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Transfer of amiprophosmethyl resistance from a *Nicotiana plumbaginifolia* mutant by somatic hybridisation

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Abstract Transfer of resistance to the phosphorothioamidate herbicide, amiprophosmethyl (APM), from the β-tubulin mutant of *Nicotiana plumbaginifolia* to the interspecific *N. plumbaginifolia (+) N. sylvestris* and to the intertribal *N. plumbaginifolia (+) Atropa belladonna* somatic hybrids has been demonstrated. Transfer to the recipient species was accomplished by: (1) symmetric hybridisation and (2) asymmetric hybridisation using γ-irradiation of donor protoplasts. Cytogenetic analysis confirmed the hybrid origin of the hybrids obtained. It was established that most of them typically inherited no more than three donor chromosomes, although it was possible to obtain symmetric hybrids in the case of symmetric fusion. Immunofluorescent microscopy analysis has shown that protoplasts of the mutant, and of the *N. plumbaginifolia (+) N. sylvestris* and *N. plumbaginifolia (+) A. belladonna* hybrids, retained the normal structure of interphase microtubule (MT) arrays and mitotic figures after treatment with 5 μM APM, whereas MTs of protoplasts of the recipients were destroyed under these conditions. It was also shown that hybrid clones contained an altered β-tubulin isoform originating from the *N. plumbaginifolia* mutant. The selected hybrid clones were characterised by cross-resistance to trifluralin, a dinitroaniline herbicide with the same mode of anti-MT action. Some of the somatic hybrids which could flower were fertile. It was established that seeds of some fertile hybrids were able to germinate in the presence of 5 μM APM. The results obtained thus support the conclusion that the technique of somatic hybridisation, especially asymmetric fusion, can be used to transfer APM resistance from the *N. plumbaginifolia* mutant to different (related and remote) plant species of the *Solanaceae*, including important crops.

Key words Amiprophosmethyl · β-Tubulin · *Nicotiana plumbaginifolia* mutant · Somatic hybridisation · Transfer of resistance

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Introduction

Somatic hybridisation can be successfully used to transfer desirable traits, especially those controlled by non-identified and uncloned genes, from mutants to different plants by overcoming the sexual-incompatibility barriers (Gleba and Shlumukov 1990; Glimelius et al. 1991). Gene transfer to recipient species by somatic hybridisation can be realised in different ways, but cybridisation and asymmetric hybridisation have been most widely used in the last decade, in particular for the transfer of herbicide resistance (Gleba and Shlumukov 1990; Glimelius et al. 1991; Galun 1995). At present, the transfer of resistance to herbicides with an antimitotic mechanism of action (Hatzios 1994), (e.g. phosphorothioamidates, dinitroanilines, carbamates) is of a particular importance. Natural mutants of *Eleusine indica* with dinitroaniline resistance (Vaughn and Vaughan 1990; Smeda and Vaughn 1994) were found and APM-resistant *Nicotiana plumbaginifolia* mutants were produced by selection in vitro (Strashnyuk et al. 1993; Blume et al. 1998).

It has been established that the target for these herbicides is tubulin and their binding with this protein inhibits the in vitro assembly of MTs (Morejohn and Fosket 1984, 1991; Morejohn et al. 1987). In the case of *E. indica*, it was shown, on the one hand, that resistance of the mutant biotype is linked with the changes in β-tubulin structure (Vaughn and Vaughan 1990; Smeda and Vaughn 1994). On the other hand, Waldin et al. (1993) showed that dinitroaniline resistance of another mutant biotype of *E. indica* was accompanied with differences in the electrophoretic properties of one of the α-tubulin isoforms. The latter alteration was confirmed by the re-

sults of a sequence analysis of tubulin cDNA clones, which also suggest the existence of an altered α-tubulin in dinitroaniline-resistant *E. indica* (Baird et al. 1996). The mutants of *N. plumbaginifolia*, resistant to the phosphorothioamidate herbicide APM, mentioned above, had an altered isoform of β-tubulin (Blume et al. 1998). They are also characterised by cross-resistance to the dinitroaniline herbicide trifluralin.

Since the mutant β-tubulin gene of APM-resistant plants has not yet been identified or cloned, we used somatic hybridisation as an alternative method to transfer the APM-resistance from a *N. plumbaginifolia* mutant to another species of the *Solanaceae*. In this paper we report the possibility of APM-resistance trait transfer from APM-resistant *N. plumbaginifolia* mutant to *Nicotiana sylvestris* (related species) and *Atropa belladonna* (remote species) by two types of somatic hybridisation: symmetric and asymmetric fusion.

Materials and methods

Plant material

The experiments used the *N. plumbaginifolia* (2n=20) mutant line resistant to 5 μM APM obtained earlier (Blume et al. 1998). Wild-type and kanamycin-resistant (100 mg/l) lines of *N. sylvestris* (2n=24) and *A. belladonna* (2n=72) were also used. Plants were grown in vitro on hormone-free MS (Murashige and Skoog 1962) media at 23–25°C.

Isolation/fusion of protoplasts and selection of APM-resistant hybrids

Mesophyll protoplasts of *N. plumbaginifolia* mutant were isolated as described by Blume et al. (1998). Protoplasts of *N. sylvestris* and *A. belladonna* were isolated from leaves of young plants by incubation in an enzyme solution containing 0.4% Onozuka R-10 (Serva), 0.2% Driselase (Sigma), 0.5 M sucrose and 5 mM $CaCl_2$, for 14 h at 25°C in the dark. To obtain symmetric somatic hybrids (Fig. 1), freshly isolated protoplasts of *N. plumbaginifolia* mutant and kanamycin-resistant *N. sylvestris* or *A. belladonna* were mixed in a 1:1 ratio and then fused by polyethylene glycol-4000 at a density 3×10^6 protoplasts/ml as described by Menczel et al. (1981). To produce the highly asymmetric somatic hybrids, *N. plumbaginifolia* protoplasts were γ-irradiated with a dose of 200 Gy (^{60}Co) before fusion. After irradiation they were washed with W5 solution (Menczel et al. 1981), mixed with recipient protoplasts of *N. sylvestris* or *A. belladonna* wild types in a 1:1 ratio and fused by the same method at a density 2×10^6 protoplasts/ml. The fusion products from combining *N. plumbaginifolia (+) N. sylvestris* were diluted and cultured in 8p medium (Kao and Michayluk 1975) for 7–10 days in the dark at 25°C; protoplasts from the combination of *N. plumbaginifolia (+) A. belladonna* were diluted and cultured in MO-1 medium (Gleba et al. 1982) under these conditions. At the stage of 3–5 divisions of heterokaryons 5 μM of APM were added in the media as same a selective pressure. In order to produce hybrids after symmetric fusion kanamycin (100 mg/l) was added simultaneously with APM. One-month later the microcolonies of *N. plumbaginifolia (+) N. sylvestris* and *N. plumbaginifolia (+) A. belladonna* were transferred to MS media, containing 1 mg/l zeatin, 0.1 mg/l indolic-3-acetic acid, 30 g/l sucrose and 7 g/l agar, for shoot regeneration of hybrid clones resistant to APM (in the presence of selective concentrations of APM and kanamycin or APM only in the media). Hybrids were rooted on MS hormone-free media. The rooted plants were eventually transferred to the greenhouse.

