Utilization of Resistant Cell Lines to 5-methyltryptophan for Cell Fusion in Rice (*Oryza sativa* L.)

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5-methyltryptophan (5MT) resistant cell lines, SAR-1, SAR-2 and SAR-3 were selected from anther derived calli of rice (*Oryza sativa* L. var. Sasanishiki). Among these selected cell lines, two (SAR-1 and SAR-3) were stable in 5MT resistance and accumulated free tryptophan at least 50 times higher than that of the unselected cells. The resistant cell lines, however, were not able to produce plants on regeneration medium. Protoplasts from SAR-3 suspension culture cells were fused using dextran method with protoplasts of 5MT sensitive cells (*Oryza sativa* L. var. Hanahikari) which had the ability to regenerate plants. Prospective somatic hybrids were selected by culture on selection medium containing 5MT and then on regeneration medium for plant formation. Three albino plants suspected to be somatic hybrids were regenerated from selected colonies. Calli, initiated from the plants, showed resistance to 5MT intermediate between that of the two parents. These results indicate that the combination of 5MT resistance and plant regeneration ability can be used as a complementary marker for selection and identification of somatic hybrids in rice plants.

KEY WORDS: *Oryza sativa* L., cell culture, 5MT resistance, protoplast fusion, somatic hybrid.

Introduction

During the last decade, many somatic hybrids were obtained through protoplast fusion. These somatic hybrids were produced using selection systems, such as biochemical markers, which allowed easy identification of hybrids on a suitable medium. Of the biochemical mutants, amino acid analog-resistance has been shown to be useful as a dominant selection marker in selecting somatic hybrids (WHITE and VASIL 1979, HARMS *et al.* 1981, 1982, KAMEYA *et al.* 1981, HORN *et al.* 1983, TORIYAMA *et al.* 1987).

Recently, plant regeneration has been reported from rice protoplasts isolated from cultured cells, that should be a prerequisite for the utilization of somatic hybridization (ABDULLAH 1986).

In this paper, we deal with selection and characterization of 5MT resistant cell lines of rice, which could not form plants on the regeneration medium. We also report that somatic hybrids were successfully obtained by fusing the 5MT resistant cell line with a different cultivar having plant regeneration ability.

Materials and Methods

Plant materials

Calli used in this study were initiated from anther culture of rice (*Oryza sativa* L. var. Sasanishiki). The anthers containing uninucleate pollen grains were placed on each of B5 (GAMBORG *et al.* 1968), AA (TORIYAMA and HINATA 1985) and N6 (CHU *et al.* 1975) agar (0.8%) medium supplemented with 2mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) to induce callus

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formation. The anther-derived calli were transferred to 40ml of AA medium, and cultured in 100ml flasks with reciprocal shaking at 120 rev/min. to establish suspension cultures. The cultures were grown in the dark at 25°C and subcultured every 2 weeks at a 1:4 dilution rate (suspension inoculum/fresh medium).

Selection of 5MT resistant cells

Six month-old subcultured cells were passed through a 200 μ m-mesh nylon net and about two hundred mg fresh weight of them were transferred to 40ml of AA liquid medium containing 50ppm 5MT. After 4 to 5 weeks, rapidly growing cells were plated on AA agar (0.8%) medium containing 50ppm 5MT in a Petri dish (90mm diameter, 15mm high) and cultured under diffuse light (3000 lux) at 25°C.

Test for 5MT resistance

Resistance to 5MT was tested by inoculating 200mg fresh weight of cells into 40ml of AA medium having different concentrations of 5MT. The growth was measured by the settled-cell volume after 10 days.

Analysis of free amino acid

Free amino acids were extracted from 12-day-old suspension cells cultured in AA liquid medium according to the method of WAKASA and WIDHOLM (1987) and analysed with an automatic amino acid analyzer, HITACHI 835-50.

Table 1. Free amino acid contents of unselected cell (SAS) and 5MT-resistant cell (SAR-3) Numbers are given as n mol/gFW

Amino acid	unselected cell (SAS)	Resistant cell (SAR-3)
ASP	51.07	254.46
THR	104.72	166.90
SER	136.12	282.11
GLU	155.93	714.80
GLY	251.67	625.80
ALA	361.65	712.48
CYS	78.39	134.64
VAL	192.40	304.71
MET	54.47	112.31
ILE	109.90	168.56
LEU	167.98	256.42
TYR	93.32	133.63
PHE	87.16	147.97
LYS	217.03	208.24
HIS	66.49	93.82
TRY	24.34	1370.75
ARG	997.51	618.38
Total	3150.15	6305.98

