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Production of a triple mutant, chlorophyll-deficient, streptomycin-, and kanamycin-resistant *Nicotiana tabacum*, and its use in intergeneric somatic hybrid formation with *Solanum melongena*

S. Toki [1,*], T. Kameya [2] and T. Abe [2,]**

[1] Institute of Biological Sciences, University of Tsukuba, Tsukuba City, Ibaraki 305, Japan
[2] Institute of Genetic Ecology, Tohoku University, Sendai City 980, Japan

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Summary. In order to produce a triple mutant, sexual crosses between a chlorophyll-deficient, streptomycin-resistant mutant of *Nicotiana tabacum* (SA) and a kanamycin-resistant transformant of *N. tabacum* (KR) were carried out. From the offspring of this cross, a triple mutant (KR-SA) was selected. In *N. tabacum* KR-SA, chlorophyll deficiency is due to recessive mutation in the nuclear genome, streptomycin resistance is due to a dominant mutation in the chloroplast genome, and kanamycin resistance is shown to be a dominant nuclear marker. Cell suspension protoplasts of *N. tabacum* KR-SA were fused with callus protoplasts of *Solanum melongena* by dextran treatment. Somatic hybrid plants were selected for streptomycin resistance and the ability to produce clorophyll in regenerated plants. By using this selection system, green plants were recovered from two colonies. When these green plants were then tested for kanamycin resistance, all analyzed plants carried this trait. In addition, the hybrid nature of these plants was confirmed by investigation of the peroxidase isozyme. The present results show that the use of *N. tabacum* KR-SA in studies of somatic hybridization makes it possible to select somatic hybrid plants easily and provides information of the *N. tabacum* genome.

Key words: *Nicotiana tabacum* – *Solanum melongena* – Somatic hybridization – Universal hybridizer

Introduction

The selection of somatic hybrid cells (or plants) from a population of unfused and homofused protoplasts is still a major area of difficulty (Harms 1983). There are many kinds of selection schemes but they often require two unique (selected) cell lines as fusion partners (Widholm 1982).

The development and use of "universal hybridizer" cell lines containing both positive and negative genetic markers allow easy isolation of somatic hybrids. Protoplasts derived from such cell lines, fused with any wild-type cell lines, produce heterokaryons that can be selectively grown and thus isolated from the parental cells. Recently, by using universal hybridizers, several somatic hybrids have been effectively obtained (Lo Schiavo et al. 1983; Pental et al. 1984, 1986; Ye et al. 1987; Brunold et al. 1987; Toriyama et al. 1987).

We have previously reported on the production of a chlorophyll-deficient, streptomycin-resistant mutant of *Nicotiana tabacum* SA (Toki and Kameya 1987). In *N. tabacum* SA, chlorophyll deficiency is due to recessive mutations at two loci in the nuclear genome, and streptomycin resistance is due to a dominant mutation in the chloroplast genome. Using this mutant for protoplast fusion experiments with *N. rustica* and *Salpiglossis sinuata*, we successfully selected interspecific and intergeneric somatic hybrids, respectively, according to their ability to produce chlorphyll and streptomycin resistance (Toki and Kameya 1988; S. Toki and T. Kameya, in preparation).

However, in the selection scheme on the use of chlorophyll-deficient, streptomycin-resistant mutant *N. tabacum* SA, there is a possibility that two types of plants will be obtained. One type has the nuclear genome of both the parents and the choroplast genome of *N. tabacum*, and the other type has the nuclear genome of the wild-type parent and the chloroplast genome of *N. tabacum*.

In this paper, we describe the production of a triple mutant by sexual crosses between *N. tabacum* SA and

* *Present address:* Department of Botany, Faculty of Science, Hokkaido University, Sapporo 060, Japan
** Chemical Regulation of Biomechanism, The Institute of Physical and Chemical Research, Wako 351-01, Japan
*** To whom correspondence should be addressed

Table 1. Composition of culture media[a]

Addenda	Medium D	Medium E	Medium F	Medium G	Medium H
Mineral salts	MS[b]	MS	MS	MS	MS
Vitamines	UM[c]	UM	UM	UM	UM
Sucrose (w/v)	3%	3%	3%	3%	3%
Glucose (w/v)	5%	5%			
2,4-D[d]	1.0 mg/l	1.0 mg/l			
NAA[e]				0.1 mg/l	0.1 mg/l
BAP[f]				1.0 mg/l	
Kinetin	0.5 mg/l	0.5 mg/l			
Zeatin			2.0 mg/l		
Streptomycin sulfate		1,000 mg/l			
Agar (w/v)			0.8%	0.8%	0.8%

[a] pH was adjusted to 5.7
[b] Murashige and Skoog (1962) formulation
[c] Uchimiya and Murashige (1974) formulation
[d] 2,4-Dichlorophenoxyacetic acid
[e] Naphthaleneacetic acid
[f] Benzylaminopurine

kanamycin-resistant *N. tabacum*. This triple mutant (denoted KR-SA) makes it possible to rapidly confirm the presence of the nuclear genome of *N. tabacum* in putative somatic hybrid plants. We also describe how this triple mutant KR-SA can be used for protoplast fusion experiments with wild-type *Solanum melongena* as well as its hybrid nature.