Primary screening of herbicide-resistant hybrids

As an initial test of hybrid lines resistant to APM and cross-resistant to trifluralin, regenerating abilities of hybrid leaf explants were tested using regeneration media in the presence of selective concentrations of APM (5 μM) or trifluralin (12 μM). Comparative experiments of the growth of parental and hybrid calli on the media for callus induction in the presence of different concentrations of APM were carried out 4 weeks after planting. Calli of both *Nicotiana* species and *A. belladonna* (nightshade) were induced on the respective media for callus induction in *Nicotiana* (Sidorov et al. 1985) and *A. belladonna* (Eapen et al. 1978). The comparative growth rates of calli were calculated by the formula:

$$W = \frac{M - m}{m} \times 100\%$$

where W=comparative rate of callus, M=4-week-old callus weight, m=initial callus weight. Three repeated experiments were carried out and mean values were defined for each of the points.

Fertility analysis

The flowering hybrid plants (30 hybrids with normal flower morphology from each of four different fusion combinations) were self-pollinated, or cross-pollinated, with corresponding parental plants (*N. sylvestris* or *A. belladonna*). The seeds obtained from self-pollinated and cross-pollinated plants were sterilised (ethanol, 5 min; sodium hypochloride, 10 min), washed three times for 10 min with sterile distilled water and planted for germination on hormone-free MS media containing 5 μM APM for an analysis of APM resistance inheritance in first progeny of hybrid plants. All the experiments were repeated three times, and in every variant about 300 seeds were used. Seed germination and the ability of seedlings to grow on the medium with 5 μM APM were examined for APM resistance.

Chromosome analysis

For cytological studies root tips of parental and hybrid plants were pre-treated with 0.5% colchicine (1 h, 10–12°C) and fixed in an ethanol/acetic acid mixture (3:1) for 12–16 h and stained with orcein (1% solution in 45% acetic acid) for 24–48 h. Squash preparations were made in 45% acetic acid to count the chromosome sets.

Immunofluorescence microscopy

Immunofluorescence staining of microtubules by TU-01 (anti-α-tubulin) monoclonal antibody in protoplasts of hybrid and parental lines was carried out as described by Blume et al. (1998). Cellular DNA was stained with 0.04% (w/v) Hoechst 33258 solution in PBS for 10 min. The coverslips were examined with a JENALUMAR-a fluorescence microscope equipped with a 100/1.30 Planachromat fluorescence oil-immersion objective.

Two-dimensional electrophoresis and immunoblotting of tubulin

Tubulin from leaves of experimental plants was purified by DEAE-chromatography as described by Blume et al. (1998). Two-dimensional PAAG electrophoresis (O'Farrell 1975) and immunoblotting (Towbin et al. 1979) of isolated tubulin from parental and *N. plumbaginifolia (+) N. sylvestris* and *N. plumbaginifolia (+) A. belladonna* hybrid lines was performed as described earlier in protocols for the analysis of APM-resistant and susceptible *N. plumbaginifolia* plants (Blume et al. 1998). TU-01 (anti-α-tubulin) and TU-06 (anti-β-tubulin) monoclonal antibodies have been used for the immunoblotting technique.

Herbicides

APM, also known as Bay NTN 6867, was obtained from Dr. J.R. Bloomberg (Agriculture Division, Miles Inc., Kansas City, USA). Trifluralin [2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine] was obtained from Dr. L. Guse (DowElanco, Greenfield, USA). Stock solutions of herbicides were prepared in dimethylsulphoxide (DMSO) and diluted directly before addition into the cooled autoclaved media.

Results

In vitro selection of somatic hybrids resistant to APM

Three hundred and twelve hybrid calli were found resistant to selective concentrations of APM and kanamycin after symmetric fusion of *N. plumbaginifolia* and *N. sylvestris* protoplasts (Table 1). Among them only 68 lines were characterised by an ability to regenerate under selective pressure conditions; 12 of these lines were taken for further analysis. Some of the selected hybrids were morphologically similar to *N. sylvestris*, others had intermediate morphology. All of them developed normally and had a higher speed of growth compared with the parental forms. As a result of the symmetric hybridisation of APM-resistant *N. plumbaginifolia* protoplasts and protoplasts of the kanamycin-resistant line of *A. belladonna*, 102 calli resistant to the given herbicide and antibiotic were obtained. Out of these 13 APM- and kanamycin-resistant hybrid plants (Table 1) were selected, four of them (NpAb-201, NpAb-202, NpAb-203 and NpAb-204) expressed morphological traits of *N. plumbaginifolia*, but nine were phenotypically related to *A. belladonna*.

As a result of γ-hybridisation of the *N. plumbaginifolia* and *N. sylvestris* protoplasts, 157 hybrid callus clones resistant to APM were selected (Table 1). Among them, 41 calli were able to regenerate, but only 17 of them could be rooted. The selected hybrids fully inherited the recipient's phenotype. After the fusion of γ-irradiated *N. plumbaginifolia* protoplasts with *A. belladonna* protoplasts, 64 hybrid callus colonies with resistance to APM were obtained (Table 1), 17 of them could be regenerated but only five hybrid plants could be rooted in vitro. The hybrids obtained were morphologically similar to *A. belladonna*. Having transferred these hybrids to a greenhouse, we noted that some of them (γNpAb-1 and γNpAb-2) had altered apical dominance, in that they did not grow normally and did not have the ability to flower. Additionally, the leaves of these plants were elongated and with twisted borders.

