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Alteration of β-tubulin in *Nicotiana plumbaginifolia* confers resistance to amiprophos-methyl

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Abstract A *Nicotiana plumbaginifolia* plant (apm5^r) resistant to amiprophos-methyl (APM), a phosphoroamide herbicide, was isolated from protoplasts prepared from leaves of haploid plants. Genetic analysis revealed that the resistance is coded for by a dominant nuclear mutation and is associated with the increased stability of cortical microtubules. Two-dimensional polyacrylamide-gel electrophoresis, combined with immunoblotting using anti-tubulin monoclonal antibodies, showed that part of the β-tubulin in the resistant plant possessed lower isoelectric points than the β-tubulin of susceptible wild-type plants. These results provide evidence that the resistance to APM is associated with a mutation in a β-tubulin gene. The APM-resistant line showed cross-resistance to trifluralin, a dinitroaniline herbicide, suggesting a common mechanism of resistance between these two classes of herbicides.

Key words Amiprophos-methyl · Resistance · Mutation · β-Tubulin · Microtubules

Introduction

Microtubules are dynamic filamentous polymers of the cytoskeleton in eucaryotic cells composed mainly of tubulin, a heterodimeric protein with similar α- and β-subunits each of about 50 kDa (Luduena et al. 1992). Microtubules are involved in a wide spectrum of cellular processes in plant cells, including cell polarization, cell-wall deposition, intracellular transport and cell division (Goddard et al. 1994). The organization of plant microtubules is highly sensitive to physiological and environmental signals; however the precise mechanisms which drive microtubular dynamics and functions are not well understood.

New data regarding microtubular organization and function can be obtained using antimicrotubular drugs, i.e. compounds which induce the disassembly of microtubules and disrupt mitosis. Several classes of antimicrotubular drugs have been described to date: colchicine and its derivatives, the vinca-alkaloids vinblastine and vincristine, and the dinitroaniline and phosphoroamide herbicides (Morejohn and Fosket 1991; Blume and Strashnyuk 1993). The last two classes are the most effective inhibitors of plant tubulin polymerization. Both of these classes act in the micromolar range of concentration and show a higher binding affinity for plant tubulins than for animal tubulins (Morejohn and Fosket 1984, 1991; Morejohn et al. 1987; Murthy et al. 1994). It has been postulated that both dinitroaniline and phosphoroamide herbicides may share a common binding site on the tubulin molecule (Ellis et al. 1994).

The use of mutant forms of microtubular proteins has been a very useful way to obtain information about the structure and different functions of microtubules and the roles of the tubulin domains in protists, fungi and mammalian cells (Oakley 1985; Blume and Strashnyuk 1993). The most widely used approach for obtaining such mutants has been the selection of cell lines resistant to antimitotic compounds (colchicine, colcemide, griseofulvin, etc.). An example of a such mutants for lower plants was the isolation of a colchicine-resistant *Chlamydomonas reinhardtii* line containing mutant tubulin polypeptides (Bolduc et al. 1988; Lee and Huang 1990). Examples of dinitroaniline resistance for higher

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plants have been described for carrot (Vaughan and Vaughn 1988) and goosegrass *Eleusine indica* (Vaughn et al. 1987). However, the molecular mechanisms of resistance in both cases still remain obscure. For example, no differences were found among tubulin genes (Mysore and Baird 1995), as well as tubulin isotypes (Waldin et al. 1992), of resistant and susceptible goosegrass. To date, no attempts have been reported to obtain such mutants *in vitro* using other species.

In this paper, we report the isolation of a *Nicotiana plumbaginifolia* line (apm5^r) resistant to 5 μM of amiprophos-methyl (APM) using leaf protoplasts and calli tissues as starting materials and gamma-irradiation as a mutagen. Genetic analysis revealed that the resistance is encoded by a dominant nuclear mutation. The cortical network of microtubules and the mitotic spindles were unaffected by 5 μM of APM in the resistant line. Two-dimensional polyacrylamide gel electrophoresis showed alterations in the pI of part of the β-tubulin subunits, which may be responsible for the observed resistance of this plant to APM.

