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The culture of manually isolated heterokaryons of *Nicotiana tabacum* and *Nicotiana rustica*

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Abstract. Protoplasts derived from leaf mesophyll tissue of *Nicotiana tabacum* were fused with cell suspension-derived protoplasts of *N. rustica*. Heterokaryons were isolated using a micromanipulator and were cultured. Nuclear fusion was observed to occur in many of these heterokaryons after culturing them for a few days. Hybrid cell division was also observed. Fluorescein isothiocyanate (FITC) staining of *N. rustica* protoplasts prior to fusion did not interfere with subsequent hybrid cell division, and the FITC fluorescence was observed to persist beyond the first division stage of hybrid cells. From a total of thirty heterokaryons which were placed in a nurse culture of protoplasts of albino *Petunia hybrida*, thirteen green colonies were subsequently obtained of which six have regenerated somatic hybrid plants. Plants were characterised for their hybrid nature by analysis of vegetative and floral morphology, isoelectric focusing pattern of leaf esterases and Fraction 1 protein. All the six plants are nuclear hybrids. Chloroplast segregation appears to have occurred in these plants, with five having the Fraction 1 protein large subunit of *N. rustica*, and one having the large subunit of *N. tabacum*. Some of the plants possess sexual fertility.

Keywords. Protoplast fusion; nuclear fusion; hybrid cell division; somatic hybrid plants; tobacco.

1. Introduction

The fusion of plant protoplasts and subsequent recovery of somatic hybrid tissue and plants has attracted much interest in recent years as a method for studying interactions between plant genomes, and as a potentially useful method for crop improvement (Cocking *et al* 1981; Harms 1983). In addition to adequate cultural capability of at least one of the parents, and efficient and non-toxic methods of protoplast fusion, a selection scheme is necessary which will enable the recovery of a small number of somatic hybrid colonies from a much larger number of colonies derived from parental protoplasts. Several approaches have been successfully utilized to overcome this problem (Evans 1983).

A selection method, of general applicability is to isolate heterokaryons after fusion and culture them. Recently we described a simple method for manually isolating heterokaryons after fusion (Patnaik *et al* 1982). In this study we report the culture of manually isolated heterokaryons between leaf mesophyll-derived protoplasts of *Nicotiana tabacum* and cell suspension-derived protoplasts of *Nicotiana rustica*. The protoplasts of both these species show high division frequencies after fusogen treatment, and plant regeneration occurs on suitable media, thus necessitating a selection scheme for hybrid recovery.

* Since deceased.

2. Materials and methods

2.1 *Plant material and protoplast isolation*

Growth of, and protoplast isolation from, *Nicotiana tabacum* cv Xanthi was as described previously for wild type *N. tabacum* cv Gatersleben (Pental *et al* 1982). Seeds of *N. rustica* cv V27 (Jinks *et al* 1981) were surface-sterilized with 20% v/v Domestos (Lever Bros., UK) for 30 min, thoroughly rinsed with sterile tap water and germinated on the surface of BGS medium (Binding 1975) in the dark at 27°C. Hypocotyls, 2 cm in height, were transferred to the surface of UM medium (Uchimiya and Murashige 1974) solidified with 0·8% w/v agar (Sigma). Callus, which was formed after 3–4 weeks, was transferred to UM liquid medium (2 g callus/100 ml medium). Cell suspensions were maintained on a rotary shaker (120 rpm, 1800 lux, 24 ± 2°C) and subcultured every 7 days. New cell suspensions were initiated every 12 weeks. To release protoplasts, cells (3 days after subculturing) were incubated in an enzyme solution containing 2% w/v Rhozyme (Röhm and Haas Ltd., Philadelphia, USA), 4% w/v Meicelase P (Meiji Seika Kaisha Ltd., Tokyo, Japan) and 0·03% w/v Macerozyme R-10 (Yakult Biochemical Ltd., Nishinamiya, Japan) with 13% w/v mannitol and CPW salts (pH 5·8) (Frearson *et al* 1973). After overnight incubation at 24 ± 2°C, the enzyme solution containing cell debris and protoplasts was passed through a 64 μm nylon seive and protoplasts were purified as for *N. tabacum* mesophyll protoplasts. Protoplasts of an albino cell line of *Petunia hybrida* cv Comanche (Patnaik *et al* 1982) were isolated as for *N. rustica* suspension.