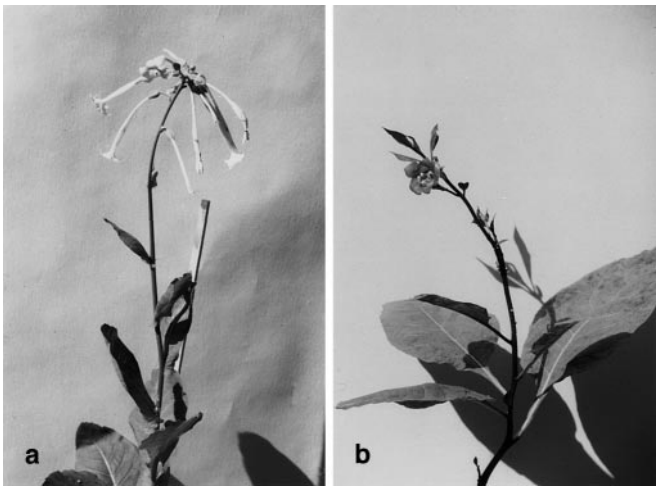

Fig. 1a, b Fertile APM-resistant somatic hybrids: **a** NpAb-107 hybrid obtained by symmetric fusion of *N. plumbaginifolia* and *A. belladonna* protoplasts; **b** γNpNs-3 hybrid obtained by asymmetric fusion of *N. plumbaginifolia* and *N. sylvestris* protoplasts

Primary screening of selected hybrid lines on APM resistance and cross resistance to trifluralin

An examination of the regeneration ability of hybrid and parental leaf explants on regeneration media with 5 µM APM was carried out as to obtain the first evidence of APM-resistance in all the hybrids obtained. Under these conditions many regenerants formed on *N. plumbagini-*

Table 1 Selection of somatic hybrids with APM-resistance

Fusion combination	Number of selected hybrid calli	Number of regenerating shoots on selective media	Number of hybrid plants picked up in the presence of APM
Symmetric hybrids			
N. plumbaginifolia apm^r(+) *N. sylvestris* Km^r	312	93	68
N. plumbaginifolia apm^r(+) *A. belladonna* Km^r	102	22	13
Asymmetric (γ-)hybrids			
γ*N. plumbaginifolia* apm^r(+) *N. sylvestris*	157	41	17
γ*N. plumbaginifolia* apm^r(+) *A. belladonna*	64	17	5

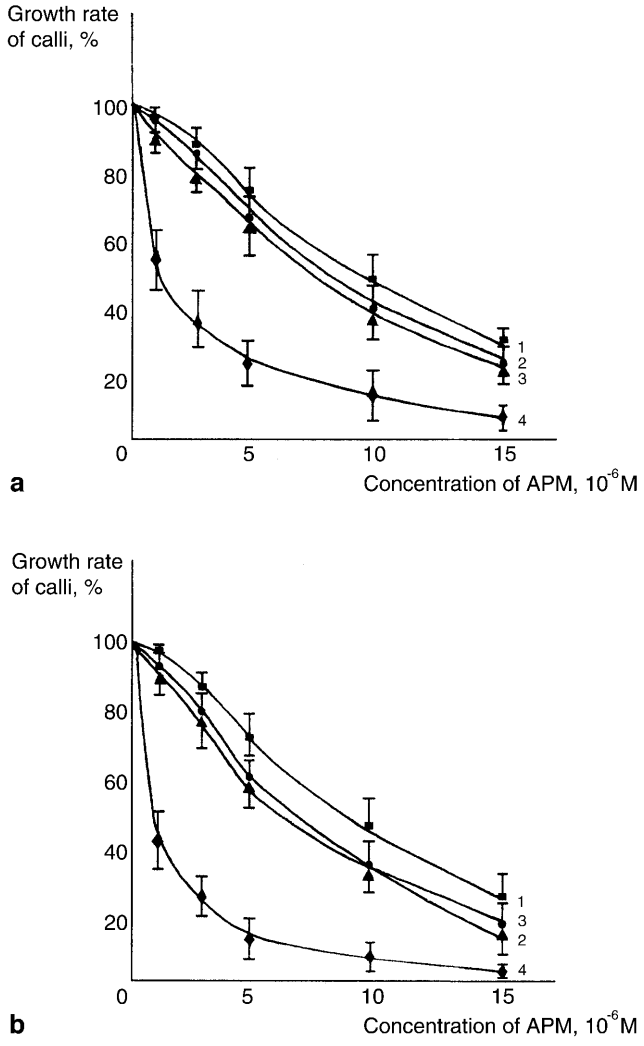

Fig. 2a, b Growth rates of calli (%) of parents and APM-resistant hybrids on media containing various concentrations of APM: **a** *1* APM-resistant *N. plumbaginifolia* mutant, *2* NpNs-1 hybrid, *3* γNpNs-3 hybrid, *4 N. sylvestris*; **b** *1* APM-resistant *N. plumbaginifolia* mutant, *2* NpAb-106 hybrid, *3* γNpAb-1 hybrid, *4 A. belladonna*

folia mutant and hybrid explants. They could grow normally as compared to *N. sylvestris* and *A. belladonna* explants, which blackened and then perished under the selective pressure.

APM resistance of hybrids was also confirmed by measuring the increase in their callus masses on media with different concentrations of herbicide (Fig. 2). Calli of hybrid and parental lines were placed on media containing from 1 µM to 15 µM APM. It was shown that the critical concentration (LD_{50}) for *N. sylvestris* callus cells was 1–1.5 µM (Fig. 2a); and for *A. belladonna* cells 0.8–1 µM of APM (Fig. 2b). For some interspecific hybrids the critical concentrations (LD_{50}) of APM were approximately 7–9 µM; and for intertribal hybrids and 7–8 µM. It was established that all the hybrids tested had a higher level of APM-resistance. Interspecific hybrids NpNs-1 and γNpNs-3 were 6–7 times more resistant to APM than *N. sylvestris* (Fig. 2a); the intertribal hybrids NpAb-106 and γNpAb-1 were nearly eight-times more resistant than *A. belladonna* (Fig. 2b).

We also observed that APM-resistant hybrids, obtained as a result of both symmetric and asymmetric fusion, were cross-resistant to trifluralin at a 12-µM concentration, which was critical for *N. plumbaginifolia*, *N. sylvestris* and *A. belladonna*. In the presence of 12-µM trifluralin, leaf explants of hybrids, similarly to APM-resistant *N. plumbaginifolia*, could generate shoots and the whole plants grew and developed normally.

Cytological analysis of hybrids

It was demonstrated that most of the *N. plumbaginifolia (+) N. sylvestris* hybrids selected after symmetric fusion contained 1–2 chromosomes of *N. plumbaginifolia* and a diploid chromosome set of *N. sylvestris* (Table 2). Some hybrids had a tetraploid set of *N. sylvestris*, and at most two *N. plumbaginifolia* chromosomes. Hybrid NpNs-12 contained 8–10 *N. plumbaginifolia* chromosomes and 24 chromosomes of *N. sylvestris*. Only one somatic hybrid,

Table 2 Chromosome sets of APM-resistant hybrids obtained by symmetric fusion

Somatic hybrid/parent[a]	Chromosome set	Chromosome number		
		N. plumbaginifolia	*N. sylvestris*	*A. belladonna*
NpNs-1	2n=24–25	1	23–24	
NpNs-2	2n=49–50	1–2	48	
NpNs-4	2n=50	2	48	
NpNs-5	2n=49–50	1–2	48	
NpNs-7	2n=48–50	1–2	47–48	
NpNs-8	2n=25–26	1–2	24	
NpNs-10	2n=25–26	1–2	24	
NpNs-12	2n=32–34	8–10	24	
NpNs-22	2n=44	20	24	
NpAb-106	2n=145–147	1–3		144
NpAb-107	2n=71–75	1–3		70–72
NpAb-202	2n=26	20		5–6
NpAb-204	2n=42	40		2
N. plumbaginifolia	2n=20	20		
N. sylvestris	2n=24		24	
A. belladonna	2n=72			72