Materials and methods

Plant material

The *N. plumbaginifolia* haploid line was used in all experiments described here. The plants were propagated in growth-room conditions at 22–25°C with white-cool illumination, and a 12–14 h photoperiod. For callus induction leaf explants of 3–5 mm width were cultivated on Murashige-Skoog's (MS) medium (Murashige and Skoog 1962) containing 1 mg/l of BAP and 1 mg/l of NAA at 25°C in the dark.

Drugs

Amiprophos-methyl (APM), also known as Bay NTN 6867, was obtained from Dr. J.R. Bloomberg (Miles Inc., Agriculture Division, Kansas City, USA). Trifluralin (a,a,a-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine) was obtained from Dr. L. Guse (Elanco Products Company, Greenfield, Indiana, USA). Stock solutions of herbicides were prepared in dimethyl sulfoxide, stored at − 20°C, and were added immediately before use to autoclaved media.

Protoplast isolation, mutagenesis and selection of APM-resistant lines

Isolation and incubation of protoplasts were performed according Gleba et al. (1984). Leaves excised from aseptic haploid *N. plumbaginifolia* plants were placed in a solution containing 0.5% Onozuka R-10, 0.5% Macerozyme R-10, 0.5% Cellulase, 0.5 M sucrose, 0.1 M $CaCl_2$ and incubated for 15–18 h at 25°C in the dark. Thereafter protoplasts were filtered through a nylon cloth, suspended by gentle shaking (100 g, 5 min) and washed twice in W5 solution (Menczel et al. 1981). Washed protoplasts were quantified using a hemacytometer, diluted to a concentration of $3–5 \times 10^4$ per ml in 8p medium (Kao and Michayluk, 1975) and incubated in diffuse light (100–400 Lk) at 25–27°C until the development of cell clusters. The cell suspension was then mounted in 0.7–0.8% agar dissolved in the 8p medium containing 0.2 M glucose, 1 mg/l of BAP and 0.1 mg/l of NAA and transferred to the growth room. Greened microcalli were transplanted on RMOP medium (Sidorov et al. 1981) for shoot induction. Thereafter root formation was induced on hormone-free MS medium or with 0.1 mg/l of NAA.

Freshly isolated protoplasts were mutagenized by gamma-irradiation at a dose of 15 Gy (^{60}Co, 0.18 Gy/s). The mutagenized protoplasts were washed in 8p medium and regenerated as described above. APM was added to the cell-cluster suspension 1 week after mutagenesis (when about 3–5 cell divisions have occurred). Plants regenerated in the presence of 3–10 μM of APM were propagated on hormone-free MS medium and used in initial tests for resistance to the herbicide.

Calli tissues were gamma-irradiated with a dose of 15 Gy and incubated on MS medium supplied with 1 mg/l of BAP, 1 mg/l of NAA and 3–10 μM of APM. Resistant clones were regenerated on APM-containing RMOP medium and then planted on MS medium as described above.

The initial tests for the APM resistance and cross resistance to trifluralin were performed using leaf explants derived from wild-type and mutagenized *N. plumbaginifolia* plants. For the tests they were planted on RMOP medium containing APM or trifluralin. The APM-resistance of wild type and selected plants were determined by a comparison of the growth rates of their calli in terms of their fresh weight after 4 weeks of growth on the medium with 5 μM of APM.

Genetic crosses

Plants resulting from genetic crosses were grown under greenhouse conditions (15–16 h photoperiod, and temperature of 21–27°C). Seeds from self-pollinated and cross-pollinated plants were sterilised in 3% H_2O_2 for 3 h, washed twice in sterilised distilled water and germinated onto hormone-free MS medium containing 5 μM of APM. Five repetitions of all experiments were made and in every variant more than 250 seeds were employed. Seed germination and the ability of seedlings to grow on the medium with 5 μM of APM were considered to provide evidence of APM-resistance.

