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Interspecific hybrid plant formation by electrofusion in *Nicotiana*

H. Morikawa [1], T. Kumashiro [2], K. Kusakari [1], A. Iida [1], A. Hirai [3] and Y. Yamada [1]

[1] Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
[2] Iwata Experiment Station, Japan Tobacco Inc., Iwata, Shizuoka 438, Japan
[3] Graduate Division of Biochemical Regulation, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan

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Summary. We obtained complete hybrid plants by electrofusion of mesophyll protoplasts from *Nicotiana glauca* and *N. langsdorffii.* Electrofocusing analysis of Fraction I proteins isolated from the leaves of these plants confirmed their hybridity. Cytological analysis indicated that the chromosome number (2n) of these plants is between 60 to 66, suggesting that they are the products of triple fusion. All plants were fertile and set viable seeds after self pollination. As we did not use an AC field for electrofusion, the present results indicate that an AC field is not essential for obtaining hybrid plants with electrofusion.

Key words: Electrofusion – Fertile somatic hybrid – Fraction I protein analysis – *Nicotiana*

Introduction

Since the first reports on successful protoplast electrofusion (Neumann et al. 1980; Senda et al. 1979; Weber et al. 1981; Zimmermann and Scheurich 1981), there have been several reports on the subsequent formation of hybrid plant calluses or shoots by this method (Bates and Hasenkampf 1985; Chapel et al. 1986; Kohn et al. 1985; Morikawa et al. 1986). However, as far as we are aware, no reports on the formation of fertile hybrid plants by electrofusion have been published.

As reported previously (Morikawa et al. 1986), by electrofusion of mesophyll protoplasts from *Nicotiana glauca* and *N. langsdorffii,* we obtained a number of hybrid calluses that grew vigorously on hormone-free medium. All hybrid callus colonies regenerated shoots after 4 months of culture and some formed roots. After more than 8 months of culture, some hybrid callus lines formed small plantlets and they developed to mature plants, flowered and set seeds.

Materials and methods

Plant materials

Hybrid callus colonies were obtained by electrofusion of protoplasts from *N. glauca* and *N. langsdorffii* in October 1984, as reported previously (Morikawa et al. 1986). Since then we have been culturing them on hormone-free medium under continuous light (ca. 1,000 lux) at 26 °C (Morikawa et al. 1986). The hormone-free medium contained mineral salts and glycine as in Murashige and Skoog medium (Murashige and Skoog 1962), vitamins as in B5 medium (Gamborg and Eveleigh 1968) and 3% sucrose (pH 5.6) solidified with 0.6% agar. The calluses were subcultured monthly. After more than 8 months of culture, some hybrid calluses formed small plantlets. The plantlets were transferred to autoclaved vermiculite in a pot under non-aseptic conditions, the plantlet and pot being covered with a plastic bag to prevent drying and cultured for 2 to 3 weeks in a culture room, as described above. After removal of the plastic bag, they were transferred to a greenhouse and cultured at 20±5 °C under natural day light. Usually, 3 to 6 months after transfer, the plants flowered, self pollinated and set seeds.

Electrofocusing analysis

Fraction I proteins were isolated from the leaves of young plants of somatic hybrid and analysed by isoelectrofocusing according to the method of Hirai (1982).

Cytology

Chromosome numbers were determined from vigorously growing root tips. Root tips were pretreated with 2 mM 8-hydroxyquinoline for 5 to 6 h, fixed with ethanol : acetic acid (3 : 1 v/v) mixture overnight, transferred into 70% ethanol and stored at 4 °C until use. After rinsing with distilled water 3 times, root tips were hydrolysed for 10 min in 1 N HCl at 60 °C, stained for 1 h in Schiff's reagent, then squashed after addition of a drop of acetocarmine. At least three mitotic figures were counted per plant to determine the chromosome number.

