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 isoenzymes 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-
ample, certain nuclear/cytoplasmic genome combinations, such as the nuclear genome of *Nicotiana tabacum* and the mitochondrial genome of *Petunia hybrida* (Bonnett and Glimeleiu 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; Babychuck 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 chloramycin 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 extracted with EcoRI were hybridized with the heterologous wheat rDNA probe cloned in pTA71 (Gerlac and Bedbrook 1979). For chloroplast DNA analysis, which was performed only for *N. tabacum (+) N. megalosiphon* somatic hybrids, total cellular DNA was digested with EcoRI and Southern blots were hybridized to a chloroplast-specific probe consisting of several restriction fragments of the *N. tabacum* chloroplast genome in plasmid pBal-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 EcoRI or BgI1 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. 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, HIDM-4 and HIDM-5 respectively, and of the transgenic parental lines, were determined by germination of surface-sterilized seed in vitro on B5 medium (Gamberg 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*) (+) *N. megalosiphon* (*kmr*) fusion experiments yielded 54 independent double-resistant calli,