Immunofluorescence microscopy

Procedures for the immunofluorescence staining of microtubules in protoplasts were adapted from Wick et al. (1981). All incubation and washing steps were carried out at room temperature. Briefly, protoplasts regenerated for 3–4 days were washed once in microtubule-stabilizing buffer (MTSB), containing 25 mM of PIPES-KOH, pH 6.9, 2 mM of EGTA, 1 mM of $MgS0_4$ and 0.2 M glucose, adhered for 3 min to poly-L-lysine-coated coverslips and fixed for 30 min in freshly prepared 3.7% (w/v) paraformaldehyde in MTSB, containing 1% (v/v) Triton X-100. The coverslips were then treated for 30 min with 0.5% (w/v) bovine serum albumin (BSA) in PBS (pH 7.0) to prevent non-specific binding of antibodies. The coverslips were incubated for 45 min with the anti-α-tubulin mouse monoclonal antibody TU-01 (Viklicky et al. 1982), diluted 1/500 in PBS containing 3% BSA. The coverslips were then washed 3-times, 5-min each, in PBS and incubated for 45 min with secondary FITC-conjugated anti-mouse antibody diluted 1/50 in PBS buffer containing 3% BSA. Cellular DNA was stained with 0.04% (w/v) Hoechst 33258 solution in PBS for 10 min. The coverslips were briefly washed in distilled water, mounted in MOWIOL 4.88 and examined with a JENALUMAR-a fluorescence microscope equipped with a 100/1.30 Planachromat fluorescence oil-immersion objective.

Preparation of tubulin samples

Tubulin from young leaves of *N. plumbaginifolia* was purified by DEAE-chromatography according Morejohn and Fosket (1982). Freshly excised leaves were crushed in liquid nitrogen and homogenized in a glass/teflon homogenizer in PM buffer (50 mM PIPES-KOH, pH 6.9, 1 mM EGTA, 0,5 mM $MgCl_2$, 2 mM GTP, 1 mM dithiotreitol), containing 0.5 mM of PMSF and 2 μM of leupeptin as a protease inhibitor. Generally, 10 ml of PM buffer per gram of fresh-leaf mass were added. The homogenate was filtered through a nylon-cloth filter and centrifuged at 10000 g for 5 min at 4°C and, thereafter at 20000 g for 30 min at 4°C. Protein quantification in the final cell extract was performed according to Bradford (1976). The cytoplasmic extract containing 4 mg of protein per ml of extract was loaded onto a DEAE-Sephadex A50 column (2.5 × 15 cm) pre-equilibrated with PM buffer to recover the tubulin-containing fraction. Unbound and weakly bound proteins were eluted with three bed volumes of 0.375 M KCI solution in PM buffer. Tightly bound proteins containing at least 75% of tubulin (as estimated by SDS-PAGE) were eluted with three bed volumes of 0.8 M KCI solution in PM buffer. The tubulin-containing fractions were precipitated by 60% $(NH_4)_2SO_4$ for 60 min. Precipitates were de-salted on a Sephadex G25 mini-column and mixed 1:1 with a two-times concentrated sample buffer containing 1.2% (w/v) Ampholine pH 3–10 (LKB Pharmacia, Uppsala, Sweden) and 2.8% (w/v) Ampholine pH 4–6.5. The samples were stored in liquid nitrogen prior electrophoresis.

Two-dimensional polyacrilamide-gel electrophoresis and immunoblotting

Two-dimensional electrophoresis was performed as described by O'Farrell (1975). Isoelectric focusing was run in vertical 0.75-mm-thick tubes at 400 V during 17 h and then at 600 V during 3 h at room temperature. The gels contained 4% (w/v) acrylamide, 0.1% (w/v) N,N′-methylene-bis-acrylamide, 1% Nonidet P-40, 9 M urea, 0.5% Ampholines pH 3–10 and 2% Ampholine pH 4–6. The catholyte was a 0.5 M solution of histidine in 1 M NaOH and the anolyte was a 0.3 M solution of glutamic acid in 1 M H_3PO_4. After isoelectric focusing the gels were washed for 10 min in equilibration buffer (2.3% SDS, 62.5 mM Tris-HCl, pH 6.8) and laid at the top of a 5% stacking gel/10% resolving polyacrylamide gel. Sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) was run at a constant voltage (100 V during the first 30 min and then at 200 V). Gels were stained overnight with 0.025% Coomassie Brilliant Blue R-250 in 50% ethanol and 10% acetic acid, destained in 7% acetic acid, and photographed through a yellow filter. Alternatively, for immunoblotting experiments proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (Towbin et al. 1979) and the membrane incubated with the mouse monoclonal antibodies TU-01 (anti-α-tubulin) and TU-06 (anti-β-tubulin) (Draber et al. 1989). Details of the immunoblotting technique are described elsewhere (Harlow and Lane 1988). Goat anti-mouse alkaline phosphatase-conjugate (Promega) was used as a secondary antibody.