Protoplast isolation, fusion, selection and culture

Protoplasts were prepared from suspension cultured cells of a 5MT-resistant cell line (SAR-3) and a 5MT-sensitive cell line, HA (*Oryza sativa* L. var Hanahikari) initiated from anther on AA agar medium supplemented with 2mg/l 2,4-D. A 0.5ml (settled cell volume) of 5-day-old cells was incubated in 10ml of an enzyme solution consisted of 2% Cellulase Onozuka R10 (Yakult Biochemicals, Nishinomiya, Japan), 0.2% Macerozyme R10 (Yakult Biochemicals), 6.5% mannitol and 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (pH 5.5) on a reciprocal shaker (60 rev/min) for 1.5 h, followed by 1.5 h of stationary incubation at 25°C. The protoplasts were passed through a 30 μm -mesh nylon net, and washed three times with a washing solution consisting of 6.5% mannitol and 0.1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (pH 5.5) by centrifugation (100 $\times$ g, 3min).

Protoplast fusion was carried out by dextran treatment (KAMEYA 1983) after mixing equal parts of protoplasts of SAR-3 and HA. These protoplasts ($2 \times 10^5/\text{ml}$) were cultured in 2ml of B5 medium supplemented with 5% glucose. After 2 weeks, 1ml fresh B5 medium containing 3% glucose was added and after another 2-3 weeks, an equal volume of AA medium containing 50ppm 5MT (selection medium) was added. After two weeks of culture in the selection medium, the cell colonies were transferred onto B5 agar (0.8%) medium without glucose. Two weeks later, colonies of about 2 to 3mm in diameter were transferred to N6 medium supplemented with 5% sucrose, 0.2mg/l indole-acetic acid, 1mg/l kinetin and 1% agarose for

Fig. 1. Selection scheme for somatic hybrids between SAR-3 and Hanahikari. Somatic hybrids were selected by using 5-methyltryptophan (5MT) and then by regeneration medium.

Fig. 2. There 5MT resistant calli developed on agar medium containing 50ppm 5MT at one month after plating. Arrows indicate 5MT resistant calli.

plant regeneration. The selection procedure for somatic hybrids is shown in Fig. 1. The suspension and protoplast cultures were grown in the dark and plant regeneration was performed under fluorescent light (3000lux) at 25°C.

Results and Discussion

By plating cell suspensions on the 5MT (50ppm) agar medium, three resistant colonies were selected (Fig. 2). The frequency of resistant colonies was about 5×10^{-7} per cell. These three resistant cell lines were designated as SAR-1, -2 and -3, and used to investigate whether 5MT resistance is stable or not. After 2 months of culture in AA medium without 5MT, SAR-1 and -3 were still resistant to 5MT but SAR-2 lost its resistance (Fig. 3). SAR-1 and SAR-3 could grow at 200ppm of 5MT, whereas growth of the unselected cell (SAS) was completely inhibited even at 50ppm. SAR-3 has been showing resistance even when grown without 5MT for more than one year (Fig. 5). Therefore, it is surely expected that they are stable 5MT-resistant cell lines.

Table 1 shows the free amino acid contents of both SAR-3 and SAS. 5MT resistant cell line, SAR-3, contained 50 times more than the normal level of free tryptophan as pointed out by WAKASA and WIDHOLM (1987). Resistance in SAR-3 is considered to be due to the accumulation of high levels of free tryptophan. The contents of the other free amino acids in SAR-3 were 1.5 to 5 times higher than those of SAS. The total amount of free amino acids in SAR-3 also increased about 2 times more than that of SAS.

Fig. 3. Stability of selected cell lines resistant to 5MT. 5MT resistance was determined after 2 months culture with or without 50ppm 5MT. SAR: SAR-3 cultured with 5MT continuously. SA (R1, R2, R3): Resistant cell lines cultured without 5MT for 2 months. SAS: unselected cell.

Fig. 4. Plant regeneration from calli selected from fusion treated protoplasts.

Fig. 5. Effect of 5MT on cell growth expressed as packed cell volume as compared to that in AA medium. The packed cell volume was determined after 10 days. SAR-3: 5MT resistant cell line after culture of 15 months without 5MT. HA: Hanahikari. SAR-3 + HA: somatic hybrid.

Fig. 6. Effect of 5MT on callus growth of SAR-3, HA and SAR-3 + HA at one month after culture.

In order to test 5MT resistance of the somatic hybrids, the callus induced from the roots was incubated in AA liquid medium with a range of 5MT concentrations. The results showed that the levels of resistance of the hybrid callus was intermediate of that of parents (Fig. 5 and 6).

From the above results, the 5MT resistance of SAR-3 can be considered to be stably inherited to the hybrids as reported in *Nicotiana* (WHITE and VASIL 1979), *Daucus* (KAMEYA *et*

Attempts to regenerate plants from SAR-3 using plant regeneration medium have been unsuccessful. Therefore, it is assumed that SAR-3 has lost plant regeneration capacity.