Materials and methods

Selection of triple mutant plants

Chlorophyll-deficient, streptomycin-resistant mutants of *Nicotiana tabacum* SA were produced by sexual crosses between streptomycin-resistant mutants of *N. tabacum* SR1 (Maliga et al. 1973) and chlorophyll-deficient mutants of *N. tabacum* Al (Toki and Kameya 1987). Kanamycin-resistant *N. tabacum* (designated as KR in this paper) was produced by the method of direct transformation, using the plasmid pCT1T3 of Uchimiya et al. (1986) and kindly provided by him.

To produce a *N. tabacum* that has triple markers, sexual crosses between SA and KR were carried out. In these crosses the SA mutant was used as female parent. Seeds from the crossed plants were germinated on filter paper tapped with water containing 400 mg/l kanamycin sulfate under continuous fluorescent light (4 W/m^2) at 25 °C, and seedlings viable on this paper were grown to the flowering stage.

To allow segregation of chlorophyll-deficient, kanamycin-resistant genotypes, F_1 plants were self-fertilized. Seeds from selfed F_1 plants were surface-sterilized with 2% (v/v) sodium hypochlorite for 15 min, followed by rinsing in sterile water. The seeds were germinated on Medium A [MS medium (Murashige and Skoog 1962) solidified with 8 g/l agar] under continuous light (4 W/m^2) at 25 °C.

The albino plants emerging in the F_2 generation were expected to be mixtures of kanamycin-resistant and -sensitive plants. They were thus transferred to selection medium (Medium B: Medium A plus 200 mg/l kanamycin sulfate). The selected plants were designated KR-SA.

Growth test

The stability of kanamycin resistance was studied in seedlings. Seeds from Al, SR1, SA, KR, and KR-SA were germinated on Medium B under continuous fluorescent light (4 W/m^2) at 25 °C. After 30 days from planting, the effect of kanamycin was determined.

Callus induction

Callus of KR-SA was induced from surface-sterilized leaves (2% sodium hypochlorite for 10 min, followed by rinsing in sterile water) on Medium C [Medium A plus 1 mg/l 2,4-D and 0.1 mg/l kinetin, with UM vitamins substituted for MS vitamins (Uchimiya and Murashige 1974)]. Callus formed from the leaf edge was transferred to liquid Medium C (Medium C minus agar), then subcultured for at least 20 passages before using for protoplast isolation. Callus of *Solanum melongena* L. (var. *shironasu*) was induced by the same method in KR-SA, but was maintained on agar Medium C.

In both cases, cultures were grown under continuous fluorescent light (4 W/m^2) at 25 °C.

Protoplast preparation and fusion

N. tabacum KR-SA protoplasts were isolated from 12-month-old suspension-cultured cells by incubation in a solution containing 10% (w/v) mannitol, 0.2% (w/v) $CaCl_2 \cdot 2H_2O$, 2% (w/v) cellulase Onozuka RS, and 0.3% (w/v) macerozyme R10 for 6 h in the dark at 25 °C. *S. melongena* protoplasts were isolated from 16-month-old callus by incubating in the same conditions.

Protoplasts were filtered through a 56 μm sieve and washed twice with washing solution [9% (w/v) mannitol, 0.1% (w/v) $CaCl_2 \cdot 2H_2O$] by centrifugation at 100 *g* for 3 min. The protoplasts were purified by floating them in a 20% (w/v) sucrose solution with centrifugation at 100 *g* for 5 min. They were brought to a density of 5×10^5/ml in washing solution, mixed in equal volumes, and fused using the dextran method of Kameya (Toki and Kameya 1988).