Results

Selection of APM-resistant lines

Wild-type haploid *N. plumbaginifolia* plants are highly sensitive to the phosphoroamide herbicide APM. Treatment of *N. plumbaginifolia* cells with 3 μM of APM resulted in a 80% inhibition of callus growth, while concentrations around 5 μM completely inhibited an increase of cell mass (Fig. 1). APM-resistant *N. plumbaginifolia* plants were selected on 3–15 μM of APM using callus tissues and protoplasts as sources of plant material (Tables 1 and 2). Cell lines showing resistance to up to 10 μM were initially obtained (Tables 1 and 2); however, those selected at a APM concentration higher than 5 μM were incapable of

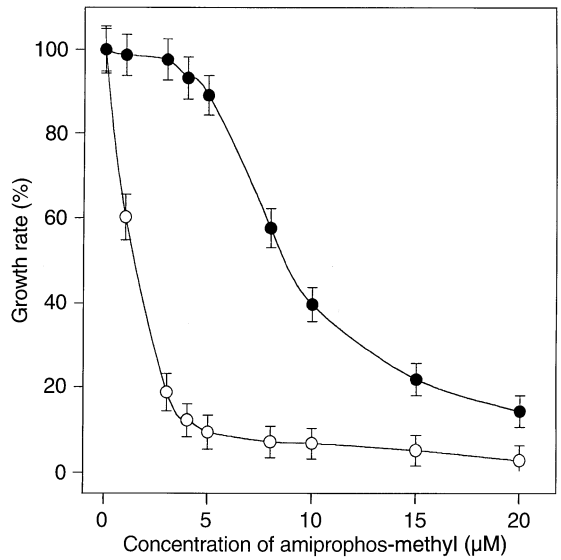

Fig. 1 Growth rates of *N. plumbaginifolia* wild type (–○–) and apm5^r (–●–) callus on MS medium containing various concentrations of APM

Table 1 Selection of APM-resistant lines from *N. plumbaginifolia* protoplasts

APM concentration (μM)	Number of selected lines	Number of lines regenerating shoots in the presence of APM
3	5	2
4	3	1
5	3	1
8	2	–
10	1	–
15	–	–

Table 2 Selection of APM-resistant lines from *N. plumbaginifolia* callus tissue

APM concentration (μM)	Number of selected lines	Number of lines regenerating shoots in the presence of APM
3	8	2
4	6	2
5	5	1
8	2	–
10	1	–
15	–	–

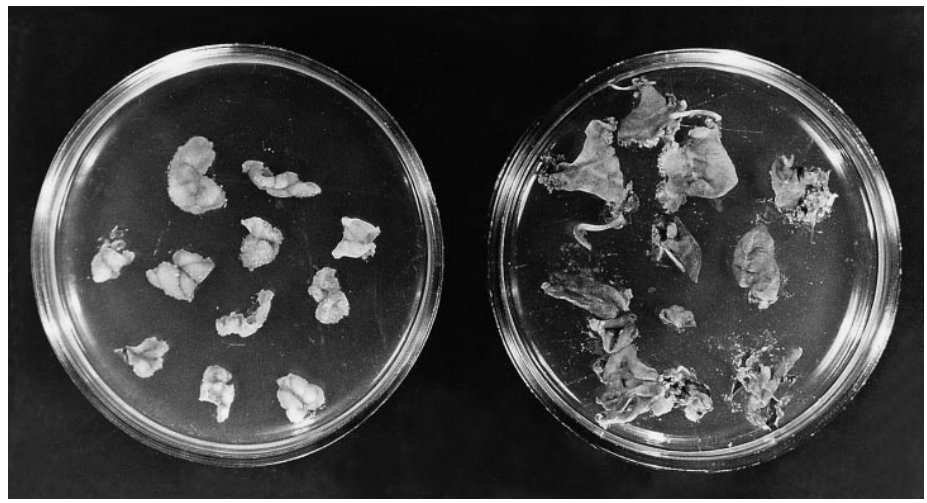

Fig. 2 Regeneration of leaf explants from wild type (left) and from apm5^r (right) plants on RMOP medium containing 5 μM of APM