[a] NpNs=APM-resistant somatic hybrids between *N. plumbaginifolia* and *N. sylvestris*; NpAb=APM-resistant somatic hybrids between *N. plumbaginifolia* and *A. belladonna*

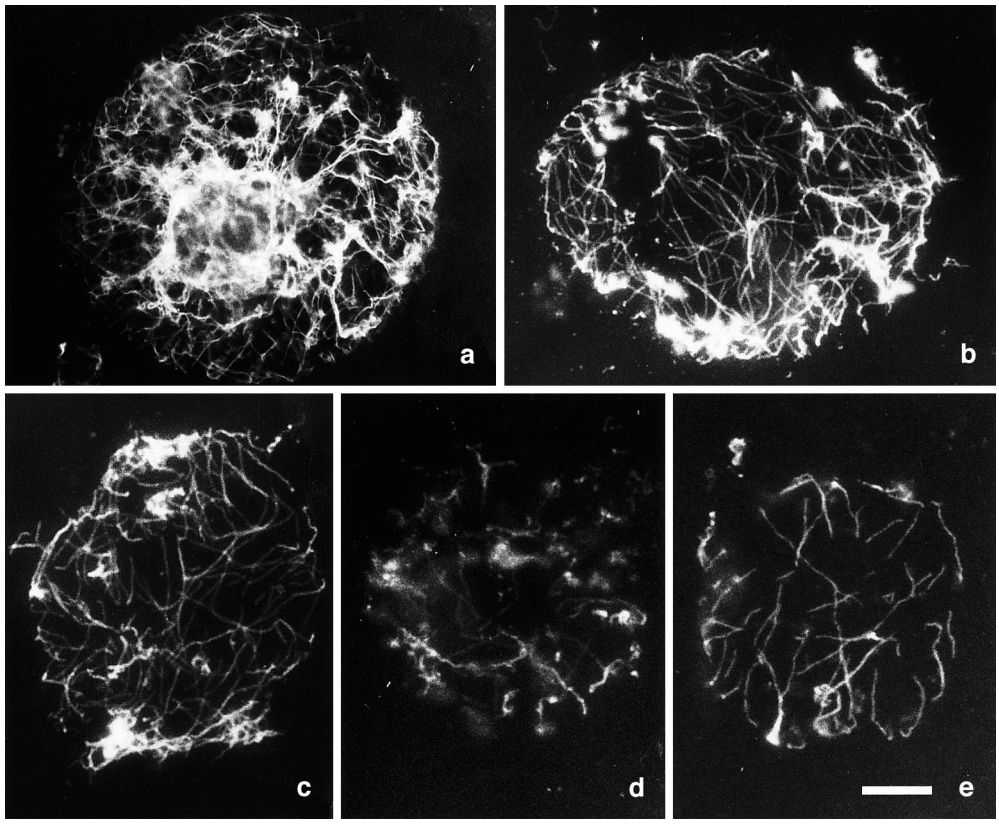

Fig. 3a–e The results of immunofluorescence microscopy of cortical MTs stained by monoclonal antibody TU-01 in protoplasts without APM-treatment (**a, c**) and after APM-treatment (**b, d, e**): **a, b** APM-resistant *N. plumbaginifolia* mutant; **c, d** *N. sylvestris*; **e** γNpNs-3 somatic hybrid. Bar=20 μm

Table 3 Chromosome sets of APM-resistant somatic γ-hybrids

Somatic hybrid/parent[a]	Chromosome set	Chromosome number		
		N. plumbaginifolia	*N. sylvestris*	*A. belladonna*
γNpNs-3	2n=25	1	24	
γNpNs-4	2n=49–50	1–2	48	
γNpNs-11	2n=25–26	1–2	24	
γNpNs-16	2n=49–51	1–3	48	
γNpAb-2	2n=146–149	2–5		144
γNpAb-4	2n=76–78	4–6		72
γNpAb-5	2n=147–148	3–4		144
N. plumbaginifolia	2n=20	20		
N. sylvestris	2n=24		24	
A. belladonna	2n=72			72

[a] γNpNs=APM-resistant somatic γ-hybrids between *N. plumbaginifolia* and *N. sylvestris*; γNpAb=APM-resistant somatic γ-hybrids between *N. plumbaginifolia* and *A. belladonna*

NpNs-22, from the clones analysed was completely symmetric, i.e. it contained a diploid chromosome set of the mutant (20 chromosomes) and a diploid chromosome set of *N. sylvestris* (24 chromosomes) (Table 2). All *N. plumbaginifolia (+) N. sylvestris* hybrids obtained after γ-hybridisation had no more than 2–3 chromosomes of the mutant, and diploid or tetraploid chromosome sets of *N. sylvestris* (Table 3).

As a result of the symmetric hybridisation of *N. plumbaginifolia* and *A. belladonna* protoplasts, morphologically different hybrids with different chromosome numbers were selected (Table 2). In particular, APM-resistant hybrids of NpAb-106 and NpAb-107 inherited an *A. belladonna* morphology and contained *Atropa* diploid or tetraploid chromosome sets and 1–3 chromosomes of *N. plumbaginifolia*. However, the NpAb-202 and NpAb-204 hybrids (morphologically similar to the mutant) contained diploid or tetraploid sets of *Nicotiana* chromosomes as well as some nightshade chromosomes (Table 2). γ-Hybrids of *N. plumbaginifolia (+) A. belladonna* possessed different numbers of chromosomes: 2 to 6 from *N. plumbaginifolia* and 72 or 144 *A. belladonna* chromosomes (Table 3).