2.2 *Protoplast fusion and isolation of heterokaryons*

Fusion between protoplasts of *N. tabacum* and *N. rustica* was carried out using the high pH/Ca^{++} method (Keller and Melchers 1973). After fusion treatment, protoplasts were cultured at a density of 5×10^4/ml in 9 cm petri dishes (Sterilin Ltd. Teddington, UK) in MS-1 medium, ((Murashige and Skoog 1962) salts supplemented with 2 mg l^{-1} NAA (α-naphthaleneacetic acid), 0·5 mg l^{-1} BAP (6-benzylaminopurine), 3% w/v sucrose (pH 5·8)) with 13% w/v mannitol. Heterokaryons were identified by the presence of chloroplasts and cytoplasmic strands, and isolated at various times after fusion using a micromanipulator and bright field optics. In some cases fluorescein isothiocyanate (FITC) staining of suspension cells was carried out to confirm the heterokaryocyte nature of isolated cells (Patnaik *et al* 1982). Isolated heterokaryons were fixed and stained with carbol fuchsin to reveal the nuclei (Kao 1975).

2.3 *Culture of isolated heterokaryons and plant regeneration*

Isolated heterokaryons were cultured in 5–10 μl microdrops of MS-1 medium with 13% w/v mannitol as previously described (Patnaik *et al* 1981). Cells were removed from droplet cultures at various intervals and were either observed using UV or stained with carbol fuchsin to reveal the state of the nuclei.

For long term culture of isolated heterokaryons, they were placed in a nurse culture of protoplasts of albino *P. hybrida* which were placed in MS-1 medium with 13% w/v mannitol 3 days previously (24 ± 2°C, continuous fluorescent light of 1000 lux). Albino protoplasts of *P. hybrida* were cultured at a density of $2{\cdot}5 \times 10^4$/ml in 4 ml of liquid

medium placed over 4 ml of medium solidified with 0·6 w/v agar in 5 cm petri dishes (A/S Nunc, Kamstrup, Roskilde, Denmark).

Individual green colonies, which were detected among albino cell colonies, were removed and placed on MS-1 medium with 4·5 % w/v mannitol. After they had grown to 0·5–1 cm in diameter these colonies were individually placed on solidified MS-2 medium (MS medium with 1 $mg\,l^{-1}$ BAP and 2 $mg\,l^{-1}$ IAA (indole-3-acetic acid)). Shoots from regenerating calli were transferred to MS medium, solidified with 0·6 % w/v agar, and with no hormones. After 4 weeks growth, plants were transferred to the greenhouse as previously described. Portions of non-regenerating calli were transferred to MS-1 medium, solidified with 0·6 % w/v agar, for isozyme analysis. Parental callus was grown under the same conditions.

2.4 *Characterization of somatic hybrids*

Regenerating plants were characterized on the basis of their vegetative and floral morphology. Pollen viability was estimated by incubating freshly dehisced pollen in a few drops of 2 % w/v acetocarmine solution.

For biochemical characterization, isoelectric focusing of the subunits of Fraction 1 protein, (Cammerts and Jacobs 1980) was carried out with some modifications. Polyacrylamide electrophoresis of total leaf proteins was carried out using a vertical electrophoresis kit (Shandon) at 10 mA/gel for 12 hr. Isoelectric focusing of Fraction 1 protein was carried out on 1·4 mm thick horizontal gels (LKB Multiphor System, Sweden) using 1·5 % Ampholine, pH 5–8 (LKB). The maximum current was 10 mA and the final voltage was maintained at 1400 V for 6 hr. The gel was fixed in a solution containing 3·5 % w/v sulphosalicylic acid (Sigma) and 12 % w/v trichloroacetic acid (BDH). Protein was visualised by staining for 10 min at 60°C in a solution comprised of 0·15 % PAGE Blue 83 (BDH) in 25 %v/v ethanol and 8 %v/v acetic acid. Destaining was carried out in the above solution without PAGE Blue 83.

