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Rare symmetric and asymmetric *Nicotiana tabacum* (+) *N. megalosiphon* somatic hybrids recovered by selection for nuclear-encoded resistance genes and in the absence of genome inactivation

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Abstract Following protoplast fusion between *Nicotiana tabacum* (*dhfr*) and *N. megalosiphon* (*nptII*) somatic hybrids were selected on the basis of dual resistance to kanamycin and methotrexate. Despite strong selection for parental nuclear-encoded resistances, only nine *N. tabacum* (+) *N. megalosiphon* somatic hybrids were obtained. A preferential loss of the parental *N. tabacum* nuclear and organelle genome was apparent in some plants in spite of the lack of genomic inactivation by the irradiation or chemical treatment of the parental protoplasts. Only six of the nine hybrids recovered possessed both parental profiles of nuclear RFLPs and isoenzymes. The remaining three hybrids were highly asymmetric with two being identical to *N. megalosiphon* except for minor morphological differences and rearranged or recombined mitochondrial DNAs (mtDNA), while the other one was distinguishable only by the presence of a rearranged or recombined mtDNA, and was therefore possibly a cybrid. Overall, eight somatic hybrids possessed rearranged or recombined mtDNAs and chloroplast inheritance was non-random since eight possessed *N. megalosiphon*-type chloroplasts and only one had *N. tabacum* chloroplasts. In contrast, using the same selection approach, numerous morphologically similar symmetric somatic hybrids with nuclear RFLPs and isozymes of both the parental species were recovered from control fusions between *N. tabacum* and the more closely related *N. sylvestris*. In spite of the low frequency of recovery of symmetric *N. tabacum* (+) *N. megalosiphon* hybrids in this study, one of these hybrids displayed a significant degree of self-fertility allowing for back-crosses to transfer *N. megalosiphon* disease-resistance traits to *N. tabacum*.

Key words *Nicotiana tabacum* · *N. megalosiphon* · *N. sylvestris* · Somatic hybrids · Organelle genome analysis · Incompatibility · Asymmetric hybrids

Introduction

Somatic hybridization by protoplast fusion, has been used to circumvent sexual crossability barriers between plant species, thus enhancing the transfer of useful agronomic traits from wild plant species to crop species. Depending upon the fusion partners, somatic hybridization may result in the production of symmetric somatic hybrids, carrying a full amphidiploid complement of both parental genomes, while in other cases the result may be asymmetric hybrids, possessing predominantly only one of the parental genomes (see review by Rose et al. 1990). For fusions between wild species donors and crop cultivars, asymmetric hybrids may be desirable since gene transfer is ideally restricted to the useful agronomic traits which may be encoded by the wild species but which the crop cultivar may lack. Retention of additional parts of the donor nuclear genome or organelle (chloroplast or mitochondrial) genomes in the case of symmetric hybrids may also lead to abnormalities, such as infertility, and may require removal via lengthy backcrosses to the crop parent.

Fusion protocols, used to recover asymmetric fusion products, typically involve inactivation of the donor-species genome using chemicals or irradiation. Unfortunately, the results of these treatments vary in terms of the degree of asymmetry obtained (see Dudits et al. 1987; Bonnema et al. 1992; Bauer-Weston et al. 1993), and the factors affecting the amount of donor DNA lost or retained are not well understood or readily controlled. However, there is increasing evidence that the genetic relatedness of the fusion partners plays a major role in the types of hybrid nuclear genomes, or heterologous nuclear and cytoplasmic genomes (i.e., the genomes of the mitochondria and chloroplast), which are compatible and therefore stable. For ex-

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ample, certain nuclear/cytoplasmic genome combinations, such as the nuclear genome of *Nicotiana tabacum* and the mitochondrial genome of *Petunia hybrida* (Bonnett and Glimelius 1990) or the *N. tabacum* nuclear genome and the *Solanum nigrum* chloroplast genome (Thanh et al. 1988), may be considered completely incompatible as they seem to be impossible to obtain. When such incompatible genomes are brought together in a heterokaryon there is a tendency for the loss of the nuclear or organellar genomes of one or other species even in the absence of genome inactivation of one of the fusion partners.

