Theor Appl Genet (1994) 88: 1017–1022

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Cybridization in *Nicotiana tabacum* L. using double inactivation of parental protoplasts and post-fusion selection based on nuclear-encoded and chloroplast-encoded marker genes

Received: 21 April 1993 / Accepted: 21 December 1993

Abstract An effective selection system preceded by double inactivation of parental protoplasts was used to transfer *Nicotiana suaveolens* Leh. cytoplasmic male sterility into a commercial tobacco (*N. tabacum* L.) breeding line. Mesophyll protoplasts from transformed plants of *N. tabacum* cultivar WZ2-3-1-1 possessing a neomycin phosphotransferase II gene were used as the nuclear donors, while those isolated from *N. suaveolens* plants carrying a chloroplast mutation for resistance to spectinomycin, induced using nitrosomethyl urea, were the cytoplasm donors in somatic cybridizations. Prior to fusion, nuclear donor protoplasts were inactivated with iodoacetamide or rhodamine 6G, while those of the cytoplasm donor were inactivated by X-irradiation. The resultant microcalli were cultured on a shoot regeneration medium containing both kanamycin and spectinomycin to select cybrids. Only regenerants that had typical characteristics of the *N. tabacum* cultivar were selected for transfer to the glasshouse. Four putative cytoplasmic male-sterile (CMS) plants, out of a total of 44 regenerated plants transferred to the glasshouse, were obtained. Intraspecific somatic transfers of the CMS trait between *N. tabacum* cultivars with distinctly-different morphologies using single inactivation and non-selective shoot regeneration medium were demonstrated. The implications of the results for practical tobacco breeding as a means of circumventing lengthy backcrossing procedures are discussed.

Key words *Agrobacterium* · Iodoacetamide *Nicotiana* protoplast fusion · Nitrosomethyl urea Rhodamine 6G · X-irradiation

Communicated by T. W. Snape

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Introduction

Protoplast fusion is now an established plant hybridization method, particularly in the genus *Nicotiana* (see e.g., Morikawa and Yamada 1992). However, the lack of widely-applicable and efficient selection systems in plants, due to a paucity of genetically and biochemically-characterized mutants, has limited the production of somatic hybrids and cybrids. Different approaches have been developed and tested to overcome this deficiency (see Pelletier and Chupeau 1984; Kumar and Cocking 1987; Sink 1991; Morikawa and Yamada 1992) but few have shown universal applicability. Genetic transformation has enabled the introduction of dominant, nuclear-encoded, selectable marker genes such as neomycin phosphotransferase II (*NPTII*) into *Nicotiana* plants (Goldman and Northrop 1976). This has led to novel, dual-antibiotic selection systems which have been described for the recovery of somatic nuclear hybrids in *Nicotiana* (e.g., Komari et al 1989; Sproule et al. 1991), *Solanum* (e.g., Masson et al. 1989), and *Medicago* (e.g., Thomas et al. 1990) species. However, the development of systems for the selection of somatic cybrids have lagged behind those for nuclear hybrids because of the absence of suitable mitochondrial and/or chloroplast marker genes that can be selected in vitro. Although in-vitro selectable plastome-encoded marker genes have been available in certain *Nicotiana* mutants, such as streptomycin resistance (Maliga et al. 1982) and chlorophyll deficiency (Menczel et al. 1987), their use is restrictive because specific cultivars are required in agriculture. Reports of the use of a plastome-targeted mutagen, nitrosomethyl urea (NMU), for obtaining antibiotic-resistant mutants of solanaceous plants (McCabe et al. 1989; Dix et al. 1990) have indicated that it is possible to develop selectable chloroplast-encoded marker genes with relative ease. Also, the "double-inactivation" protoplast fusion technique (Sidorov et al. 1981) is now a proven procedure for establishing cybrid plants and effecting organelle transfer (Pelletier and Chupeau 1984; Kumar and Cocking 1987; Morikawa and Yamada 1992). The double-inactivation method exploits the

"donor-recipient" technique (Zelcer et al. 1978), in which the nuclei of one fusion partner are inactivated by ionizing irradiation, whilst treatment of the other fusion partner with a toxin, such as iodoacetate, inactivates the cytoplasm.