Fertility of hybrids and inheritance of APM resistance in their first progeny

Initially, fertile hybrids after self-pollination were selected. Thirty five plants, including five parental lines and

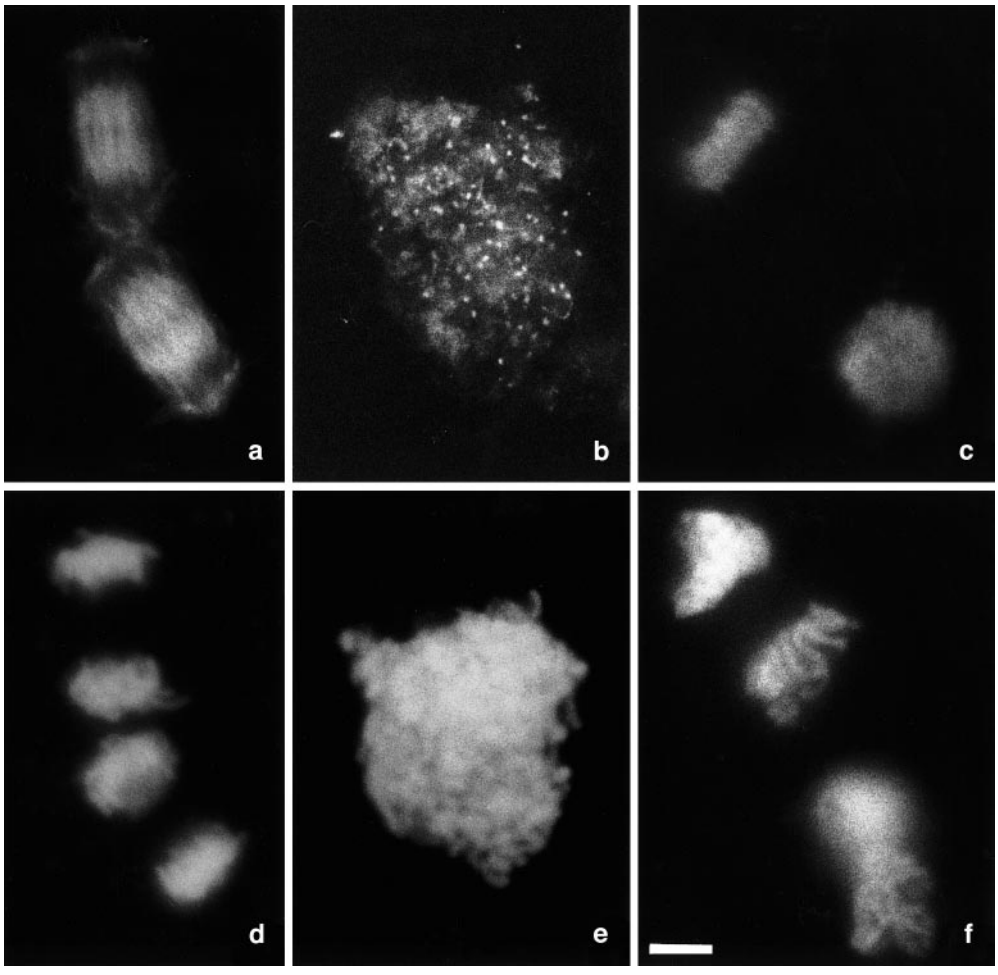

Fig. 4 The results of MT visualisation by monoclonal antibody TU-01 (**a, b, c**) and DNA staining by Hoechst 33258 (**d, e, f**) in regenerating protoplasts after APM-treatment: **a, d** APM-resistant *N. plumbaginifolia* mutant; **b, e** *N. sylvestris*; **c, f** γNpNs-3 somatic hybrid. Bar=10 μm

30 hybrid lines, were analysed. Among the analysed hybrid plants of *N. plumbaginifolia (+) N. sylvestris*, hybrids NpNs-4, NpNs-5, NpNs-7 and γNpNs-16 were infertile. It was established that the hybrid lines NpAb-204, γNpAb-2, γNpAb-5 of the *N. plumbaginifolia (+) A. belladonna* combination were also infertile. It was observed that hybrid NpAb-204, morphologically similar to *N. plumbaginifolia*, had a flower anomaly: four- or six-petal flowers formed, whereas *N. plumbaginifolia* had five-petal flowers.

Later on, the hybrid plants which were fertile after self-pollination, were cross-pollinated with the corresponding recipient line (*N. sylvestris* or *A. belladonna*). However, it was found that hybrids, especially intertribal hybrids, produced significantly less seeds after self-pollination and cross-pollination than parental plants. The seeds obtained after self-pollination and backcrossing (approximately 300 from each combination) were planted on media with 5 μM APM. Approximately 56.8–93.4% of the analysed seeds, obtained after self-pollination, could germinate on herbicide-free media, and from 43.2% to 78.2% of these seeds could germinate in the presence of APM. The percentage of germinated seeds, obtained after cross-pollination with *N. sylvestris*, was 32.7–87.8% on herbicide-free medium and 26.7–63.2% on the medium with APM, respectively. Seeds after backcrossing of the NpAb-107 hybrid with nightshade were not obtained. Seeds of *N. sylvestris* and *A. belladonna* could not be germinated in the presence of 5 μM APM.

The analysis of hybrids by immunofluorescent microscopy

The analysis of interspecific hybrids and parental plants showed that cortical MTs of APM-resistant *N. plumbaginifolia* (Fig. 3b) and APM-resistant *N. plumbaginifolia (+) N. sylvestris* protoplasts (Fig. 3e) preserved their native structure in interphase after APM treatment (5 μM, 2 h). However, the cortical MTs of *N. sylvestris* protoplasts were destroyed under these conditions (Fig. 3d).

We have also studied the sensitivity of the mitotic MTs of hybrids and parents and have shown that complete disruption of mitotic spindles of regenerating *N. sylvestris* protoplasts occurs after 2 h treatment with 5 μM APM. However, the mitotic structures of mutant and hybrid cells, like the cortical MT networks, were resistant to APM action and were not destroyed under these conditions. The results of MT visualisation by anti-