Although somatic incompatibilities may limit the number of stable fusion products arising following certain hybridizations, the advent of powerful hybrid selection approaches based on selection for the expression of introduced selective-agent resistance genes has permitted the recovery of rare stable fusion products. An example is the recombined hybrid chloroplast genome recovered in the presence of selective pressure for a chloroplast-encoded resistance gene (Medgyesy et al. 1985; Thanh and Medgyesy 1989). Similarly, selection for nuclear-encoded resistance genes associated with one or both parental genomes has also been used to select somatic hybrids (Komari et al. 1989; Sproule et al. 1991; Babiychuck et al. 1992; Donaldson et al.1993,1994). The feasibility of using this approach for the selection of relatively rare nuclear hybrids between somatically incompatible parental species has, so far, however, not been fully exploited. We previously used this type of selection approach to recover hybrids between transgenic methotrexate-resistant *N. tabacum* (tobacco) and several transgenic wild *Nicotiana* species, each carrying a kanamycin resistance gene (Sproule et al. 1991; Donaldson et al. 1993, 1994). In the present study the same approach was used in attempts to produce somatic hybrids between *N. tabacum* and *N. megalosiphon* in order to transfer disease-resistance traits from the wild species to tobacco. The pattern of organelle inheritance was also examined for all of the hybrids recovered. Somatic hybrids between these species have not been described previously. The relatively low frequency of recovery of hybrid plants, as well as the asymmetric nature of some of the fusion products between these distantly related species, is compared with the results for similar fusions between *N. tabacum* and the more closely related *N. sylvestris*.

Materials and methods

Plant material

The transgenic parental genotypes which were fused included a methotrexate-resistant *N. tabacum* cv Delgold (*dhfr*) and either a kanamycin-resistant *N. megalosiphon* or *N. sylvestris* carrying chimaeric neomycin phosphotransferase (*nptII*) genes which were introduced via *Agrobacterium*-mediated transformation of leaf discs as described previously (Dijak et al. 1991). Selective agent-resistant back-cross progeny (from crosses to the respective untransformed genotype) were germinated in vitro and used as donors of leaf-mesophyll protoplasts for fusions.

Protoplast fusion and recovery of double-resistant fusion products

PEG-mediated fusion of leaf-mesophyll protoplasts of *N. tabacum* (*dhfr*) and *N. megalosiphon* (*nptII*) or *N. tabacum* (*dhfr*) and *N. sylvestris* (*nptII*) was performed essentially as detailed previously (Sproule et al. 1991; Donaldson et al. 1993). Protoplasts were first plated on control medium in the absence of selective agents, or on medium with either kanamycin or methotrexate, followed by transfer, 4 weeks after fusion, to regeneration medium containing both selective agents (150 mg/l of kanamycin and 2 mg/l of methotrexate). Calli which were resistant to both selective agents were subcultured at regular intervals on regeneration medium (as above) until either regeneration occurred or a complete loss of vigour was noted. Somatic hybrids were either propagated in vitro by regeneration of leaf pieces on regeneration medium with both selective agents or alternatively they were propagated in the greenhouse by rooting of axial cuttings.

RFLP, isoenzyme and organellar genome analysis

Isolation of total cellular DNA and Southern-blot hybridization analysis was performed as described previously (Donaldson et al. 1994). For RFLP analysis of nuclear DNA, Southern blots of total cellular DNA restricted with *Eco*R1 were hybridized with the heterologous wheat rDNA probe cloned in pTA71 (Gerlach and Bedbrook 1979). For chloroplast DNA analysis, which was performed only for *N. tabacum* (+) *N. megalosiphon* somatic hybrids, total cellular DNA was digested with *Eco*R1 and Southern blots were hybridized to a chloroplast-specific probe consisting of several restriction fragments of the *N. tabacum* chloroplast genome in plasmid pBa1-9, which was kindly provided by E. Galun (Aviv et al. 1984). For mitochondrial DNA analysis, also performed only for *N. tabacum* (+) *N. megalosiphon* somatic hybrids, total cellular DNA was digested with *Eco*R1 or *Bgl*1 and Southern blots were hybridized with mtDNA sequences encoding the heterologous wheat *cytochrome B* (*cytB*) gene (Boer et al. 1985), kindly provided by L. Bonen.

Detection of peroxidase and glutamate oxaloacetate transaminase (GOT) isozymes in leaf extracts of parental species and the somatic hybrids was performed following native polyacrylamide-gel electrophoresis, which was carried out as described previously (Donaldson et al. 1993, 1994).

Morphology and fertility

Male-fertility was evaluated from the frequency of pollen stainable in 1% acetocarmine. The percentage was determined as the mean value (±SD) for at least three individual flowers per somatic hybrid with 1000 grains per anther per flower scored. Flower length was measured as the distance from the sepal base at the pedicel to the tip of the corolla lobes. The selective agent-resistance phenotypes of selfed progeny of the asymmetric and symmetric *N. tabacum* (+) *N. megalosiphon* somatic hybrids, HDM-4 and HDM-5 respectively, and of the transgenic parental lines, were determined by germination of surface-sterilized seed in vitro on B5 medium (Gamborg et al. 1968) with 2% (w/v) sucrose and supplemented with either 150 mg/l of kanamycin, 10 mg/l of methotrexate or else with no selective-agent. Germination of selfed-seed in soil was also tested.