In this paper we describe *Nicotiana* somatic hybridizations in which a double-inactivation procedure was used in combination with a stringent selection system based on a nuclear-selectable marker gene inserted into the nucleus donor by *Agrobacterium*-mediated transformation with a NMU-induced chloroplast-encoded selectable marker gene in the cytoplasm donor parent. The practical goal of our work was to transfer *N. suaveolens* cytoplasmic male sterility (CMS) into a commercial *N. tabacum* breeding line in one manipulation.

Materials and methods

Plant materials

Seeds of *N. tabacum* [cvs WZ2-3-1-1 (WZ), CMS Burley 21 (CMS Bu21)] and *N. suaveolens* were obtained from the Plant Breeding Department, Tobacco Research Board, Harare, Zimbabwe. *N. tabacum* cv CMS Bu21 is an alloplasmic tobacco line possessing *N. suaveolens* cytoplasm which confers male sterility. The reason for including the CMS Bu21 material as one of the protoplast sources in the planned series of somatic fusions was that intraspecific WZ + CMS Bu21 products were more likely than interspecific fusions to result in useable plants that would be recovered more easily and more rapidly. This was because cultural conditions necessary for cell and plant regeneration would probably be similar to those used for WZ, the main breeding line of interest into which CMS was to be introduced. It was also more likely that the electrofusion parameters necessary for fusing intraspecific combinations would be easier to devise than those required for fusing interspecific combinations. Seeds of these cultivars and species were surface-sterilized with 70% ethanol for 5 s followed by a 20 min immersion in a 10% (v/v) 'Brobat' bleach (Jeyes, UK) solution containing 0.01% (v/v) 'Tween 20' (BDH, UK), followed by three rinses of 5 min each in sterile water. Seeds of each cultivar or species were germinated on 20 ml of plant-growth-regulator-free Murashige and Skoog salts medium (MS)containing Murashige and Skoog (1962) salts and vitamins, 3% (w/v) sucrose and 0.8% 'Technical Grade 3' agar (Oxoid, UK) dispensed into 100 ml jars. The seeds were germinated at 25°C in growth rooms, maintained under 16-h photoperiods, supplied by Philips TLD 50 W/84HF fluorescent tubes producing mean irradiances, at plant level, of 55–62 $\mu E\ m^{-2}\ s^{-1}$. Axenic plants were maintained by nodal or shoot-tip subcultures once every 6 weeks onto fresh 80 ml of agar-solidified plain MS medium contained in 300-ml honey jars.

Transformation of nucleus donor

The procedures followed during the preparation of the bacterial strains and plant transformation were based on standard protocols (Maniatis et al. 1982). A transformation plasmid was mobilized from *Escherichia coli* strain MC1022 into *A. tumefaciens* strain LBA4404 (Hoekema et al. 1983), a disarmed octopine-type *Agrobacterium* strain that has chromosomal streptomycin- and rifampicin-resistance mutations. This mobilization was achieved by standard tri-parental mating (Bevan 1984).

N. tabacum cv WZ shoots were transformed using a standard leaf-disc procedure (Horsch et al. 1985). Seeds from nine kanamycin-resistant plantlets were harvested and their transformation indicated normal growth of seedlings on an antibiotic selection medium consisting of plain MS medium supplemented with 100 mg l^{-1} of geneticin. Seeds of previously-transformed *N. tabacum* cv Xanthi, obtained from Dr.Vicky Buchanan-Wollaston (Plant Molecular Biology Laboratory, Department of Biochemistry and Biological Sciences, Wye College) and germinated on the same antibiotic medium, also grew normally (green) while those of the non-transformed *N. tabacum* cv AWR became white. The segregation ratios of green and white seedlings determined by scoring *N. tabacum* cv WZ seedlings raised from seeds collected from one of the six regenerated plants were approximately 3:1 (Matibiri 1993). Normal green seedlings were rescued from the selective antibiotic medium and maintained in 80 ml of plain MS medium in honey jars as sources of protoplasts.

Induction of chloroplast-encoded antibiotic resistance

This was achieved using the leaf-disc procedure of McCabe et al.(1989) in which mutations were induced using 5-mM NMU solutions and antibiotic resistant adventitious shoots recovered by selecting cultures against 100 mg l^{-1} of spectinomycin.