For isoelectric focusing of leaf esterases, and callus esterases, plant extracts were made as for Fraction 1 protein. Samples of 100 μg protein (in 30–40 μl), measured by the method of Lowry *et al* (1951) using lysozyme (Sigma) as standard, were applied to filter paper wicks 2 cm from the cathode on pre-cast thin gels (LKB PAG plate, pH 3·5–9, 5 %-acrylamide, catholyte 1 M NaOH, anolyte 1 M H_3PO_4). Gels were run on the multiphor horizontal gel kit (LKB) for 4 hr at 4°C (maximum current of 15 mM and at a final and constant voltage of 1400V). Esterase bands were visualised following a modified procedure of Smith *et al* (1970), using 0·1 M sodium phosphate buffer, pH 7.

3. Results

Frequencies of interspecific protoplast fusion, calculated as the percentage of heterokaryons existing among intact parental protoplasts, were normally within the 1–2 % heterokaryon formation range using the described fusion procedure. Post fusion division frequencies were between 25 and 30 % for *N. tabacum* mesophyll protoplasts and between 35 and 40 % for *N. rustica* suspension protoplasts.

3.1 *Observations on nuclear behaviour and cell division of isolated heterokaryons*

The majority of heterokaryons, isolated and fixed immediately after fusion, were binucleate although some multi-nucleate heterokaryons were observed.

A time course was carried out on the division of parental protoplasts and heterokaryons. Suspension culture-derived protoplasts were observed to commence division 24 hr after fusion, although the majority divided within 2–3 days. *N. tabacum* protoplasts were observed to begin division after 48 hr with the majority of first divisions occurring within 3–5 days. Division of heterokaryons was observed 2–4 days after fusion.

In one experiment 52 heterokaryons were isolated after fusion and cultured in 5–10 μl microdrops. After 24 hr in culture, nuclear staining revealed that 34 of these heterokaryons had undergone nuclear fusion. At the time of fixation, the heterokaryons still exhibited the morphological markers of cytoplasmic strands (from the suspension parent) and chloroplasts (from the mesophyll parent). These heterokaryons did not exhibit any signs of mitosis at this stage although cell wall formation was indicated by the oval shape of the heterokaryons.

In another experiment 62 heterokaryons were isolated after fusion and cultured in 5–10 μl microdrops. In this case the suspension protoplasts had been labelled with FITC prior to fusion. Within 4 days of culture, 28 of these heterokaryons had divided at least once. Combined FITC and chlorophyll fluorescence was observed under UV light. Such double fluorescence persisted beyond the first division stage of heterokaryon-derived cells.

3.2 *Continued culture of isolated heterokaryons*

A total of 30 heterokaryons were isolated from a mass culture 1 day after fusion. None of the heterokaryons had divided at this stage but all had changed to an oval shape and were intact, indicating cell viability. These heterokaryons were placed in an albino nurse culture of protoplasts of *P. hybrida*. After 6 weeks, a total of thirteen green colonies were observed among the albino cells.

3.3 *Regeneration and plant morphology*

Regeneration of shoots was attempted as described in §2. Of the 13 colonies which were recovered, 6 regenerated shoots, which subsequently rooted and were transferred to the greenhouse (lines 5, 6, 9, 11, 12 and 13). The height of regenerated plants at flowering, was not uniform in the population of plants but was between that of *N. tabacum* and *N. rustica*. Leaf dimensions were also variable (table 1). The stem colour of all these plants was green, like the *N. tabacum* parent and unlike the recessive cream yellow stem colour of this variety of *N. rustica* (Jinks *et al* 1981).

The flowers of the six different plants were intermediate between the two parental flowers with regard to corolla shape and dimensions (figure 1, table 1). Several flower colours were also observed (table 1). Pollen viability was non-uniform in the population and was over 20% for three of the plants (table 1).

Certain abnormalities were present in these hybrids which were not present in either parent. Some leaves of all the hybrid plants exhibited occasional light green or yellow sectors. Flowers of all the hybrids occasionally exhibited abnormalities such as a split corolla or a stamen fused with the corolla. The flowers had a tendency to drop prematurely. However, a small number of seed pods were recovered in some selfing experiments. Those of line 5 showed 64% germination and those of line 6, 58% germination.

Table 1. Vegetative and floral morphology of *N. rustica* × *N. tabacum* somatic hybrid plants.