Results

Comparison of frequency of recovery of double-resistant (kanamycin + methotrexate) fusion products between *N. tabacum* (+) *N. megalosiphon* and *N. tabacum* (+) *N. sylvestris*

The *N. tabacum* (mtx^r) (+) *N. megalosiphon* (km^r) fusion experiments yielded 54 independent double-resistant calli,

Table 1 RFLP, isozyme analysis, fertility and organelle composition of the nine *N. tabacum* (+) *N. megalosiphon* somatic hybrids

Somatic hybrid	Flower length (mm)	Isoenzyme analysis[a]	rDNA RFLP analysis[b]	% Male-fertility	Self-fertility[c]	cp genome[d]	mt genome[d,e]
N. tabacum	71 ± 1.3	*N. tabacum*	*N. tabacum*	92 ± 8.0	+	T	T
N. megalosiphon	81 ± 3.4	*N. meg.*	*N. meg.*	93 ± 5.5	+	M	M
HDM-1	62 ± 1.2	Mix	Hybrid	20 ± 10.0	–[s]	M	R
HDM-2	44	Mix	Hybrid	3 ± 2.9	–	M	R
HDM-3	65 ± 1.9	Mix	Hybrid	54 ± 15.8	–[s]	M	R
HDM-4	79 ± 6.5	*N. meg.*	*N. meg.*	32 ± 16.7	+	M	R
HDM-5	86 ± 5.5	Mix/hybrid	Hybrid	12 ± 9.6	+	M	T
HDM-6	72 ± 4.3	Mix/hybrid	Hybrid	2 ± 1.4	–	T	R
HDM-7	80 ± 3.7	*N. meg.*	*N. meg.*	35 ± 10	+	M	R
HDM-8	69 ± 4.1	Mix/hybrid	Hybrid	0	–	M	R
HDM-9	66 ± 2.8	*N. meg.*	*N. meg.*	60 ± 4.5	+	M	R

[a] Isoenzymes identical to those of parental *N. megalosiphon*, a mix of both parental types, or a mix plus unique (hybrid) bands were observed

[b] Probing with the rDNA probe revealed the presence of either only *N. megalosiphon*-specific hybridizing bands, or both parental-specific bands (hybrid)

[c] [S] Small, shrivelled non-viable seed only

[d] Analysis of the chloroplast or mitochondrial genome compositions revealed patterns of hybridization consistent with the presence of *N. tabacum* (*T*) or *N. megalosiphon* (*M*) chloroplasts or mitochondria or

[e] The presence of a rearranged or recombined mitochondrial DNA (*R*)

41 from fusions plated initially on the control and 13 from fusions plated initially on medium with methotrexate. However, only nine calli regenerated in the presence of both selective agents; the remainder eventually lost vigour in spite of survival through several subcultures in the presence of both methotrexate and kanamycin. In contrast, 53 double-resistant calli were recovered from fusions between the more closely related *N. tabacum* (*mtx*^{r}) and *N. sylvestris* (*km*^{r}) and all but two of these calli regenerated. In contrast to the calli from the *N. tabacum* (+) *N. megalosiphon* fusions, the *N. tabacum* (+) *N. sylvestris* calli regenerated rapidly without an extensive callus proliferation phase and all of the 44 plants grown to maturity had a hybrid morphology.

Fertility and morphological analysis

The results of flower length, nuclear RFLP and isozyme analysis, male-fertility levels and organelle composition for all of the *N. tabacum* (+) *N. megalosiphon* somatic hybrids are summarized in Table 1. The nine hybrids can be loosely grouped into one of four distinct types on the basis of morphology and the results of RFLP and isoenzyme analysis. These include, firstly the three highly asymmetric hybrids, HDM-4, HDM-7 and HDM-9, secondly the three *N. megalosiphon*-like hybrids with 'tobacco-pink' flowers, i.e., HDM-1, HDM-2 and HDM-3, thirdly the two abnormal somatic hybrids, HDM-6 and HDM-8, and finally a fourth type represented by HDM-5 which was the only fertile, more symmetric somatic hybrid. Figure 1a shows the morphology of two of the three highly asymmetric somatic hybrids HDM-7 and -9 (HDM-4 is identical to parental *N. megalosiphon*) while examples of the more symmetric hybrids are shown in Fig. 1b. Only HDM-4 was morphologically indistinguishable from the parental *N. megalosiphon* except for a reduction in male-fertility. HDM-7 and HDM-9 are also similar to parental *N. megalosiphon,* but have either an altered leaf morphology (HDM-7) or a shorter growth habit and smaller leaves and flowers (HDM-9). The three asymmetric somatic hybrids were self-fertile but the yield and viability of selfed-seed from HDM-7 and HDM-9 was low. The three hybrids HDM-1, HDM-2 and HDM-3 also appeared very similar to *N. megalosiphon* until they reached maturity. The flowers of these hybrids were closer in size to *N. megalosiphon* but the flowers were pink as in *N. tabacum* and all three hybrids were infertile. In contrast, HDM-5, HDM-6 and HDM-8 had a hybrid morphology as shown in Fig. 1b for HDM-5 and HDM-6 only. HDM-5 was closer to parental *N. tabacum* in growth habit and leaf and flower morphology than the others and it was the only self-fertile symmetric somatic hybrid. HDM-6 and HDM-8 were both stunted and bore abnormal, deformed leaves. Somatic hybrid HDM-6, which was the only hybrid with tobacco chloroplasts, had an abnormal anther position in all the flowers examined, as shown in Fig. 1c. Levels of male-fertility were quite low in most cases but varied from undetectable in HDM-8 to 60±4.5 in HDM-9.