Four green spectinomycin plants were recovered, rooted, and transferred to the glasshouse. Reciprocal crosses were made between these and wild-type *N. suaveolens* plants. When seeds from the reciprocal crosses were mature they were dried in darkness at 25 °C, surface-sterilized and aseptically germinated on 10 ml of agar-solidified basal MS medium supplemented with 50 mg l^{-1} of spectinomycin. Seedlings were scored for bleaching after 2 weeks. All seedlings from crosses in which the regenerated plants had been used as maternal parents grew into normal, green plants. The other seedlings and those of wild-type *N. suaveolens* became white or consisted of variegated leaves. Spectinomycin-tolerant shoots were rescued and subcultured individually into glass jars containing 20 ml of plain non-selective agar-solidified MS medium.

Protoplast isolation

Leaf mesophyll protoplasts were isolated from fully expanded leaves of in-vitro grown *N. suaveolens* (spectinomycin-resistant), *N. tabacum* cvs WZ (transformed) and CMS Bu21, as previously described (Matibiri 1993). Leaves were cut into strips of less than 2 mm width and incubated in a PIM solution [(0.1% (w/v) Cellulase Onozuka R-10, 0.05% (w/v) Macerozyme R-10, MS salts and vitamins and 10% (w/v) mannitol at pH 5.8]. They were incubated overnight (12–14 h) at 25 °C in the dark on a slow oscillatory table (Gallenkamp, UK). Protoplasts were filtered through 100-m steel mesh sieves, collected by centrifugation at 42 *g* for 10 min and resuspended in 1 ml of 10% (w/v) mannitol and purified by floatation and centrifugation at 42 *g* for 10 min over 20% (w/v) sucrose. They were then resuspended in 10% mannitol and centrifuged down at 42 *g* for 10 min and resuspended again in 10% (w/v) mannitol. This washing and centrifugation process was repeated twice. Protoplast viabilities were determined using FDA (Widholm 1972) and the presence of primary cell walls on the protoplasts was detected using Calcofluor White M2R (Davey et al. 1974). The protoplast densities in the suspensions were adjusted to 1.5×10^5 ml^{-1}.

Inactivation of protoplasts

Inactivation was carried out using a double inactivation strategy similar to the ones described by Zelcer et al. (1978) and Ichikawa et al. (1987) in which a combination of irradiation and iodoacetamide treatments were employed to inactivate the nuclear and cytoplasmic components, respectively, of the different *Nicotiana* fusion partners.

Mesophyll protoplasts of the nucleus donor (transformed *N. tabacum* cv WZ) were incubated in a 3 g l^{-1} of iodoacetamide or 35 mg l^{-1} of rhodamine 6G solution in 10% (w/v) mannitol at a density of 1.5×10^5 ml^{-1} for 5 min. After treatment, the protoplasts were washed twice by centrifugation and resuspension in 10% (w/v) mannitol. The protoplast suspension density was adjusted to 1.5×10^5 ml^{-1}.

Nuclei of the cytoplasm donor mesophyll protoplasts of *N. suaveolens* (spectinomycin-resistant) and *N. tabacum* cv CMS Bu21 were inactivated by a 200 Gy X-irradiation dose, applied at a rate of 2 Gy min^{-1}, using a Torex 150 X-ray inspection system (Torr X-ray Corporation, California) operating at 5 mA and 150 kV. After irradiation, the protoplast suspension densities were again adjusted to 1.5×10^5 ml^{-1}.

Protoplast fusion and culture

All protoplast pre-fusion treatments were performed as described above. *N. suaveolens* and *N. tabacum* cv CMS Bu21 protoplast pre-fusion treatments were: (1) control (coded '*N. suav*' and 'CMS Bu21', respectively), and (2) a 200 Gy X-irradiation dose (coded 'X-*N. suav*' and 'X-CMS Bu21', respectively). *N. tabacum* cv WZ protoplast pre-fusion treatments were: (1) control (coded 'WZ'), (2) 3 g l^{-1} of iodoacetamide for 5 min (coded 'iodo-WZ'), and (3) 35 mg l^{-1} of rhodamine 6G for 5 min (coded 'rhod-WZ').