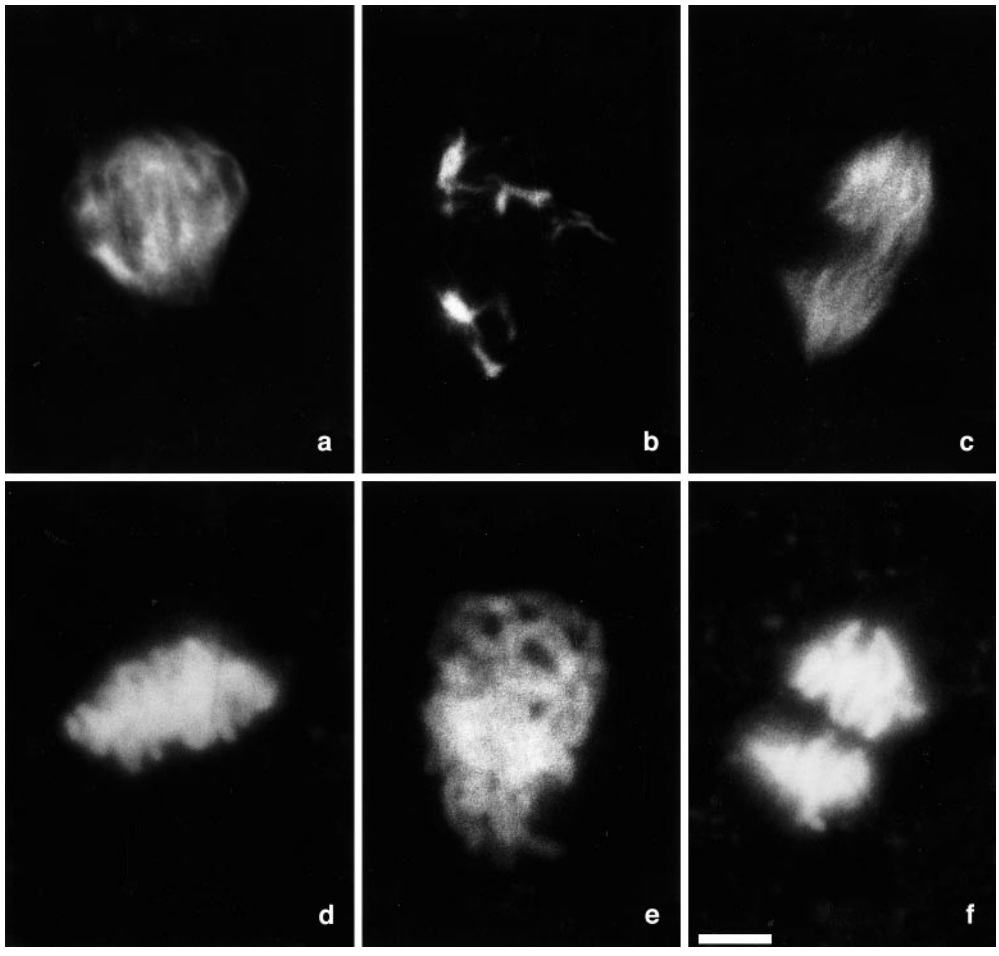

Fig. 5 The results of MT visualisation by monoclonal antibody TU-01 (**a, b, c**) and DNA staining by Hoechst 33258 (**d, e, f**) in regenerating protoplasts after APM-treatment: **a, d** APM-resistant *N. plumbaginifolia* mutant; **b, e** *A. belladonna*; **c, f** NpAb-107 somatic hybrid. Bar=10 μm

body and DNA staining by Hoechst 33258 in regenerated protoplasts of the APM-resistant mutant, *N. sylvestris,* and the APM-resistant hybrid, γNpNs-3, after APM treatment are presented in Fig. 4. It was demonstrated that the mitotic spindles of two neighbouring mutants' cells remained undamaged after the herbicide action (Fig. 4a). DNA location (chromosomes at the poles) in these cells is shown in Fig. 4d. Complete disruption of *N. sylvestris* cell mitotic spindles after APM treatment and, as consequence of this, the chaotic disposition of genetic material in the cells, is demonstrated in Fig. 4b and e, respectively. The mitotic structures of regenerating protoplasts of the γNpNs-3 hybrid were insensitive to APM action. In particular, undamaged phragmoplasts of γNpNs-3 cells, as seen from various positions, are shown on Fig. 4c; the DNA disposition of these cells is given in Fig. 4f.

Analysis of intertribal *N. plumbaginifolia+A. belladonna* hybrids and the recipient line of *A. belladonna* also demonstrated the full disruption of mitotic MTs in nightshade regenerating protoplasts after 2 h of APM-treatment (Fig. 5b). As shown in Fig. 5a and c the dividing cells of APM-resistant *N. plumbaginifolia* and the NpAb-107 hybrid did not remain in prometaphase and kept the normal form of the mitotic spindle. The analysed protoplasts were also stained additionally by Hoechst for the detection of DNA disposition in cells (Fig. 5d, e, and f).

Biochemical analysis of hybrid lines

Tubulin isoforms of parental and hybrid plants were compared by two-dimensional electrophoresis (Fig. 6) and identified as designated on the electrophoregrams by following immunoblotting using the monoclonal antibodies against α- and β-tubulins (TU-01 and TU-06, respectively). Changes in the polypeptide composition were easily detectable in stained gels. Although no apparent alterations in the molecular weight of the α- and β-tubulin of the APM-resistant mutant were observed, some part of the β-tubulin from the mutant line possessed a more acidic pH than those from control plants (Fig. 6a, b). Immunoblotting with the above-mentioned antibodies confirmed these results (data not shown). The analysis of tubulin constitution from the hybrid lines has also revealed additional altered β-tubulin isoforms typical for the *N. plumbaginifolia* mutant coincidental with α- and β-tubulin isoforms of the recipient in analysed interspecific and intertribal hybrids. For example, the position of the altered β-tubulin isoform (*β-tubulin) of an APM-resistant mutant, compared with other tubulin iso-

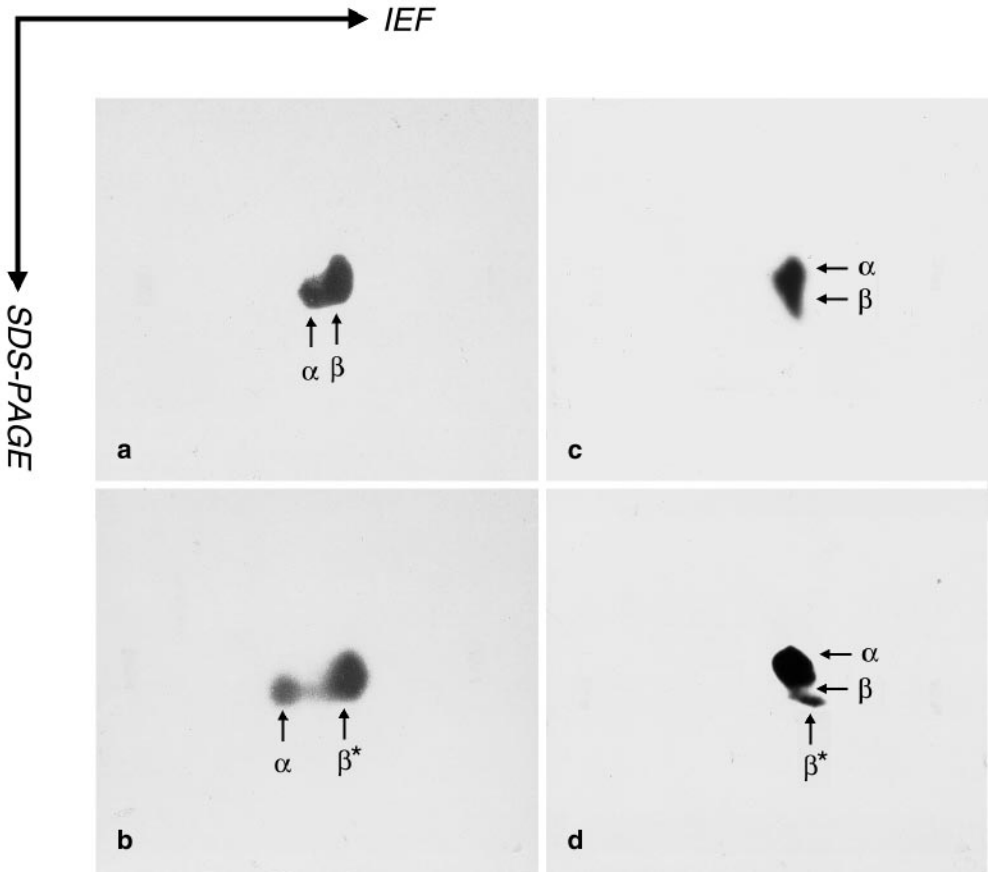

Fig. 6 The results of two-dimensional electrophoresis (Coomassie Brilliant Blue-stained gels) of tubulins isolated from: **a** control APM-sensitive *N. plumbaginifolia*; **b** APM-resistant *N. plumbaginifolia* mutant; **c** *A. belladonna*; **d** NpAb-107 somatic hybrid. Only areas of electrophoregrams containing tubulins are shown

forms in *N. plumbaginifolia (+) A. belladonna* hybrid (NpAb-107) resistant to APM, is shown in Fig. 6d. *β-Tubulin has a more acidic isoelectric point (Fig. 6d).