In contrast to the rare *N. tabacum* (+) *N. megalosiphon* hybrids, the many *N. tabacum* (+) *N. sylvestris* somatic hybrids which were recovered were morphologically homogeneous; although some stunted plants with leaf abnormalities were observed, all 44 hybrids grown to maturity had the same intermediate leaf and flower morphology (flowers shown in Fig. 1d). Male-fertility values which were determined for 32 of the *N. tabacum* (+) *N. sylvestris* hybrids showed an average value of 84±9.3% fertility with individual values ranging from 61 to 96% viable pollen.

Fig. 1a–d Morphology of 'symmetric' and 'asymmetric' *N. tabacum* (+) *N. megalosiphon* and *N. tabacum* (+) *N. sylvestris* somatic hybrid flowers and leaves. **a** Upper and lower, asymmetric hybrids: flowers and leaves of, from left to right, *N. tabacum*, the two highly asymmetric *N. tabacum* (+) *N. megalosiphon* somatic hybrids HDM-9 and HDM-7, and *N. megalosiphon*. Asymmetric somatic hybrid HDM-4 appears identical to the *N. megalosiphon* parent and is therefore not shown. **b** Upper and lower, symmetric hybrids: flowers and leaves of, from left to right, *N. tabacum*, *N. tabacum* (+) *N. megalosiphon* somatic hybrids HDM-5, HDM-6, HDM-1, HDM-2 and *N. megalosiphon*. **c** Flower of HDM-6 (left) showing abnormal anther position compared with flower of *N. tabacum* (right). **d** Flowers of tobacco (left), *N. sylvestris* (centre), and a representative *N. tabacum* (+) *N. sylvestris* somatic hybrid (right). All of the 44 somatic hybrids between *N. tabacum and N. sylvestris* were very homogeneous morphologically, in contrast to the varying phenotypes present amongst the nine *N. tabacum* (+) *N. megalosiphon* somatic hybrids

Nuclear RFLP, isoenzyme analysis, and retention or loss of *N. tabacum* nuclear-markers

N. tabacum (+) *N. megalosiphon somatic hybrids.* Hybridization of genomic DNA with the heterologous wheat rDNA probe revealed patterns of hybridization which were unique to each parent (Fig. 2). However, the six somatic hybrids HDM-1, -2, -3, -5, -6, and -8 each possessed all of the hybridizing bands of both the *N. tabacum* and the *N. megalosiphon* parent, although for HDM-8 hybridization to one of the *N. megalosiphon*-specific bands was very faint. In HDM-8 the reason for both the one faint *N. megalosiphon*-specific hybridizing band and the presence of unique hybridizing bands (which were seen also in HDM-7, as shown, and in HDM-9, which is not shown) are unclear and it could not be ruled out that DNA was partially digested. The highly asymmetric hybrids HDM-4, -7, and -9 possessed *N. megalosiphon*-specific bands but none of the *N. tabacum*-specific bands [see Fig. 2, lanes 6 and 9; HDM-9, which is not shown in the figure, had a pattern identical to HDM-7, in which some unique hybridizing bands appeared to be present (see Fig. 2, lane 9)]. Somatic hybrid HDM-4 had a pattern of hybridization identical to a parental type, i.e., identical to *N. megalosiphon.*

The results of isoenzyme analysis for the peroxidase (data not shown) and glutamate oxaloacetate transaminase (GOT) isozymes (shown in Fig. 3) revealed three different patterns amongst the somatic hybrids. HDM-1, HDM-2 and HDM-3 (the *N. megalosiphon*-like hybrids with 'tobacco-pink' flowers) possessed all of both parental GOT isozymes (Fig. 3, lane 4), and no unique GOT bands, and all of the *N. tabacum* peroxidases and only one of the *N. megalosiphon* peroxidases. The situation for HDM-5, HDM-6 and HDM-8 (the morphologically hybrid plants) was different when compared to the previous three as they possessed all of both parental-specific GOT bands plus one novel band (Fig. 3, lane 6) and all of the peroxidases of both parental *N. tabacum* and *N. megalosiphon*. Results for the three highly asymmetric hybrids, HDM-4, -7 and -9, showed that only parental *N. megalosiphon* peroxidases and GOT isozymes (Fig. 3, lanes 5, 7, 9) could be detected in extracts of these plants while none of the parental *N. tabacum* isozymes were evident.