	Flowering height (1) (to nearest 10 cm)	Leaf index (2) length/width	Largest leaf dimensions		Flower colour (3)	Flower corolla Length (cm) (4)	Pollen viability (%)
			length (cm)	Max. width (cm)			
N. tabacum var. Xanthi	130	1·8 ± 0·1	23	13	Neyron rose (55C)	4·1 ± 0·1	95
N. rustica var. V27	80	1·5 ± 0·1	25	17	Barium yellow (10C)	1·8 ± 0·1	100
N. rustica × *N. tabacum* somatic hybrids—							
H 5	110	2·1 ± 0·3	25	12	Neyron rose (56B)	2·9 ± 0·2	21
H 6	130	1·8 ± 0·2	29	16	Orient pink (36C)	2·8 ± 0·3	38
H 9	150	2·1 ± 0·2	27	13	Neyron rose (56A)	2·9 ± 0·1	17
H 11	160	1·8 ± 0·1	22	12	Orient pink (36A)	2·8 ± 0·2	43
H 12	140	1·9 ± 0·2	28	14	Orient pink (36 D)	2·9 ± 0·1	11
H 13	90	2·1 ± 0·3	22	11	Amber yellow (18B)	2·1 ± 0·2	4

(1) Plants grown to flower stage of compost (2) Six fully expanded leaves, from the primary stem of each plant, were measured. Leaves formed before the onset of flowering were assessed. (3) Assessed by comparison with Royal Horticultural Society, London, Colour Chart. Numbers in parenthesis represent actual colour bar reference. (4) Average of ten, normally formed flowers.

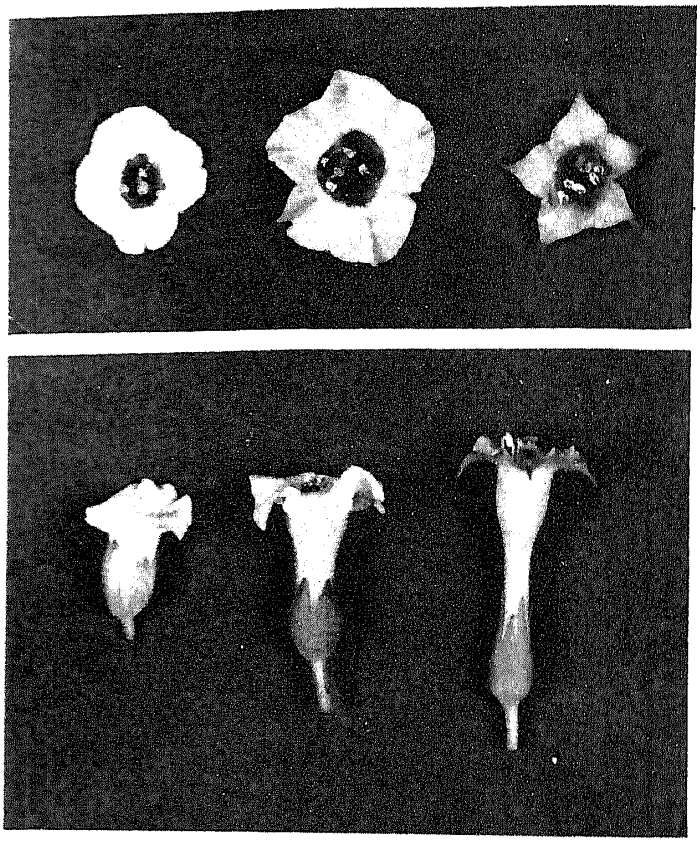

Figure 1. Floral morphology of *N. rustica* and *N. tabacum*, and somatic hybrid selected by heterokaryon isolation. Left to right.
N. rustica cv V27; somatic hybrid (line 5); *N. tabacum* cv Xanthi (× 0·65).

3.4 *Isoelectric focusing of the subunits of Fraction* 1 *protein*

The two species showed differences in polypeptide band profiles for both the large and small subunits of Fraction 1 protein (figure 2Ai, 2Aii). All the six hybrids had the small subunit polypeptides of both parents suggesting the presence of both nuclear genomes (figure 2Aii). Each hybrid exhibited the large subunit polypeptides of one or other parent suggesting that chloroplast segregation to have occurred. Five of the hybrid plants possessed the chloroplast Fraction 1 protein profile of *N. rustica* and one line (line 6) possessed the chloroplast Fraction 1 profile of *N. tabacum* (figure 2Ai).

