. 1 b).

Peroxidase isozyme pattern was markedly different between calli of the selected and non-selected lines (Fig. 2). In the non-selected line, three weak bands were present, whereas in the selected one, two strong bands and one intermediate band of the staining intensity where the mobility was different from that of the non-selected were detected; the two weak bands in the non-selected line were absent in the selected line. No difference was observed between peroxidase band patterns of the calli cultured on MS and N6 media and samples from each

Table 2. Plant regeneration from rice calli of selected line at 15th culture passage after 24 months

Basal medium	2,4-D (mg/l)	Shoot or GS initiation[a]		Shoot initiation[a]	
		No.	%	No.	%
MS	2.0	0	0	0	0
	1.0	3	17	0	0
	0.5	7	39	0	0
	0.1	12	67	11	61
	0	17	94	15	83
N6	2.0	9	50	0	0
	1.0	11	61	1	6
	0.5	14	78	2	11
	0.1	17	94	10	55
	0	15	83	10	55

MS, Green shoot-bud primordium
[a] In each case, 18 calli were examined

Table 3. Plant regeneration with root initiation after transfer of differentiated calli to the same regeneration medium (MS) at 16th culture passage[a]

2,4-D (mg)	Shoot or GS initiation		Shoot initiation		Root initiation	
	No.	%	No.	%	No.	%
0	18	100	12	67	10	55
0.1	18	100	17	94	18	100
0.5	16	88	12	67	12	67

[a] In each case, 18 calli were examined
GS, Green shoot-but primordium

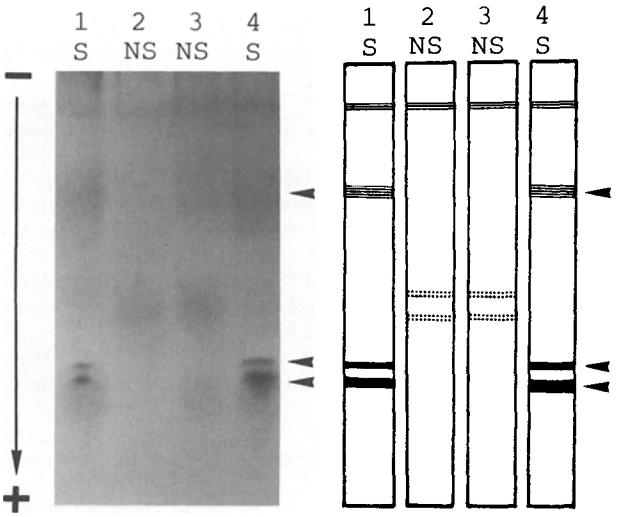

Fig. 2. Peroxidase isozyme pattern of two callus lines cultured on MS (*lanes 1 and 2*) and N6 media (*lanes 3 and 4*). *Lanes 1 and 4:* selected line (S); *lanes 2 and 3:* non-selected line (NS). *Arrows* on the right indicate specific peroxidase bands appearing in selected calli

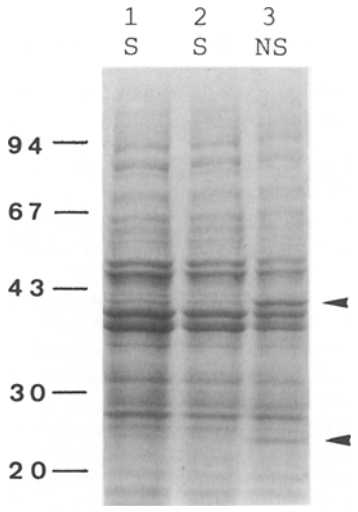

Fig. 3. SDS-PAGE pattern of the two callus lines cultured on MS (*lanes 1 and 3*) and N6 (*lane 2*) at 5th culture passage. *1,2:* selected line (S); *3:* non-selected line (NS). Numbers on left represent molecular weight in Kilodaltons of standard. *Arrows* of the right indicate specific polypeptide in non-selected line

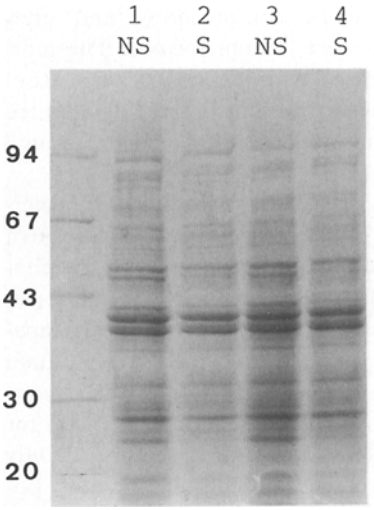

Fig. 4. SDS-PAGE pattern of the two callus lines cultured on MS (*lanes 1 and 2*) and N6 (*lanes 3 and 4*) at 15th culture passage. *1, 3:* non selected line (NS); *2, 4:* selected line (S)

strain. In addition to the peroxidase, acid phosphatase and leucine aminopeptidase were analyzed, but there was no difference between selected and non-selected lines.