Protoplast fusion and selection of somatic hybrids between SAR-3 and HA were carried out according to the scheme in Fig. 1. Fused protoplasts could not be identified because it was impossible to distinguish SAR-3 from HA in morphology of isolated protoplasts.

From the preexamination for culture of SAR-3 protoplasts, it was found that colony formation was greatly influenced by both 5MT concentration and the time of addition of selection medium (Table 2). In the present experiments, the fusion-treated protoplasts were cultured in B5 medium without 5MT for 5 to 6 weeks, then transferred to selection medium containing 25ppm 5MT, which caused death of HA protoplasts. After about 2 weeks, 570 colonies (more than 2mm in diameter) were recovered from 2×10^5 protoplasts. These colonies were transferred to regeneration medium and 3 albino plants differentiated (Fig. 4). Since HA protoplasts could not develop to colonies and SAR-3 colonies did not produce any organs under the present experimental conditions (Table 3), these albino plants are highly suspected to be the somatic hybrids between SAR-3 and HA.

Table 2. The effect of 5MT concentration and time of applying selection medium on the growth of SAR-3 protoplast culture

Culture period (weeks) before 5MT addition	Liquid media for protoplast culture	5MT concentration (mg/l)				
		0	5	10	25	50
2 C	NO ₃	+	—	—	—	—
	B5	++	—	—	—	—
	AA	+	+	—	—	—
3-4 C	NO ₃	++	—	—	—	—
	B5	+	—	—	—	—
	AA	+	+	+	—	—
5-6 C	NO ₃	++	++	++	+	+
	B5	++	++	++	++	+
	AA	+	+	+	+	+

++: Vigorous growth, +: A little growth, —: No growth

NO₃: B5 medium without (NH₄)₂SO₄ was supplemented with 2.95 g/l KCl.

AA: Toriyama *et al.* 1985

B%: Gamborg *et al.* 1968

Table 3. Regeneration frequency of colonies obtained from selection medium (B5 medium containing 25ppm 5MT)

Cultivar	Repetition	Number of colonies transplanted to regeneration medium	Number of colonies produced		
			Green plant	Albino plant	Root
SAR-3	2	300	0	0	0
HA	2	0	0	0	0
SAR-3 + HA	5	570	0	3	5

SAR-3: Sasanishiki (5MT resistant calli)

HA: Hanahikari (5MT sensitive calli)

1981) and *Sinapis* (TORIYAMA *et al.* 1987). Our results also demonstrate that the combination of 5MT resistance and plant regeneration capacity is an effective selection marker or somatic hybridization in rice plants.

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イネの細胞融合における5-メチルトリプトファン耐性細胞株の利用

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5-メチルトリプトファン (5 MT) 耐性細胞株をイネ (品種, ササニシキ) の薬培養由来の懸濁培養細胞から, 50 ppm の 5 MT を含む液体培地及び寒天培地で選抜したところ, 3 系統の細胞株 (SAR-1, 2, 3) が得られた. この耐性株の出現頻度は, およそ 5×10^{-7} であった. これらのうち 2 系統 (SAR-1, 3) は安定した耐性を示したが, 1 系統 (SAR-2) は, 二ヶ月間, 5 MT を含まない培地で培養したところ耐性を消失した. 安定した耐性を示した SAR-3 の遊離アミノ酸含量を分析したところ, トリプトファンが 5 MT で選抜しなかった親株より, 50 倍増加していることがわかった. この SAR-3 株からの植物再分化を試みたが, 成功せず, それ故, この株は, 長期培養のため, 再分化能力を失ったものと推定した.

SAR-3 株の 5 MT 耐性形質を植物体に導入するため, 再分化能力があり, 5 MT 感受性の細胞株 (ハナヒカリ) との細胞融合を行った. 両プラトプラストをデキストラン溶液中で混合し, 融合処理を行った. コロニーの直径が 200 μm ぐらいになるまで 5 MT 無添加培地で培養した. その後, 5 MT 25 ppm を含む選抜培地で 2 週間培養し, 盛んに増殖してきたコロニーを選び, 再分化培地で培養したところ, およそ 2×10^5 個のプロトプラストから, 3 個のアルビノ植物が得られた. これらの植物からカルスを作成し, 5 MT 耐性を調査したところ, SAR-3 とハナヒカリの中間型を示した. 本実験の対照として用いた SAR-3 は再分化せず, ハナヒカリにおいては, 細胞分裂はほとんど観察されず, コロニー形成には到らなかったため, 得られたアルビノ個体は雑種であると推定された.

本実験の結果は他植物 (*Nicotiana*, *Dacus*, *Sinapis*) で報告されているように, 5 MT 耐性と再分化能のマーカーがイネの細胞融合においても有効であることを示している.