Discussion

The transfer of APM resistance from the *N. plumbaginifolia* mutant into *N. sylvestris* did not cause particular complications, since there are a number of references to the somatic hybridisation of *Nicotiana* (Spangenberg et al. 1990; Donaldson et al. 1993; Hung et al. 1993; Trick et al. 1994, Trick and Bates1996). Almost all APM-resistant hybrids, resulting from symmetric fusion, were morphologically similar to *N. sylvestris* (analogous leaf forms, flower structure, etc.), although some of them possessed intermediate phenotypes combining the morphological traits of both species, which confirmed the results on the selection of somatic hybrids in this combination (Hung et al. 1993). It is known that, after symmetric somatic hybridisation of different plant species, spontaneous nuclear and cytoplasmic material elimination occurs in fusion products. Since in experiments with symmetric fusion the hybrids with intermediate or *N. sylvestris* morphology were selected, we can deduce, perhaps, that in *N. plumbaginifolia (+) N. sylvestris* hybrids the elimination of *N. plumbaginifolia* nuclear- or plasmagenes prevailed.

To achieve the partially directed transfer of nuclear material, inactivation of one of the partners in the fusion combination by lethal or super-lethal γ-ray, x-ray or UV doses is usually employed (Gleba and Shlumukov 1990). In our experiments we γ-irradiated *N. plumbaginifolia* protoplasts with a dose of 200 Gy, taking into account the results of earlier work on asymmetric somatic hybridisation in *Nicotiana* (Bates et al. 1987). As a result of γ-hybridisation all *N. plumbaginifolia (+) N. sylvestris* hybrid plants inherited the recipient phenotype.

The transfer of APM resistance from the *N. plumbaginifolia* mutant to remote species of *A. belladonna* by somatic hybridisation was more complicated, as these plants belong to different tribes of the family *Solanaceae*. *N. plumbaginifolia* belongs to the *Cestreae* tribe of the *Cestroideae* subfamily, and *A. belladonna* belongs to the *Lycineae* tribe of the *Solanoideae* subfamily (D'Arcy 1979). It is known that after fusion of protoplasts of remote species an alteration in the differentiation processes and in cell regeneration often occurs due to a misbalance of the genomes or to the nuclear-cytoplasmic incompatibility of the species. Although *N. plumbaginifolia* and *A. belladonna* plants are phylogenetic remote, they are characterised by somatic congruency (Babiychuk et al. 1992).

In our experiments, after symmetric hybridisation of protoplasts of APM-resistant *N. plumbaginifolia* and kanamycin-resistant *A. belladonna,* hybrids with *Nicoti-*

ana and nightshade morphology were found as indicated above. In this case it can be suggested that, as the direct advantageous elimination of nuclear material or plasmagenes of one of the partners did not occur in *N. plumbaginifolia (+) A. belladonna* hybrids, it is perhaps more an accidental rather than a regular process. All intertribal hybrids obtained after γ-hybridisation inherited the *A. belladonna* morphology, as would be expected after such hybridisation. We note that these are the first results on asymmetric somatic hybridisation where *A. belladonna* was used as a recipient and *Nicotiana* as a donor.

The next step in our investigation was the primary test of APM resistance in interspecific and intertribal hybrids, which included verification of the regeneration ability of hybrid leaf explants in the presence of 5 μM APM. It was established that the shoots formed only on hybrid and *N. plumbaginifolia* mutant explants under herbicide pressure. This confirmed the results on the regeneration ability of *N. plumbaginifolia* mutant leaf explants in the presence of APM, published earlier by Blume et al. (1998). It was also established that calli of some of the analysed hybrids were 6–8 times more resistant to APM as compared with *N. sylvestris* and *A. belladonna*. We can presume that the resistance of hybrid lines involves the transfer of APM resistance from the *N. plumbaginifolia* mutant.

The availability of APM-resistant somatic hybrids prompted studies to determine the cross-resistance to other herbicides dissimilar to APM, but which also disrupt mitosis. Such compounds could be the dinitroaniline herbicides, which are widely used in agriculture. The molecular interaction of dinitroanilines with microtubule proteins has been extensively studied. Some of the reports indicate that dinitroanilines and phosphorothioamidates share a common target site, which involves tubulins (Morejohn and Fosket 1991; Ellis et al. 1994). We have shown that all hybrids selected by us were cross-resistant to trifluralin, like APM-resistant *N. plumbaginifolia* mutants with altered β-tubulin (Blume et al. 1998).

Subsequent cytological analysis of the root-tip meristematic cells of regenerated plants was aimed at establishing direct evidence for the hybrid nature of selected lines. In these experiments we took into account that, in the processes of culturing and plant regeneration, the chromosomes of somatic hybrids, in particular, can undergo strong reconstruction and significant changes in comparison with the chromosomes of the initial plant species (Larkin and Scowcroft 1981). In our case cytological analysis was facilitated by the fact that the chromosomes of *N. plumbaginifolia*, *N. sylvestris* and *A. belladonna* are very different visually, and these differences were used for the definition of the genomic constitution of hybrids. As described earlier, the *N. plumbaginifolia* diploid has 20 telocentric or acrocentric different-sized chromosomes (Hung et al. 1993), and diploid cells of *A. belladonna* have 72 metacentric chromosomes, which are significantly shorter and thinner than those of *N. plumbaginifolia* (Gleba et al. 1988).

The results of cytological analysis showed that APM-resistant *N. plumbaginifolia (+) N. sylvestris* somatic hybrids regenerated after symmetric fusion (with the exception of the NpNs-22 hybrid) inherited a small donor chromosome number and a diploid or tetraploid recipient chromosome set. These data are also evidence for the preferable segregation of *N. plumbaginifolia* chromosomes in this fusion combination. Our assumption is supported by known data on the spontaneous genome elimination of the *N. plumbaginifolia* donor (from 33% to 67%) on the selection of high asymmetric hybrids between *N. plumbaginifolia* and *N. tabacum* without any pre-treatment of *N. plumbaginifolia* cells (Agoudgil et al. 1990). The hybrids in the same combination produced by γ-hybridisation inherited no more than 1–3 *N. plumbaginifolia* chromosomes in all cases. But this regularly lowered inheritance of donor material can be considered as a result of the damage of most chromosomes following γ-irradiation of the respective protoplasts.