Equal volumes of parental protoplasts for each fusion combination were mixed in a centrifuge tube. The protoplast fusion combinations and the number of times they were carried out are listed in Table 1. Protoplast fusions were carried out using the electrofusion equipment and fusion parameters described elsewhere (Matibiri 1993). Fusion chambers made in 5-cm-diameter plastic Petri dishes with 2-mm-diameter brass electrodes spaced 3 mm apart were used. Dielectrophoresis was induced by application of an AC field of 50 V cm^{-1} and an 0.5 MHz oscillation frequency. After 60 s, to allow for alignment and collection of protoplasts into "pearl" chains, fusion was induced by the application of two 0.7-kV cm^{-1} DC pulses, each of 50 µs duration. The AC field strength was gradually reduced and switched off about 30 s after application of the DC pulses. Fusion products were collected from the inter-electrode spaces approximately 2 min after application of the DC pulses. Single drops of the fusion products were placed in each of the central nine wells of a 25-well plastic Petri dish (Sterilin, UK). Three drops of PCM (Matibiri 1993) were added to each drop of fusion products and unused wells of the Petri dish were each filled with 1.5 ml of sterile water. Each Petri dish was sealed with Nescofilm and incubated at 25C in darkness. Nine drop cultures were obtained per electrofusion session for each protoplast fusion combination. Appropriate non-fused control protoplasts (both inactivated and non-inactivated parental protoplasts and their appropriate mixtures) were also cultured whenever a batch of fusion combinations were electrofused and incubated.

After 4 days culture, three drops of PCM were added to each protoplast-suspension drop culture. When protoplast-derived cells had formed microcalluses just visible to the naked eye, 1 ml of ACM [MS salts and vitamins, 3 mg l^{-1} NAA, 0.1 mg l^{-1} BAP, 3% (w/v) sucrose, 8% (w/v) mannitol and pH 5.8] was added to each culture well. The calluses were kept in darkness at 25C until most of them were larger than 1.5 mm^3 in volume.

Selection of cybrids

Calluses from each culture well were then transferred onto agar-solidified MSR medium containing 75 mg l^{-1} of kanamycin and 75 mg l^{-1} of spectinomycin for shoot induction. Green shoots that regenerated were transferred to rooting medium (growth regulator-free solid MS medium) containing 50 mg l^{-1} of kanamycin and 50 mg l^{-1} of spectinomycin. Well-rooted plantlets were removed from their culture vessels and transferred to glasshouses.

Table 1 Numbers of culture wells of control protoplasts and products of fusion combinations showing cell division after 10 d culture (A); numbers of culture wells showing callus growth after 21 days culture (B) and total numbers of shoots > 3 mm length regenerated from calluses after 21 days culture on MSR media (C); numbers of regenerated shoots discarded at time of transfer to glasshouse (D); numbers of plants surviving to flowering in glasshouse (E) and numbers of male-sterile plants obtained (F)

(1) Controls	A	B	C	D	E	F
*N. suav	18	18	67	N/A	20[d]	0
*X-*N. suav*	2	0	0	0	0	0
*WZ	18	17	86	N/A	20[d]	0
*Iodo-WZ	0	0	0	0	0	0
*Rhod-WZ	0	0	0	0	0	0
*CMS Bu21	18	10[a]	79	N/A	20[d]	20
*X-CMS Bu21	6	3[a]	12	N/A	10[d]	10
(2) Fusion combinations						
**N. suav* (+) WZ	9	5	19	7	10	0
**N. suav* (+) iodo-WZ	3	3	23	8	13	1[e]
**N. suav* (+) rhod-WZ	11	0	0	0	0	0
**X-*N. suav* (+) WZ	7	2	12	3	9	1[f]
**X-*N. suav* (+) iodo-WZ	4	3	20	3	12	2[g]
**X-*N. suav* (+) rhod-WZ	11	0	0	0	0	0
**CMS Bu21 (+) WZ	18	15	63	23[b]	33[b]	0
**CMS Bu21 (+) iodo-WZ	7	1[a]	10	3[b]	6[c]	1[h]
**CMS Bu21 (+) rhod-WZ	9	2[a]	0	0	0	0
**X-CMS Bu21 (+) WZ	10	4[a]	12	6[b]	6[b]	0
**X-CMS Bu21 (+) iodo-WZ	7	3	27	14[b]	11[b]	0
**X-CMS Bu21 (+) rhod-WZ	15	10	98	41[b]	50[b]	0