3.5 *Isoelectric focusing of leaf esterases*

Both *N. tabacum* and *N. rustica* possessed leaf esterases unique to each other with respect to their isoelectric points. All six of the hybrid plants exhibited a largely additive effect of these bands (figure 2B).

3.6 *Analysis of non regenerating cell lines*

Isoelectric focusing of callus esterases (not shown) revealed only one unambiguous band present in *N. rustica* callus and not in *N. tabacum* callus. All of the seven non-

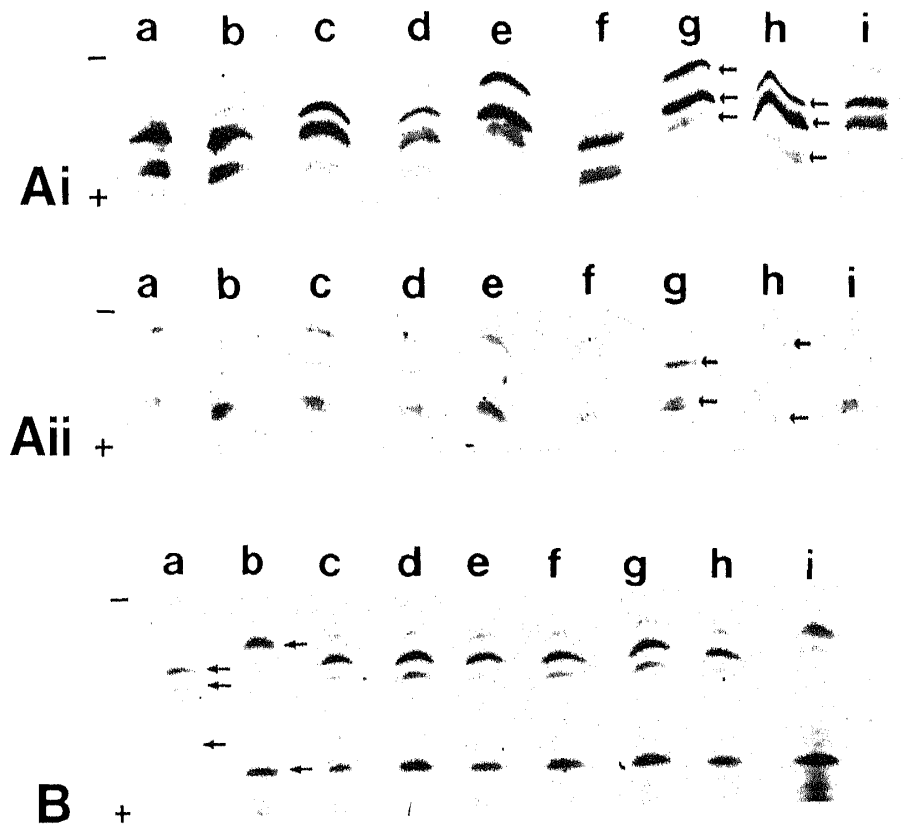

Figure 2. Biochemical analysis of *N. rustica* × *N. tabacum* somatic hybrids. (A) Fraction 1 protein analysis (**Ai**) Large subunit (chloroplast encoded) polypeptides. (**Aii**) Small subunit (nucleus encoded) polypeptides. (**a**) Hybrid Line 13. (**b**) Hybrid Line 12. (**c**) Hybrid Line 11. (**d**) Hybrid Line 9. (**e**) Hybrid Line 6. (**f**) Hybrid Line 5. (**g**) *N. tabacum* cv Xanthi. (**h**) *N. rustica* cv V27. (**i**) Physical mixture of *N. tabacum* and *N. rustica*. (Arrows indicate relative positions of parental bands. Uppermost bands in channels c and d of the large subunit (Ai) proved to be non reproducible and were considered artifacts). (**B**) Isoelectric focusing zymogram of leaf esterases. (**a**) *N. tabacum* cv Xanthi. (**b**) *N. rustica* cv V27. (**c**) Hybrid Line 5. (**d**) Hybrid Line 6. (**e**) Hybrid Line 9. (**f**) Hybrid Line 11. (**g**) Hybrid Line 12. (**h**) Hybrid Line 13. (**i**) Physical mix of *N. tabacum* and *N. rustica*. (Arrows indicate parental bands).

regenerating cell lines possessed this band. However, all of these lines grew on MS-1 medium as green callus, like *N. tabacum* and unlike the creamy-yellow colour of *N. rustica* under these conditions. This latter feature of *N. rustica* is attributed to the genetically recessive 'yellow' character which this plant exhibits (Hamill 1983). Thus there is some evidence that all of these seven non-regenerating cell lines possessed the nuclear genomes of both parents.