Culture of protoplasts and plant regeneration

After fusion, the protoplasts (1×10^5) were cultured in 4 ml of protoplast culture medium (Medium D, Table 1) in plastic petri dishes at 25 °C. During the first 30 days, petri dishes were incu-

Table 2. The results of crosses between *N. tabacum* SA × *N. tabacum* KR

Cross	No. of incubated seeds	No. of germinated seeds	Observed ratios		Expected ratios		χ^2	*P*
			green	albino	green	albino		
F_1	92	86	86	0	1	0	0.000	1.0
F_1 selfed	375	367	342	25	15	1	0.119	0.5–0.75

F_1 seeds were germinated on filter paper tapped with water containing 400 mg/l kanamycin sulfate. F_1 selfed seeds were germinated on Medium A (see text)

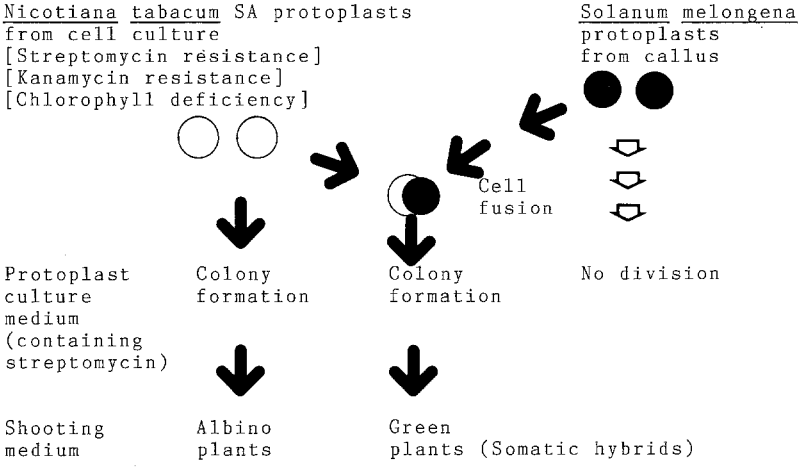

Fig. 1. Selection scheme for hybrids between *Nicotiana tabacum* KR-SA and *Solanum melongena*

bated in the dark and then transferred to continuous light (4 W/m^2). After 14 days of culture, the cell suspension was diluted with an equal volume of selection medium (Medium E, Table 1), according to the selection scheme in Fig. 1. After 30 days of culture, colonies consisting of 20–50 cells were diluted with four times as much volume of Medium E [concentration of glucose was reduced to 2.5% (w/v)]. After growth, colonies about 1–2 mm in diameter were transferred to shooting medium (Medium F, G, Table 1). Regenerated shoots were transferred to a rooting medium (Medium H, Table 1).

Analysis of peroxidase isozyme

Segments of 100 mg of young leaf were homogenized with 200 µl of 50 m*M* Tris-HCl buffer (pH 6.7) at 4 °C. After centrifugation at 2,000 *g* for 10 min, 20 µl of the supernatant was used for thin-layer, polyacrylamide gel isoelectroforesis (Okazaki and Hinata 1984). The gels were stained according to Yamamoto and Momotani (1971).

Kanamycin sensitivity of hybrid plants

The callus of putative somatic hybrids was induced from leaves on Medium C, then transferred to the same medium containing 200 mg/l kanamycin sulfate.

Neomycin phosphotransferase assay [NPT (3′)II assay]

Leaves (20 mg) were homogenized in 50 µl buffer, and 30 µl of each extract was electrophoresed through a polyacrylamide gel and assayed for NPT(3′)II activity, using the method of Schreier et al. (1985).

Results and discussion

Production of the KR-SA triple mutant

The progenies of crosses between SA and KR were all green plants in the presence of 400 mg/l kanamycin sulfate (Table 2). Therefore, they have the ability to produce chlorophyll and are resistant to kanamycin (and also resistant to streptomycin, because streptomycin resistance is coded in the chloroplast DNA of SA).

F_1 hybrids were selfed and the progeny were scored for green to albino plant segregation. The results, shown in Table 2, indicate that the albino trait is determined by two recessive genes. The albino plants that emerged in the F_1 progeny were transferred to selection medium B, which contained 200 mg/l kanamycin sulfate. Approximately 75% of these plants grew normally on this medium and the remaining ones died. The former were expected to be chlorophyll-deficient, kanamycin-resistant, and streptomycin-resistant mutants.

However, the former were most likely to be mixtures of plants that were homogenous and heterogenous as to the kanamycin-resistant gene. Therefore, a progeny test was undertaken on them, and homogenous plants were selected and designated as KR-SA.