[a] Presence of fungal contamination
[b] Burley-type plants
[c] Five of the six plants were Burley types
[d] Randomly selected from regnerated plants
[e] Coded CMS YMZ4
[f] Coded CMS YWZ3
[g] Coded CMS YWZ1
[h] Coded CMS YWZ5
N/A, not applicable
* Total number of culture wells was 18 and shoot induction was carried out on antibiotic-free media;
** Total number of culture wells was 36 and shoot induction was carried out on media with spectinomycin and kanamycin;

Results

Using the electrical parameters of electrofusion mentioned above together with widely-spaced brass rod electrodes ensured that large numbers of fusion products with few multicell fusions were obtained. Heterofusion events between mesophyll protoplasts of *N. tabacum* cv WZ and of *N. suaveolens*, when they were assessed, occurred with a frequency ranging from 1 to 15%, although values varied between batches of protoplast mixtures. Heterofusion frequencies between *N. tabacum* cvs WZ and CMS Bu21 mesophyll protoplasts could not be assessed because of the similar morphologies of their protoplasts. The plating efficiencies obtained with control protoplasts after 10 days of culture were usually 15–30%.

The combined results are summarized in Table 1. Neither *N. tabacum* cv WZ protoplasts, treated with either iodoacetamide or rhodamine 6G, divided; nor did X-irradiated *N. suaveolens* give rise to callus colonies. The latter contrasted with X-irradiated *N. tabacum* cv CMS Bu21 protoplasts, from which 12 plants were regenerated (Table 1). In addition, relatively-high numbers of plants with typical CMS Bu21 phenotypes were obtained from calluses produced by fusion products in which *N. tabacum* cv CMS Bu21 was one of the parents. In all cases where rhodamine 6G-treated *N. tabacum* cv WZ protoplasts were employed in fusions with other protoplasts, no regenerated plants with *N. tabacum* cv WZ phenotypes were obtained. Cultures of kanamycin-resistant *N. tabacum* cv WZ protoplasts, spectinomycin-resistant *N. suaveolens* protoplasts, and mixtures of the two, did not yield any shoots under the double-antibiotic resistance selection procedures used.

Cultures derived from fusion products in which *N. tabacum* cv CMS Bu21 was one of the parents did not regenerate shoots on the selective shoot-induction medium. Shoot formation on the selective medium was observed only on callus produced from fusions between *N. tabacum* cv WZ protoplasts possessing the nuclear-encoded *NPTII* gene and *N. suaveolens* protoplasts carrying the cytoplasmically-encoded spectinomycin resistance. In all cases, the shoots arose from green-callus colonies or islands of green callus surrounded by white calluses. Large numbers of shoots were regenerated from calluses produced by CMS Bu21 (+) WZ fusion combinations because, for all CMS Bu21 fusion combinations, shoot induction was not carried out on selective media.

In order to favour recovery of the desired cybrid plant phenotype, any regenerated plants that did not have the characteristics of *N. tabacum* cv WZ were discarded at the time of transfer to the glasshouse. Since *N. tabacum* cv WZ is a flue-cured (Virginia) tobacco cultivar it was easy to distinguish regenerants that had air-cured (Burley) features, as exhibited by *N. tabacum* cv CMS Bu21. Burley cultivars have white stems and white leaf veins set at relatively wide angles to the midribs. When fully-expanded, Burley cultivar leaves are yellowish green in colour. Flue-cured cultivars, on the other hand, have darker green stems and leaves, the veins of which are at more acute angles to the midribs. Most of the regenerated *N. suaveolens* plants, and some of the plants which appeared to be nuclear hybrids (with thick lanceolate leaves and very prominent midribs) amongst those plants regenerated from *N. suaveolens* (+) *N. tabacum* cv WZ protoplast fusion-derived calluses, were discarded at the time of transfer of other regenerates to the glasshouse. All those discarded from CMS Bu21 (+) WZ fusion-derived shoots had typical Burley characteristics.

No visual morphological differences between plants regenerated from in vitro (control) cultures and those planted from seeds of *N. tabacum* cvs WZ and CMS Bu21, or of *N. suaveolens*, could be identified. From all the regenerants transferred to the glasshouse only one sectorial chimera (which remained stable through to flowering) was observed. It was derived from a X-*N. suav* (+) WZ protoplast fusion combination.