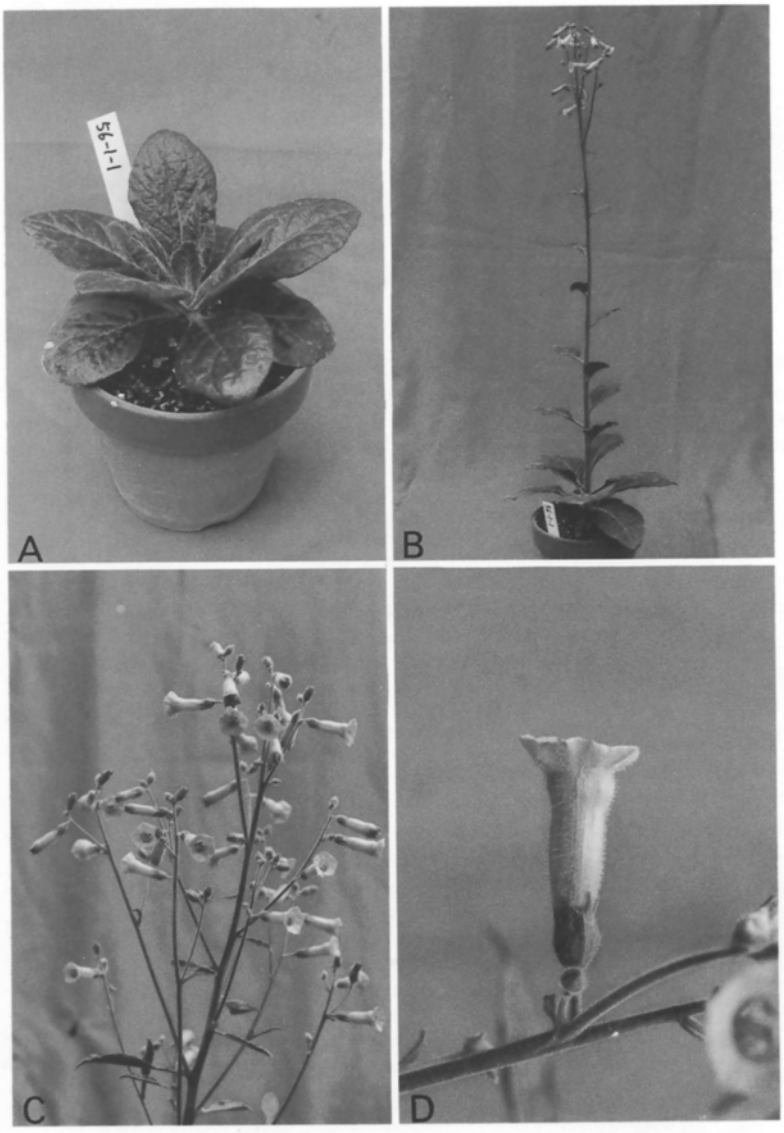

Fig. 1 A–D. Typical pictures of young and mature plants and flowers of somatic hybrids of *N. glauca* and *N. langsdorffii.* **A** about 3 months after transfer to a greenhouse (Table 1, line 56-1, no. 1); **B** about 4 months after transfer to a greenhouse (line 56-1, no. 1); **C, D** about 5 months after transfer to a greenhouse (line 17-2)

Results and discussion

We cultured more than 90 hybrid callus (teratoma) lines of *N. glauca* and *N. langsdorffii* on hormone-free medium which were obtained by electrofusion in October 1984, as reported previously (Morikawa et al. 1986). After more than 8 months of culture, some callus lines formed small plantlets which developed to mature plants. Figure 1 shows a typical picture of the hybrid plants thus obtained. The morphology of young (A) and mature (B) plants and flowers (C and D) were distinct from parental plants but similar to that of amphidiploid plants obtained from colchicine treatment of sexual hybrids of *N. glauca* × *N. langsdorffii.* Smith et al. (1979) also reported that mature plants from protoplast fusion of these *Nicotiana* species by polyethylene glycol method were morphologically similar to true amphidiploid plants. At present we have obtained seven complete hybrid plants like the one shown in Fig. 1 B, and all have set seeds upon self pollination (see Table 1).

We next analysed the hybridity of these plants by electrofocusing analysis of the Fraction I proteins from leaf extracts. Typical results are shown in Fig. 2. Lanes G and L correspond to *N. glauca* and *N. langsdorffii* respectively, and lanes 1 to 5 to somatic hybrid plants obtained by electrofusion while lane 6 is from a sexual hybrid with *N. glauca* as a mother plant. Clearly, these electrically-produced hybrid plants had the small subunit (SS) polypeptides of the proteins of *N. glauca* and *N. langsdorffii.* Similar results were obtained with other plants (data not shown). These results indicate that the information in the two different nuclear genomes of parental tobacco species was expressed in all of these hybrid plants and confirm their hybridity.

It should be noted that in general the bands due to the small subunit of *N. langsdorffii* type were more distinct than those of *N. glauca* with all the hybrid plants obtained in this study (Fig. 2). This was also the case with the hybrid calluses, as reported previously (Morikawa et al. 1986). These results indicate that in the hybrids the nuclear SS genes from *N. langsdorffii* are more prominent in quantity and/or expression than are those from *N. glauca.*

Interestingly, the large subunit (LS) polypeptides of the proteins of these hybrid plants appeared to be *N. langsdorffii* type (Fig. 2). This indicates that chloroplast genomes in these plants are only from *N. langsdorffii* or that chloroplast LS genes from *N. glauca* in the plants, if any, were not expressed sufficiently to be detected. Recently, Chapel et al. (1986) also reported that in the hybrid calluses produced by electrofusion of *N. glauca* and *N. langsdorffii* protoplasts, the large subunit polypeptides were of the *N. langsdorffii* type. It would be interesting to study the sorting out of *N. glauca* chloroplast genomes during the culture of hybrid calluses or the regeneration of plants.