4. Discussion

4.1 *Isolation of heterokaryons*

We have demonstrated that, by using a micromanipulator and capillary pipette, coupled to a specially constructed syringe, it is possible to isolate viable heterokaryons, which in *N. rustica* and *N. tabacum* have given rise to somatic hybrid plants at a frequency of six out of the thirty heterokaryons cultured. This was facilitated by placing

the heterokaryons in a nurse culture of albino *Petunia hybrida* protoplasts demonstrating that protoplasts of a species of a different genus can act as a nurse culture for heterokaryons.

In previous studies viable heterokaryons have been successfully isolated by a variety of methods. Kao (1977) used a dilution plating technique to isolate heterokaryons of soybean and *N. glauca* in wells of Cuprak dishes. In this case cells derived from fusions were successively diluted until one heterokaryon occupied a well which was then registered. The high initial fusion frequency (39 % heterokaryon formation) and the use of a complex medium capable of supporting division of single cells were important factors in the isolation of hybrid colonies. Gleba and Hoffmann (1978) used a hand-held micropipette to isolate heterokaryons of *Arabidopsis thaliana* and *Brassica campestris* fusions, but in this case only fusion products divided and thus isolation of dividing heterokaryon-derived cells was greatly facilitated. The isolation of heterokaryons of *N. tabacum* mesophyll and *N. rustica* suspension protoplasts carried out in this study is more comparable to the isolation of *Atropa belladona* and *N. chinensis* heterokaryons (Gleba *et al* 1982). In this case, one type of parental protoplast was derived from mesophyll cells and the other from suspension cells; however, no mention was made of the fusion frequencies. In our experiments working with fusion frequencies around 1–2 %, we found that isolation with a fixed micropipette is much more convenient than isolation with a hand-held micropipette.

Hein *et al* (1983) have produced hybrid calli by isolation of heterokaryons of *N. paniculata* and *N. tabacum cnx* and *N. sylvestris* and *N. tabacum cnx* using a fixed micropipette. In this case fusion frequency was around 3 %, comparable to frequencies observed between *N. rustica* and *N. tabacum*. Isolated heterokaryons were cultured in a nurse culture of protoplasts of *N. tabacum cnx* parent which is a mutant unable to utilize nitrate as the sole source of nitrogen. Selection of the hybrid calli was accomplished by plating the entire culture on medium with nitrate as the sole source of nitrogen in which case the *N. tabacum cnx* colonies failed to grow but the hybrid calli proliferated. As the auxotrophic line of *N. tabacum cnx* was used both as a fusion parent and a nurse culture, and a selection medium was employed, the isolation of heterokaryons did not need to be stringent so as to exclude any parental *N. tabacum* protoplasts. In a similar study, Menczel *et al* (1978) used an albino, kanamycin resistant cell line of *N. sylvestris* both as a fusion parent and as a nurse culture for fusions with *N. knightiana* leaf mesophyll-derived protoplasts.

4.2 *Initial response of heterokaryons*

By isolating heterokaryons and culturing them in small droplets, we were able to follow the behaviour of nuclei at different time intervals. It seems that nuclear fusion occurs rapidly, within the first day of post-fusion culture, and before obvious signs of mitosis and cell division. These observations were not consistent with reports on nuclear fusion in animal cell heterokaryons where nuclear fusion has been reported to occur at mitosis (Harris 1974). However, nuclear fusion during interphase has been observed in plant heterokaryons by other workers. For example, in protoplasts fusion of *Pisum sativum* and *Glycine max* (Constabel *et al* 1975), and of *Daucus carota* and *Hordeum vulgare* (Dudits *et al* 1976) nuclei were observed to fuse within 24 hr of post fusion culture. Electron microscopic observation of soybean homokaryons revealed connections between interphase nuclei which may have preceded and facilitated nuclear fusion

(Fowke *et al* 1975). The situation appears analogous to a report on nuclear fusion following sexual fertilization in *Gossypium hirsutum*, where nuclear fusion occurred *via* nuclear endoplasmic reticulum coalescence and membrane bridge formation (Jensen 1964).