We determined whether the highly asymmetric hybrids retained both parental nuclear-encoded selective markers to maturity by looking at the resistance phenotypes of their respective selfed progeny. For somatic hybrid HDM-4, 18/18, 14/20 and 0/15 were the fractions of seedlings surviving in vitro on control, kanamycin- and methotrexate-containing medium respectively, and thus the *dhfr* gene from the tobacco parent was most likely lost in this hybrid or else was not transmitted to the progeny. In contrast, the symmetric hybrid HDM-5 showed 17/17, 21/25 and 11/20 seedlings surviving on control, kanamycin- or methotrexate-containing medium respectively, suggesting that both the parental *N. megalosiphon nptII* gene and the *N. tabacum dhfr* gene were stable in this hybrid and were transmitted to the progeny. Segregation data for the parental *N. tabacum* and *N. megalosiphon* were, respectively, 18/18, 0/18, 18/20 and 20/20, 13/16 and 0/20 for plating on control, kanamycin- or methotrexate-containing medium respectively. Unfortunately, in the case of HDM-7 and -9, seeds did not germinate even on control plates, therefore no results were obtained. Some of these seeds were capable of germination in soil but unfortunately these were not tested for their resistance phenotype (s).

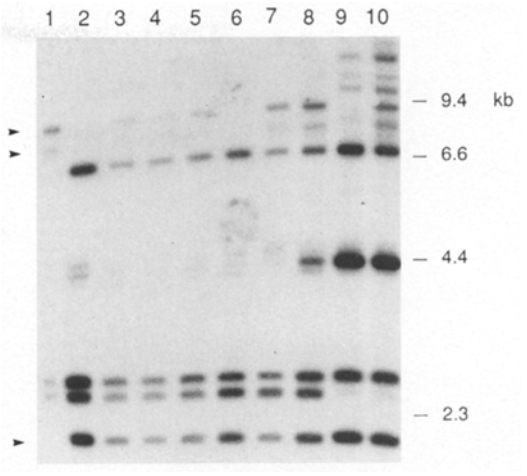

Fig. 2 Southern-blot hybridization of a heterologous wheat nuclear ribosomal DNA probe with total cellular DNA isolated from *N. tabacum* (*dhfr*), *N. megalosiphon* (*nptII*) and selected somatic hybrids and digested with *Eco*R1. *Lanes* from left to right are (*1*) *N. tabacum* (*dhfr*), (*2*) *N. megalosiphon*, (*3*) HDM-1, (*4*) HDM-2, (*5*) HDM-3, (*6*) HDM-4, (*7*) HDM-5, (*8*) HDM-6, (*9*) HDM-7, (*10*) HDM-8. The *arrows* on the left indicate the positions of some of the polymorphic *N. tabacum*-specific (*top two arrows*) and *N. megalosiphon*-specific (*bottom arrow*) hybridizing bands. The somatic hybrids HDM-1, -2, -3, -5, -6, and -8 all show species-specific hybridizing bands from both parents while HDM-4, -7, and -9 (data not shown) do not show any of the *N. tabacum*-specific hybridizing bands. The positions of molecular-size markers are indicated, in kilobases, on the right

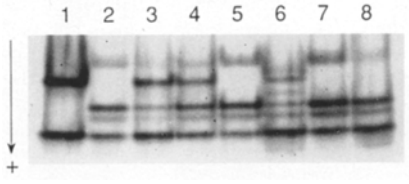

Fig. 3 Native polyacrylamide-gel electrophoresis (5% acrylamide) of leaf glutamate-oxaloacetate transaminases of the parental species and selected *N. tabacum* (+) *N. megalosiphon* somatic hybrids. *Lanes* from left to right are (*1*) *N. tabacum*, (*2*) *N. megalosiphon*, (*3*) a 1:1 mixture of leaf extracts of *N. tabacum* and *N. megalosiphon*, (*4*) HDM-2, (*5*) HDM-4, (*6*) HDM-5, (*7*) HDM-7, (*8*) HDM-9. The result in *lane 4* showing a mix of parental bands was also obtained for HDM-1 and HDM-3. The result in *lane 6* showing a mix plus a unique isozyme band for HDM-5 was also obtained for HDM-6 and -8. The asymmetric hybrids in *lanes 5, 7, and 8* do not contain the *N. tabacum*-specific isozyme band. The direction of migration is towards the anode indicated at the lower left of the figure