After 21 weeks of growth in the glasshouse, almost all plants were in full flower and final assessments of flower morphology were carried out. Five totally male-sterile plants were identified. Except in the case of the CMS Bu21 (+) iodo-WZ fusion combination, all other male-sterile plants were obtained where double inactivation of parental protoplasts and the stringent selection system had been employed. Male-sterile plants were identified after detailed examination of their flowers and comparing their structures to those of fertile *N. tabacum* cv WZ and conventionally-bred male-sterile *N. tabacum* cv CMS WZ (possessing *N. suaveolens* cytoplasm), planted in the glasshouse as controls. The CMS *N. tabacum* cultivar and the putative CMS regenerants containing *N. suaveolens* cytoplasm, showed feminization of anthers similar to that described previously (Schweppenhauser and Mann 1968). in that the anthers appeared as green stigmatoids and usually one of them had a style that was much longer than those of the others in the flower and could be seen hanging out from the flower. Within batches of plants regenerated from each fusion combination, the recovered male-sterile plants flowered 3–10 days after the other fertile plants. All the recovered male-sterile plants, except for CMS YWZ4 [obtained from X-*N. suav* (+) WZ] had gross morphologies identical to those of the control *N. tabacum* cvs WZ and CMS WZ plants. In comparison to control plants CMS YWZ4 was squat with thick, rather flat (but not smooth), dark green leaves with prominent midribs and a tightly-packed flower head. All male-sterile plants were pollinated with *N. tabacum* cv WZ pollen for seed collection.

Discussion

Stringent selection systems have been used in the past to favour recovery of particular nuclear recombinations. For example, *Agrobacterium*-transformed parental *N. tabacum* lines were used to select for dual antibiotic-resistant nuclear hybrids by Komari et al. (1989). A similar approach was employed to obtain *N. tabacum* (+) *N.debneyi* nuclear hybrids (Sproule et al. 1991) and Malone et al. (1992) fused an *N. tabacum* line with albino and streptomycin-resistant chloroplasts to a wild-type *N. plumbaginifolia* line. These latter workers also managed to induce a leaky chloroplast recombination after culturing the fusion products on streptomycin-containing media in darkness. The current work has shown, for the first time to our knowledge, that unique nuclear-cytoplasmic combinations can be obtained if suitable selectable markers are available in the desired cytoplasmic and nuclear genomes. The combination of positive selection genes used, provided a convenient combination of markers for efficient selection of cybrids. The results also showed that cybridization can be obtained by immobilizing the undesired components of only one of the parents. Further, it was also proven that cybridization occurs, though at a low frequency, even when there is no selection towards it. In addition, the choice of parental species and cultivars permitted visual selection of regenerated plants with the desired phenotype at an early stage and before they had been transferred to the glasshouse.

The approximately 3:1 Mendelian segregation ratios of green to white seedlings, obtained when seeds of transformed *N. tabacum* cv WZ were germinated on a medium containing geneticin, suggested that a single copy of the *NPTII* gene had been successfully inserted by the *A.tumefaciens* transformation vector into the *N. tabacum* cv WZ nuclear genome. The *NPTII* gene is the selection marker most frequently used in plant transformation studies and has been shown to be a powerful marker in protoplast fusion (Brunold et al. 1987). This gene system, together with the chloroplast-encoded spectinomycin gene produced by standard chemical mutagenesis approaches, facilitated the development of the efficient dual selection system employed in the current work for selecting asymmetric fusion

products, as has been done for nuclear somatic hybrids by others (Komari et al. 1989; Sproule et al. 1991).