4.3 *Relevance of fluorescein isothiocyanate staining*

As has been shown previously (Patnaik *et al* 1982), combined FITC and chlorophyll fluorescence within the heterokaryon can be useful for confirming the heterokaryocyte nature of fusion products isolated using bright field optics. This double fluorescence may be useful for the manual isolation of heterokaryons in cases where the presence of cytoplasmic standards and chloroplasts are insufficient markers for unambiguous identification of heterokaryons. Moreover, the observation that this double fluorescence persists beyond the first division of heterokaryons may be valuable for recovering viable and dividing hybrid cells. In future, combined FITC and chlorophyll fluorescence could be used for automated cell sorting using fluorescence-activated cell sorters (Redenbaugh *et al* 1982).

4.4 *Characterization of somatic hybrids*

The six somatic hybrids of *N. tabacum* and *N. rustica* produced in this study showed variable morphology and vigour as previously reported for somatic hybrids of these species (Douglas *et al* 1981a). Some seed set was observed in four of the hybrids, and the progeny are currently being analysed for hybrid characteristics.

4.5 *Chloroplasts constitution of the hybrids*

The isoelectric focusing patterns of Fraction 1 protein given evidence for the presence of one of other parental chloroplast type being present in the hybrid plants. Five hybrids appeared to possess the plastids of *N. rustica* and one hybrid possessed the plastids of *N. tabacum*. Chloroplast sorting out is a generally observed phenomenon in somatic hybrids (Chen *et al* 1977; Scowcroft and Larkin 1981). In one previous report on somatic hybridization between *N. rustica* and *N. tabacum*, the chloroplast segregation pattern was found to be random (Iwai *et al* 1980). In this case, both the parent protoplasts used for fusion were derived from mesophyll tissue. In another report on the somatic hybridization between these two species, there appeared to be a bias for *N. rustica* chloroplasts (Douglas *et al* 1981b). In this case protoplasts of both parents were derived from cell suspensions. In our study, where *N. rustica* parental protoplasts were derived from suspension cells and *N. tabacum* parental protoplasts from mesophyll tissue, there is an apparent bias for *N. rustica* chloroplasts in the hybrids. This may suggest a competetive advantage for *N. rustica* plastids, in such hybrids, when the *N. rustica* parental cells have been grown heterotrophically. However, the existence of a hybrid plant, in this study, which possesses the chloroplasts of *N. tabacum* shows that fully differentiated plastids can be retained and the observed segregation pattern may simply be due to a random assortment as has been demonstrated for other somatic hybrids, both intra and interspecific (Chen *et al* 1977; Scowcroft and Larkin 1981).

4.6 *General applicability of manual isolation of heterokaryons for somatic hybridization studies*

A large number of dicotyledon species are amenable to protoplast isolation, culture and plant regeneration (Vasil and Vasil 1980; Lu *et al* 1982; Davey and Kumar 1983), and a number of cases have been suggested where somatic hybrids could be of practical utility (Evans *et al* 1981; Evans 1983; Ahuja *et al* 1983). Since the number of mutants available for complementation selection is low (Cocking *et al* 1981) there is clearly a need to develop methods for the isolation of heterokaryons to produce somatic hybrids.

The production of somatic hybrids, between species which are difficult or impossible to cross sexually, could be approached in two steps as was carried out in this study. The first step involves the observation of nuclear fusion and hybrid cell division using a sample of isolated heterokaryons. The use of double fluorescence, as was used in this study, may be helpful in the identification of dividing hybrid cells. Such observations may provide valuable information on the extent of nuclear fusion and reveal any incompatibility that may exist at the initial stages of culture. In the second step, the remaining heterokaryons would be cultured, either in microdrops or in a nurse culture, to assess their growth and development. Such a two step approach has been shown, in this study, to be valuable for recovering somatic hybrids of *N. tabacum* and *N. rustica*, and should be applicable to hybridization of other species which have no markers for complementation selection of hybrids in culture.

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