Differences of the protein band after SDS-PAGE separation between the selected and non-selected calli were observed at the 5th and 15th culture passage. Polypeptides of 24 and 42 K which were clearly observed in the calli of non-selected lines, were absent or very weak in those of selected lines (Fig. 3). Other bands were similar between calli of the selected and non-selected lines. In the selected line, polypeptide patterns between calli cultured on MS and N6 media were also similar. Polypeptide profiles of the calli which were subcultured both at the 5th and 15th culture passage were almost similar (Fig. 3 and 4).

Discussion

The ability of plant regeneration and the type of differentiation (embryogenesis and organogenesis) considerably varied with genotypes used in rice cultures (Abe and Futsuhara 1984). In the experiment where the aim was to obtain efficient plant regeneration from rice callus cultures, embryogenic calli were initiated from mature seed and immature embryo (Heyser et al. 1983) and root section (Abe and Futsuhara 1985). In this experiment, however, a highly regenerative callus line was selected from the calli initiating from seminal roots. The selected calli differentiated numerous shoot-buds without roots. The sector with green shoot-bud differentiated from original callus even on the medium with 2 mg/l 2,4-D, although at the 2,4-D concentration (2 mg/l), no sign of green shoot-bud differentiation has been detected in the callus of *japonica* rice, Aichiasahi (Inoue and Maeda 1980). The

shoot-buds proliferated as microtellering and grew vigourously, but roots were seldom formed. The morphogenetic pattern showed typical organogenesis. Root formation was attained by transferring the differentiated shoots into the medium supplemented with 0 or 0.1 mg/l 2,4-D.

It is important to note that many plants were regenerated by organogenesis after a long-term callus culture of rice. On the other hand, in *Citrus* a shift in the morphogenetic pattern from shoot-bud to embryoid formation in the long-subcultured callus tissues had been accompanied by the change in requirement of a different cytokinin (Chaturvedi and Mitra 1975). In this experiment, however, the calli which had been selected and subcultured for 7 months or more after callus induction formed only shoot-buds. The calli retained the high regenerative potentiality and their morphogenetic pattern was typical caulogenesis, although it has been reported that shoot organogenesis from rice callus was almost completely inhibited with increased age of cultures (Inoue and Maeda 1980). The results also show that efficient plant regeneration was attained from a long-term subculture by organogenesis as well as by somatic embryogenesis in an earlier report (Abe and Futsuhara 1985).

In the callus cultures of *Solanum* species (Swarnkar et al. 1986) and tobacco (Thorpe and Gaspar 1978), an increase was found in the specific activity of peroxidase in shoot- and root-forming calli, implying the involvement of the enzymes related to the process of organogenesis. In rice callus cultures, Saka and Maeda (1974) demonstrated changes in isozyme pattern of α-amylase, and increase in activities of α-amylase and RNase in the shooting region. The present investigation demonstrated that calli of the selected line have a strong peroxidase band compared with the non-selected line, and suggested that the ability of shoot-bud formation in rice callus cultures was related to the peroxidase zymogram pattern. This also suggested that auxins in the selected calli have been oxidized due to a higher activity of peroxidases than those in the non-selected ones and, hence, the endogenous auxins in the selected calli were assumed to be dissolved because of the stronger activity of peroxidase, resulting in lowering of auxin/cytokinin ratio as in sugar beet callus described by Kevers et al. (1981). Consequently, these highly active peroxidases may increase the ability of shoot-bud formation and may change morphogenic pattern.

Additional biochemical changes were that the two protein bands (24 and 42 K) characteristic of the non-selected calli were lost in the selected calli. Although the functional significance of loss of the polypeptides is not clear, it might be considered to be associated with the ability of the callus to differentiate shoot-bud. Changes in peroxidase isozyme pattern and other protein bands and different responses to the 2,4-D concentration seem to be a reflection of some genetic variation which has occurred during the long-term culture and selection.

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