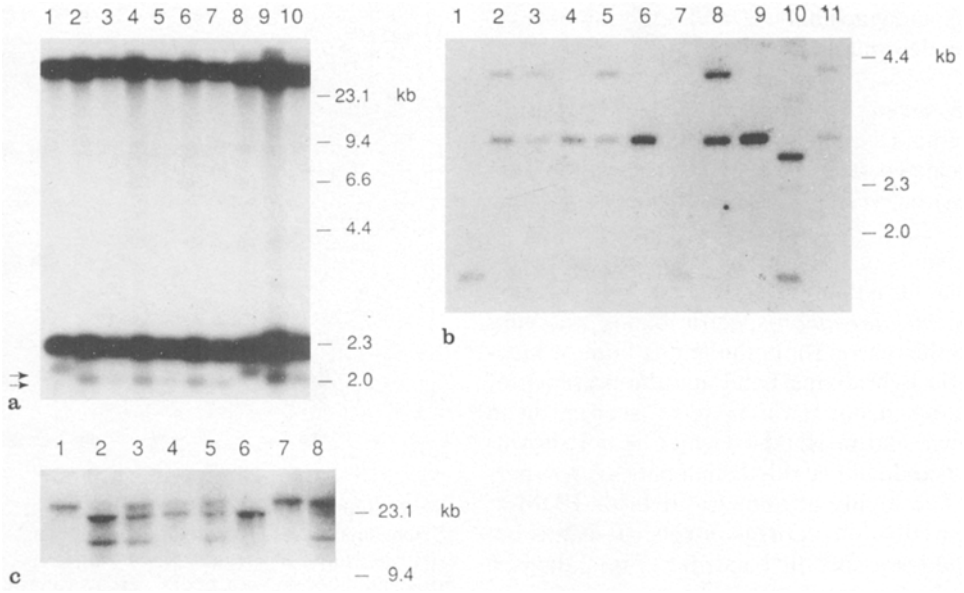

Fig. 4a–c Analysis of organelle genomes in the *N. tabacum* (+) *N. megalosiphon* somatic hybrids. **a** Chloroplast inheritance: hybridization of the cpDNA sequences (in pBa1-9) to total cellular DNA of the parental species and somatic hybrids digested with *Eco*R1. *Lanes* are (*1*) *N. tabacum,* (*2*) *N. megalosiphon,* (*3*) HDM-1, (*4*) HDM-2, (*5*) HDM-3, (*6*) HDM-4, (*7*) HDM-5, (*8*) HDM-6, (*9*) HDM-7, (*10*) HDM-8. The two *arrows* at the lower left of the figure point to the *N. tabacum*-specific hybridizing band (*top arrow*) and the *N. megalosiphon*-specific hybridizing band (*bottom arrow*). Somatic hybrid HDM-9, not shown in the figure, gave a pattern of hybridization indicating the presence of *N. megalosiphon* chloroplasts. **b, c** Mitochondrial genome analysis; total cellular DNA was digested with either *Eco*R1 or *Bgl*1 and probed with a heterologous *cytB* probe. **b** *Eco*R1-digested DNA. *Lanes* are (*1*) *N. tabacum,* (*2*) *N. megalosiphon,* (*3*) HDM-1, (*4*) HDM-2, (*5*) HDM-3, (*6*) HDM-4, (*7*) HDM-5, (*8*) HDM-6, (*9*) HDM-7, (*10*) HDM-8, and (*11*) HDM-9. **c** *Bgl*1-digested DNA. *Lanes* are (*1*) *N. tabacum,* (*2*) *N. megalosiphon,* (*3*) HDM-1, (*4*) HDM-2, (*5*) HDM-3, (*6*) HDM-4, (*7*) HDM-5, (*8*) HDM-6. Positions of molecular-size markers are indicated, in kilobases, on the right side of the figures

N. tabacum (+) *N. sylvestris somatic hybrids.* Probing of genomic DNA using the nuclear rDNA probe pTA71 revealed that a different pattern of hybridization was obtained for each of the parental species. When pTA71 was used to probe the somatic hybrids, both *N. tabacum*- and *N. sylvestris*-specific bands were seen for all 19 individual *N. tabacum* (+) *N. sylvestris* hybrids which were examined (data not shown). In addition, examination of peroxidase isozymes, performed for 29 of the hybrids, revealed that all hybrids possessed peroxidases from both parental species while no unique bands were detected (data not shown).

Organellar DNA analysis

N. tabacum (+) *N. megalosiphon somatic hybrids.* Results of probing *Eco*R1 digests with the chloroplast probe pBa1-9 (Fig. 4a) showed that eight of the somatic hybrids possessed chloroplasts from *N. megalosiphon* while only somatic hybrid HDM-6 (Fig. 4a, lane 8) had *N. tabacum* chloroplasts. The probe hybridizes to three different *Eco*R1 fragments in the parental species, including two higher-molecular-weight bands which are not polymorphic between the parents and a third-lower-molecular weight fragment which is polymorphic. Therefore, only these bands, which are marked by arrows in the figure were used to determine chloroplast type in the somatic hybrids.