Three to five days after planting, tobacco seeds started to germinate on medium containing 200 mg/l

Fig. 2. The regeneration of a green plant from the somatic hybrid between *N. tabacum* KR-SA and *S. melongena*

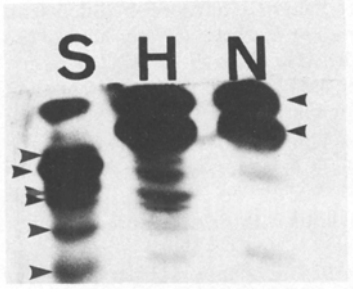

Fig. 3. Isozyme patterns of peroxidase. *N: N. tabacum* KR-SA; *H:* a somatic hybrid from H1 colony; *S: Solanum melongena*

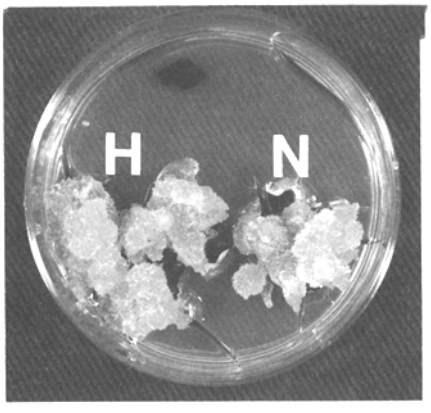

Fig. 4. Kanamycin-resistant test of *N. tabacum* KR-SA (*N*) and a somatic hybrid plant (*H*)

kanamycin sulfate. The effect of kanamycin was observed on the color and growth of seedlings. In green plants, cotyledons of kanamycin-sensitive seedlings (SR1) turned yellow, whereas cotyledons of resistant seedlings (KR) remained green. In albino plants, growth of kanamycin-sensitive seedlings (Al and SA) was inhibited, whereas that of KR-SA seedlings was not.

It has been proven that the chlorophyll-deficient trait is determined by two recessive genes (Toki and Kameya 1987) and that the kanamycin-resistant trait is determined by a single dominant gene (Uchimiya et al. 1986). Therefore, theoretically, one can expect to obtain the desired triple mutant with a ratio of 1/64 in the SA × KR F_1 progeny. The results, obtained from SA × KR F_1 self-fertilization and their progeny test, confirmed our expectations.

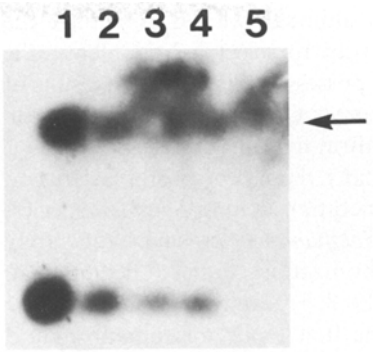

Fig. 5. Neomycin phosphotransferase assay. *Lane 1:* a positive control (*N. tabacum* KR-SA); *lanes 2* and *3:* somatic hybrids from H1 colony; *lane 4:* a somatic hybrid from H2 colony; *lane 5:* a negative control (SR1). *Arrow* indicates an endogenous enzyme

Protoplast fusion and selection of somatic hybrids

N. tabacum KR-SA protoplasts and *S. melongena* protoplasts were fused by dextran treatment. The fusion frequency was ca. 10%. The heteroplasmic fusion products could not be identified because the parent protoplasts had similar morphology.

After 2 months of culture, colonies (1–2 mm in diameter) appeared on selection Medium E. One hundred and twelve colonies were transferred to shooting Medium F. From these 17 colonies regenerated several albino shoots and 2 colonies (NS1, NS2) regenerated green shoots. When 96 colonies were transferred to shooting Medium G, no colonies regenerated any shoots.

The green shoots regenerated from selected colonies were expected to be somatic hybrids according to the selection scheme in Fig. 1. Consequently, they were transferred to rooting Medium H. On this medium, only one shoot derived from the NS1 colony produced roots (Fig. 2).

Isozyme analysis on leaves was performed for putative somatic hybrids derived from the NS1 colony (Fig. 3). The bands of peroxidase in these plants were a combination of the parental bands.

The callus of putative somatic hybrid plants deriving from an NS1 colony was resistant to kanamycin (Fig. 4). NPT (3′)II activity was detected in the leaf extracts of putative somatic hybrid plants deriving from NS1 and NS2 (Fig. 5).

In this experiment, although we did not utilize the kanamycin resistance trait to select hybrid plants, the obtained hybrid plants possessed this trait. The results of isozyme analysis, kanamycin resistance test, and green color of the plants confirm hybridity of these plants.

There have been many reports of protoplast fusion and hybrid plant regeneration among *Solanum* species, but the nontuberous *Solanum* species have been rarely utilized in somatic hybridization studies (Gleddie et al. 1986: Sihachakr et al. 1988; T. Kameya, in press). As far as we know, this is the first report of somatic hybrid formation between *N. tabacum* and *S. melongena*.

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