Table 1 summarizes cytological information obtained from seven somatic hybrid plants. The chromosome number (2n) of the plants was between 60 and 66 (see also Fig. 3). The parental diploid chromosome numbers are 24 for *N. glauca* (GG) and 18 for *N. langsdorffii* (LL). A simple addition gives a chromosome number of 42 and the products of triple fusion may have a chromosome number of 60 or 66:

LL+GG=42 chromosones; LL+LL+GG=60 chromosomes; GG+GG+LL=66 chromosomes.

The present result suggests that at least six out of seven hybrid plants are the products of a triple fusion of the GG+GG+LL type and that the loss of chromosomes during culture gave rise to aneuploid types. On the other hand, one may expect, from the results of the analysis of the subunits of the Fraction I proteins, that the hybrid plants would be LL+LL+GG type (see above). In order to explain this apparent "discrepancy" more detailed studies are needed on how or why triple fusion-products are preferentially formed during electrofusion and/or on how or why they are selected during prolonged culture.

Interestingly, Smith et al. (1976) also reported a chromosome number of 54 to 64 for the somatic hybrid plants produced by the polyethylene glycol method. In contrast, for similarly produced somatic hybrid plants, Chupeau et al. (1978) reported 42 to 80 and Carlson et al. (1972) 42.

In our electrofusion work, we did not use an AC field to make protoplast pairs but made protoplasts contact each other by placing about ten layers of mixed protoplasts in an electrofusion chamber and applied a

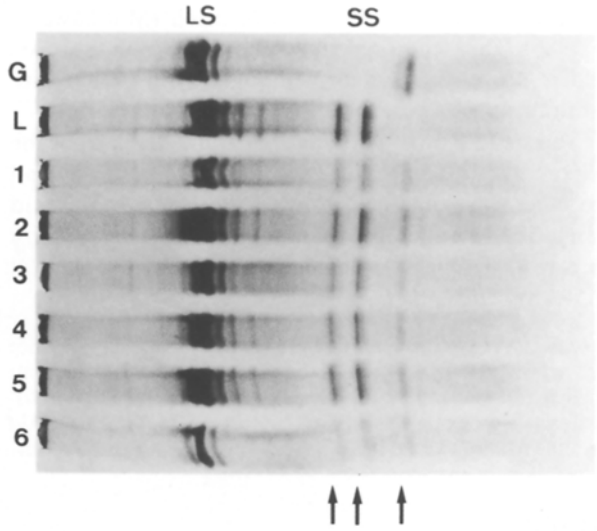

Fig. 2. Photographs of gels after electrofocusing of Fraction I proteins obtained from leaves of *N. glauca* (G), *N. langsdorffii* (L), five somatic hybrid plants (*lanes 1–5*) and sexual hybrid plants (*lane 6*). *Lanes 1–5* correspond to lines 56-2, no. 1, 56-2, no. 2, 56-1, no 1, 56-1, no. 2 and 54, respectively, in Table 1. LS and SS represent the bands due to large and small subunit polypeptides of Fraction I proteins, respectively

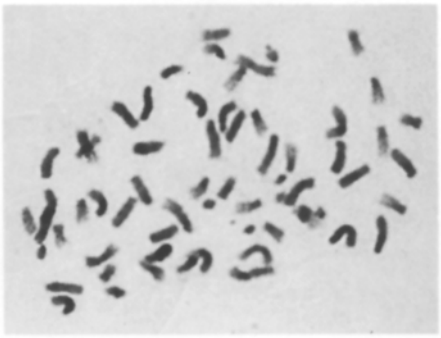

Fig. 3. The 63 chromosomes of line 17-2 during metaphase

Table 1. Cytological information on seven mature hybrid plants produced by electrofusion of protoplasts of *Nicotiana glauca* and *N. langsdorffii*

Line no.	2n	Subunits of FIP[a]		Viable seeds produced
		SS	LS	
17-1	66	G+L	L	+
17-2	63	G+L	L	+
54	66	G+L	L	+
56-1, no. 1	66	G+L	L	+
56-1, no. 2	64	G+L	L	+
56-2, no. 1	60	G+L	L	+
56-2, no. 2	66	G+L	L	+

[a] FIP, SS and LS = Fraction I protein and its small and large subunits, respectively

single DC pulse to induce electrofusion (Morikawa et al. 1986). Thus, the present result indicates that an AC field is not essential for obtaining hybrid plants with electrofusion.

The frequency of germination of the seeds from the somatic hybrid plants was very high (almost 100%) and progenies were obtained from each of the seven hybrid plants. The morphology of the selfed progenies was very similar to that of the regenerant plants. Cytological studies are currently being carried out.

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