Under the conditions used to immobilize the cytoplasm of *N. tabacum* cv WZ, Rhodamine 6G was probably cytotoxic. No plants were obtained from control rhodamine 6G-treated *N. tabacum* cv WZ protoplasts or from fusion combinations in which these treated protoplasts were used (Table 1). Control iodoacetamide-treated *N. tabacum* cv WZ protoplasts did not regenerate callus but, in fusion combinations with *N. suaveolens* and *N. tabacum* cv CMS Bu21 protoplasts, plants were regenerated from the derived calli. Further, iodo-WZ protoplasts when fused with *N. suav* and with X-*N. suav* yielded one and two male-sterile plants, respectively. Thus, iodoacetamide appeared to be an effective treatment for blocking the cytoplasm of the nucleus donor without impairing the incumbent nucleus. Because putative CMS plants were obtained with iodoacetamide-treated *N. tabacum* cv WZ protoplasts fused with non-irradiated *N. suaveolens* and non-irradiated *N. tabacum* cv CMS Bu21 protoplasts, it was apparent that treating the nucleus donor of a fusion combination with iodoacetamide is all that may be required to obtain male-sterile plants with the correct combination of cytoplasm and nucleus. This has been reported by others (Terada et al. 1987; Wright et al. 1987). A single male-sterile plant was obtained from each of the *N. suav* (+) iodo-WZ and from X-*N. suav* (+) WZ combinations. CMS YWZ4, obtained from the fusion combination *N. suav* (+) iodo-WZ was different from the other male-sterile plants. This may be because it contained some nuclear genetic material from *N. suaveolens*; alternatively its different morphology might have been due to a blue mould (*Peronospora tabacina* Adam) infection during early stages of growth. However, the latter seems unlikely since control plants affected by this disease did not have any such characteristics. It is well known that irradiation results in fragmentation of nuclear DNA but does not impede incorporation of fragments into the nucleus of the somatic hybrid (Dudits et al. 1987; Gleba et al. 1988; Famelaer et al. 1989). However, the nuclear genome of CMS YWZ4 appears to be that of a normal *N. tabacum* cultivar according to peroxidase isozyme banding patterns (Matibiri 1993). The DNA profiles of the mitochondrial and chloroplast genomes of the putative CMS plants have yet to be studied in subsequent generations.

It was interesting that a male-sterile plant was regenerated from callus derived from fusions between *N. tabacum* cvs CMS Bu21 and iodo-WZ protoplasts. This was obtained despite the fact that the CMS Bu21 protoplasts had not had any treatment to block their nuclei and the male-sterile plant was not induced on media containing selective agents. This suggested that cybridization can be obtained even when it is not the favoured process during somatic hybridization. It has been reported that most somatic hybrids randomly re-distribute their cytoplasms and re-individualize their nuclei at mitosis (Pelletier and Chupeau 1984), thus giving rise to various cybrid combinations. Although no visual differences could be discerned between regenerated and control plants of *N. suaveolens* and of *N. tabacum* cvs CMS Bu21 and WZ raised from seed, it is known that changes, other than purely morphological ones, can occur during the process of in-vitro culture (Larkin and Scowcroft 1981).

From the results presented in Table 1, it is difficult to provide an estimate of the cybridization frequencies which resulted from the fusion and selection system described here since more detailed nuclear, mitochondrial and chloroplast DNA analyses of all plants regenerated are still required. In addition, in order to save time and space, selection of plants with desirable phenotypes was carried out at the time of transferring regenerated plants to the glasshouse. Undoubtedly many more "interesting" plants would have been recovered had all of the regenerated antibiotic-selected shoots been transferred to the glasshouse.

For generating the cybridizations described, protoplasts were prepared from progeny of the transformed *N. tabacum* cv WZ plants. In order to have saved time and resources, protoplasts could have been isolated from the transformed plants themselves. Only approximately 3–4 months would have been required to obtain and grow transformed plants large enough to be used as sources of protoplasts. Similarly, chloroplast-encoded drug resistance could have been induced simultaneously, using NMU in the cytoplasm donor plants, and resultant drug-resistant plants could have been ready for use in protoplast isolations within the same period. About 6 months would be required from the somatic hybridizations to the final evaluations of regenerated plants in the glasshouse. A further 4–6 weeks would be needed to perform a single backcross pollination on the putative cybrids and to allow for seed maturity and collection. In conclusion, a CMS line of a desirable commercial tobacco cultivar would be ready for field testing within 12 months using the techniques and experimental protocols described in this paper.

Acknowledgements The senior author would like to thank the Tobacco Research Board, Zimbabwe, for granting him study leave and the Archie Henderson Memorial Trust of Zimbabwe for their generous sponsorship. The assistance of Dr. Vicky Buchanan-Wollerston in all aspects of the tobacco transformation work described is gratefully acknowledged and the excellent technical support provided by Mrs. Susan Farris of UAPS and by Mr. Brian Wilson and Mr. Charles Kemp of the Horticulture Section, Wye College, is much appreciated.

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