Intertribal hybrids obtained after symmetric fusion had a different morphology and different chromosome sets, as described above. In our experiments the presence of different chromosome sets of *N. plumbaginifolia (+) A. belladonna* could perhaps provide evidence that the preferential chromosome elimination of either partner in this fusion combination does not take place, and is more likely to be an accidental rather than a systematic process. Nevertheless, only a small number of alien chromosomes is necessary for further normal growth of these hybrid plants: in the case of the NpAb-202 and NpAb-204 hybrids, 2 or 5–6 *A. belladonna* chromosomes respectively; for the NpAb-106 and NpAb-107 hybrids 1–3 *N. plumbaginifolia* chromosomes. But all analysed γ-hybrids of *N. plumbaginifolia (+) A. belladonna* inherited only a small number of *N. plumbaginifolia* chromosomes. Perhaps, some of the selected γ-hybrids have also inherited fragments of the *N. plumbaginifolia* chromosomes, rather than complete donor chromosomes, which would be of interest to study further. The results obtained show that γ-hybridisation is a preferable way to transfer the definitive trait controlled by uncloned nuclear gene(s) from one plant to another.

We estimated that some analysed hybrids were infertile. As a rule, such hybrids are characterised by large numbers of chromosomes, which is, perhaps, the main reason for their inability to function normally and form reproductive organs. All the infertile interspecific hybrids analysed (NpNs-4, NpNs-5, NpNs-7 and γNpNs-16) had tetraploid chromosome sets of *N. sylvestris* and 1–3 chromosomes of *N. plumbaginifolia*. The infertile intertribal hybrids γNpAb-2 and γNpAb-5 also possessed large numbers of chromosomes with tetraploid recipient sets. The seeds obtained after self- and cross-pollination of the hybrids had a comparatively high germination rate in the presence of APM. As reported earlier by Blume et al. (1998), APM resistance in respective *N. plumbaginifolia* mutants is inherited as a nuclear dominant homozygotic trait. Inheritance of this trait in the first progeny of hybrids is obviously a complicated

process. It might involve an anomalous chromosome status in some hybrids, the absence in some cases of homologous pairs of chromosomes, transferred from *N. plumbaginifolia*, or complicated alterations occurring during meiotic cell divisions in the hybrids' reproductive organs. It was very difficult to evaluate the type of segregation after seed germination of the analysed hybrids, because almost each of the hybrids had its own individual index. However, it was demonstrated that APM-resistance in fertile hybrids could be inherited in their first progeny.

It is known that APM binds effectively with isolated plant tubulin and inhibits the polymerisation of tubulin in MTs in vitro (Morejohn and Fosket 1991). Immunofluorescence microscopy of the MT arrays in the interphase and mitotic cells of *N. plumbaginifolia (+) N. sylvestris* and *N. plumbaginifolia (+) A. belladonna* hybrids pre-treated with APM was therefore carried out to determine the resistance of their MTs to APM action. The initial tests showed that cortical MTs of interphase protoplasts of the APM-resistant *N. plumbaginifolia* mutant, as well as both interspecific and intertribal hybrids, were resistant to treatment with a selective concentration of APM. Similar results for the initial mutant were presented earlier (Blume et al.1998) where it was shown that this stability of the MT network is directly related to the appearance of an altered β-tubulin subunit (Blume et al. 1998). The cortical MTs of *N. sylvestris* and *A. belladonna* protoplasts were completely destroyed by such a treatment, a typical result for cells of many plant species susceptible to APM. It is known that 1–3 μM of this herbicide can completely de-polymerise both cortical and mitotic MT arrays in cells of such plants within 1 h (Falconer and Seagull 1987).

Some observations indicated that mitotic arrays, such as the pre-prophase band, the division spindle and phragmoplast, are more sensitive to anti-MT compounds' action (Hoffman and Vaughn 1994), so the next step of our research was aimed at studying the APM influence on the MTs of regenerating protoplasts of hybrids and their parents. As was shown, the mitotic arrays of interspecific and intertribal hybrids cells, like those of mutant cells, were resistant and not injured after 2 h of APM treatment (Figs. 4 and 5), as compared with *N. sylvestris* and *A. belladonna.*

The results of the immunofluorescent analysis indicate that the cortical and mitotic MTs of APM-resistant hybrid lines are resistant to APM action, unlike those of recipient lines of *N. sylvestris* and *A. belladonna*. The resistance of the MTs in the hybrids can be explained by the availability of mutant β-tubulin subunit in the composition of their arrays (as in the case of the *N. plumbaginifolia* mutant), which could be expressed as a consequence of mutant β-tubulin transfer from the APM-resistant *N. plumbaginifolia* line.

In order to reveal the mutant β-tubulin isoform in the MTs of *N. plumbaginifolia (+) N. sylvestris* and *N. plumbaginifolia (+) A. belladonna* the purified tubulins from these plants and parental lines have been resolved by two-dimensional electrophoresis with subsequent identification of the respective isoforms by immunoblotting. It has been demonstrated that the analysed hybrids possessed an additional β-tubulin subunit isoform, which in electrophoretic mobility and isoelectric point was related to the mutant β-tubulin isoform of APM-resistant *N. plumbaginifolia* (Blume et al. 1998). The appearance of this additional β-tubulin isoform on the tubulin composition of hybrids could therefore be connected with the transfer of the mutant β-tubulin gene from the *N. plumbaginifolia* donor and its subsequent expression. We can also assume that all interspecific and intertribal hybrids obtained after symmetric and asymmetric hybridisation inherited a mutant β-tubulin gene, which provided the APM resistance of the hybrid plants.

The present work has thus demonstrated the possibility of APM resistance transfer from the respective *N. plumbaginifolia* mutant with an altered β-tubulin to *N. sylvestris* (related species) and *A. belladonna* (remote species) by two methods of somatic hybridisation: symmetric and asymmetric fusion. Both provide for the successful transfer of herbicide resistance into the given plant species. We can conclude that asymmetric hybridisation using γ-irradiation can be the most effective method for the transfer of the APM-resistance trait from the *N. plumbaginifolia* mutant to another plant species. In future the hybrids which inherit the minimal chromosome number from APM-resistant *N. plumbaginifolia*, especially those which possess only one chromosome, could be used for the identification and cloning of the β-tubulin mutant gene (it is easier to identify the mutant gene on a donor plant chromosome and to isolate it) with the purpose of transferring it into important crops in order to obtain plant varieties with resistance to a given herbicide.

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