The heterologous *cytB* mtDNA probe yielded parental species-specific patterns of hybridization for the parents when used to probe total cellular DNA restricted with either *Eco*R1 or *Bgl*1. Analysis of the genomic DNA of the parents and the somatic hybrids is shown in Fig. 4b and c for *Eco*R1 and *Bgl*1 digests respectively. A pattern of hybridization identical to the *N. megalosiphon* parent was seen following the probing of *Eco*R1 digests of HDM-1, -3, -6 and -9 (Fig. 4b, lanes 3, 5, 8 and 11), but for *Bgl*1 digests (Fig. 4c, lanes 3, 5, 8; HDM-9 data not shown) the pattern of hybridization was unlike either parent and thus consistent with the presence of a rearranged or recombined mtDNA in these hybrids. HDM-2, -4, and -7 shared a unique pattern of hybridization, unlike either parent, for *Eco*R1 digests (Fig. 4b, lanes 4, 6, and 9) and these results, together with those for probing of *Bgl*1 digests (Fig. 4c, lanes 4 and 6; HDM-7 data not shown), also indicate the presence of a rearranged or recombined mtDNA. HDM-8 showed a different unique pattern of hybridization for the probing of *Eco*R1 digests (Fig. 4b, lane 10) also consistent with a rearranged or recombined mtDNA, while results of the probing of *Bgl*1 digests for this hybrid revealed a pattern similar to the *N. tabacum* parent. HDM-5 showed a pattern of hybridization identical with the *N. tabacum* parent for the probing of both *Eco*R1 and *Bgl*1 digests (Fig. 4b, c, lane 7) and thus this was the only somatic hybrid for which no evidence for rearrangements in the mtDNA was found.

N. tabacum (+) *N. sylvestris somatic hybrids.* The maternal ancestral parent of tobacco was an ancient *N. sylvestris* species and previous reports have shown that the cytoplasmic genomes of present-day *N. tabacum* and *N. sylvestris* are virtually indistinguishable (Nagy et al. 1983; Aviv et al. 1984); therefore, we did not attempt to examine the cytoplasmic genomes of the somatic hybrids.

Discussion

This report describes the first somatic hybrid plants between *N. tabacum* and the wild species *N. megalosiphon*. Both the difficulty encountered in the recovery of these hybrids and their characteristics suggest that considerable genomic incompatibility may exist between the parental species. Regeneration of somatic hybrids resistant to both selective agents was not efficient as only 9 of the 54 selected calli regenerated and the other 45 double-resistant calli eventually lost vigour in culture. These calli are considered unlikely to be escapes from one of the selective agents because of the absence of growth in parental control platings and the failure to observe escapes in numerous other hybrid selection experiments which we have conducted with other *Nicotiana* spp. using the same selection approach (Sproule et al. 1991; Donaldson et al. 1993, 1994). In addition, the *N. tabacum* (+) *N. sylvestris* somatic hybridizations led to 53 calli, of which all but two regenerated in the presence of both selective agents and all of the 44 plants grown to maturity were hybrid in morphology. Thus, the numerous *N. tabacum* (+) *N. megalosiphon* fusion-derived calli which were obtained most likely carried resistance genes for both selective agents initially, but one or both resistance genes were lost by chromosome elimination at a later stage in callus development. Alternatively, the non-regenerating calli may have been more-symmetric hybrids in which further development was arrested, as has been reported for certain nuclear-cytoplasmic types (see Rose et al. 1990). Presumably, relatively stable nuclear-cytoplasmic genome combinations allowed the retention of both nuclear-encoded resistance genes and regeneration in the presence of both selective agents for nine of the calli. However, the recovery of at least one plant, HDM-4, in which the *N. tabacum* nuclear marker seemed to have been lost (since it was not transmitted to the progeny) suggests that chromosome elimination was incomplete following removal from selection, i.e., at the rooting stage. Alternatively, a chimaeric callus may have supported the survival of a non-hybrid sector which gave rise to HDM-4.

Results of an analysis of morphology, nuclear RFLPs and organelle composition for the nine plants recovered provides evidence that preferential elimination of *N. tabacum* chromosomes may have occurred in some plants in spite of the lack of chemical or irradiation pre-treatments to inactivate the parental genome. Firstly, both the morphological characteristics of the three highly asymmetric hybrids (HDM-4, HDM-7, HDM-9) and the loss of the *N. tabacum*-specific methotrexate-resistance phenotype in HDM-4 progeny strongly suggest that extensive elimination of *N. tabacum* chromosomes occurred in these hybrids. The lack of *N. tabacum*-specific nuclear RFLP markers and isoenzymes in HDM-4, HDM-7 and HDM-9 was also established; however, the presence of a small portion of the *N. tabacum* nuclear genome is not ruled out, especially as chromosome numbers were not examined. Secondly, the predominance of *N. megalosiphon* chloroplasts in eight of the nine somatic hybrids also indirectly suggests the dominance of the *N. megalosiphon* nuclear genome in these plants, as discussed below. HDM-5, HDM-6, and HDM-8 may contain a more balanced composition of genetic material from both parents compared to HDM-1, -2, or -3, since the results of GOT analysis with the former three hybrids showed the presence, not only of a mixture of parental GOTs, but also of a novel band. In addition HDM-5, HDM-6 and HDM-8 were judged to be more intermediate in morphology in contrast to the latter three which were very similar to parental *N. megalosiphon*.

Our findings support other evidence which indicates that the degree of genetic relatedness, and thus the genomic compatibility, between the two nuclear genomes and between the nucleus and the heterologous cytoplasmic genomes of the fusion partners influences the final nuclear/cytoplasmic genome content of the resulting plants. Both the preferential elimination of the parental *N. tabacum* nuclear genome and the possibly related under-representation of *N. tabacum* chloroplasts in some of the somatic hybrids probably reflects the genomic incompatibility between these distantly related species (see Goodspeed 1954 for a review of phylogenetic relationships among *Nicotiana*). Incompatibility causing unstable fusion products, and affecting regeneration and fertility, may be a result of ineffective nucleo-cytoplasmic or nuclear-nuclear interactions. In both cases the elimination of parental chromosomes could lead to a more functional hybrid. Spontaneous chromosome elimination has been reported previously for intertribal fusions between *Solanum tuberosum* and *N. plumbaginifolia* in which non-regenerable hybrid calli were obtained (Gilissen et al. 1992), and for hybridizations of more distantly related Brassicaceae species (Sundberg and Glimelius 1991) as well as for *N. tabacum* (+) *Atropa belladonna* somatic hybrids (Babiychuk et al. 1992).

Amongst the nine somatic hybrids which were recovered there was a preferential retention of *N. megalosiphon* chloroplasts in 8/9 somatic hybrids. Parental chloroplast populations typically segregate in hybrids resulting in somatic hybrids with one or the other parental chloroplast type (see review by Rose et al. 1990). In the absence of certain extenuating factors which are sometimes thought to influence segregation, nuclear-cytoplasmic or alternatively nuclear-nuclear incompatibilities may result in preferential retention of one or other of the parental species chloroplasts (see Donaldson et al. 1993, 1994). For example, preferential elimination of *N. tabacum* chromosomes and/or duplication of the *N. megalosiphon* chromosomes in the hybrids, either of which could result in dominance of the *N. megalosiphon* genome in the nucleus, may have caused the biased retention of *N. megalosiphon* chloro-

plasts, due to plastid input bias (Butterfass 1988). The abnormal floral morphology present in HDM-6 (shown in Fig. 1c) may be due to a nuclear incompatibility with the *N. tabacum*-type chloroplasts, which were present only in this hybrid, but could also be due to the particular parental nuclear genes combined in this, but not in the other hybrids. The only trait which has been tentatively attributed to incompatible chloroplast-nuclear interactions is chlorophyll-deficiency (Kushnir et al. 1991), and while HDM-6 is somewhat chlorotic, so is HDM-8 which possesses *N. megalosiphon* chloroplasts. The majority of the hybrids had non-parental profiles for mtDNA analysis, consistent with recombination between parental genomes, which is frequently observed in somatic hybrids (Belliard et al. 1979; Nagy et al. 1983). Since we did not further characterize the mtDNAs we cannot conclude whether there were more *N. megalosiphon*-specific than *N. tabacum*-specific sequences present, which might be expected if the *N. megalosiphon* nuclear genome is predominant in the hybrids. Although the only difference detected between HDM-4 and the parental *N. megalosiphon* (apart from the reduced male-fertility) was the presence of a non-parental mtDNA, it is probably at least a cybrid and possibly a hybrid (and thus may also contain some nuclear *N. tabacum* DNA) since mitochondrial DNA rearrangements at least for *N. tabacum* and *N. plumbaginfolia* have been shown to occur as a result of a heteroplasmic state and not usually as a consequence of tissue culture (Nagy et al. 1983).

Sexual hybridization of *N. tabacum* and *N. megalosiphon* is possible with difficulty and has been used in attempts to transfer resistance to the causative agent of the blue mold pathogen from the wild species into tobacco breeding lines (Trancheva 1989). The results of the present study show how strong selective pressure can overcome the crossability barrier between distantly related species and that transfer of limited amounts of genetic material may have resulted in some cases due to extensive chromosome elimination. It is possible that the use of agents which favour one or the other chloroplast or mitochondrial DNA type could be used to influence which parental nuclear genome is retained since, as has been suggested by Derks et al. (1992), particular nuclear genes are required to maintain stable nuclear-cytoplasmic compatibilities in certain rare nucleo-cytoplasmic types. It should be emphasized, however, that while somatic incompatibility may favour the formation of asymmetric hybrids it is not at all clear that the precise amount of donor material retained can be controlled. Thus isolated gene transfer via transformation technology may be a more efficient means of moving important genes from wild species into crop species.

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