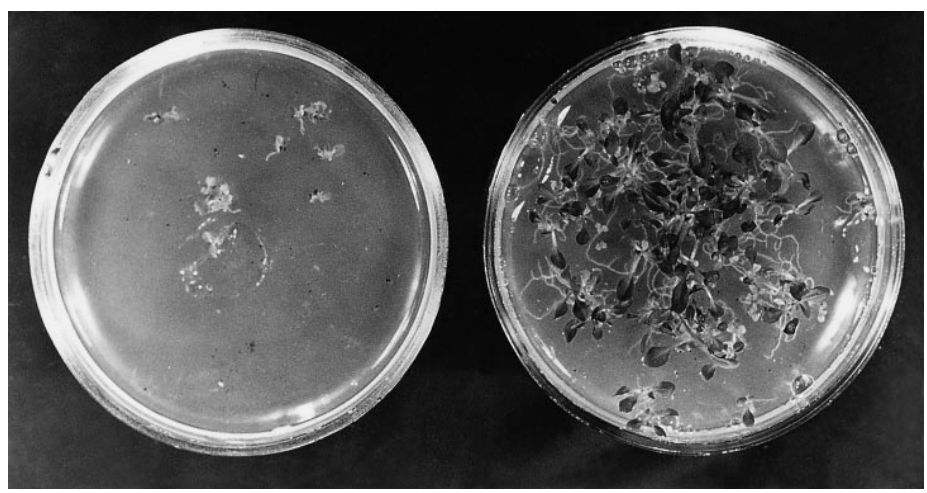

Fig. 3 Germination of seeds and growth of seedlings obtained from self-pollinated wild type (left) or self-pollinated apm5^r (right) *N. plumbaginifolia* plants on hormone-free MS medium containing 5 μM of APM

shoot and/or root regeneration (Tables 1 and 2). As a result of selection using leaf protoplasts as a starting material, four lines were obtained able to regenerate on medium with 3–5 μM of APM. The regenerated plants were indistinguishable from wild type plants. The mutation frequency was $2.2–3.8 \times 10^{-7}$. In the case of selection using callus tissue as a starting material, five lines were obtained, capable of regenerating on medium with 3–5 μM of APM. In this case the mutation frequency was $3.3–6.7 \times 10^{-6}$. One of the lines resistant to 5 μM of APM, apm5^r, and showing more rapid growth and regeneration potential, was analysed further. Leaf explants from this line were regenerated on RMOP medium containing 5 μM of APM after several passages *in vitro* (Fig. 2). The resulting callus tissue was 10–12 times more resistant to APM than callus tissue derived from wild type *N. plumbaginifolia* plants (Fig. 1). While 5 μM of APM almost completely inhibited the growth of the control callus, it only slightly (10%) reduced growth of the apm5^r callus. APM concentrations around 10 μM significantly (more than 50%) inhibited apm5^r callus growth.

Genetic analysis of the apm5^r line

The inheritance of the APM-resistance trait in the apm5^r lines was examined by self- and cross-pollination of apm5^r plants. Seed harvested from self-pollinated or cross-pollinated wild and apm5^r plants were aseptically germinated on the hormone-free MS medium containing 5 μM of APM (Fig. 3, Table 3). The trait resistant to APM was transmitted to all progeny obtained from the self-pollination of apm5^r plants (Table 3). Analogous results were obtained from the analysis of the F_1 progeny produced from the cross-pollination of apm5^r plants to wild type plants. The resistant seedlings grew quickly and developed normally on the medium containing 5 μM of APM. The analysis of F_2 progeny, obtained from self-pollination of the F_1, demonstrated that of the 281 individuals examined, 208 proved to be resistant to APM and 73 were sensitive to the herbicide. Some sensitive seeds germinated on the selective medium but their seedlings formed anomalous leaves and did not develop roots. By backcrossing of F_1 progeny to wild type plants it was established that 140 out of

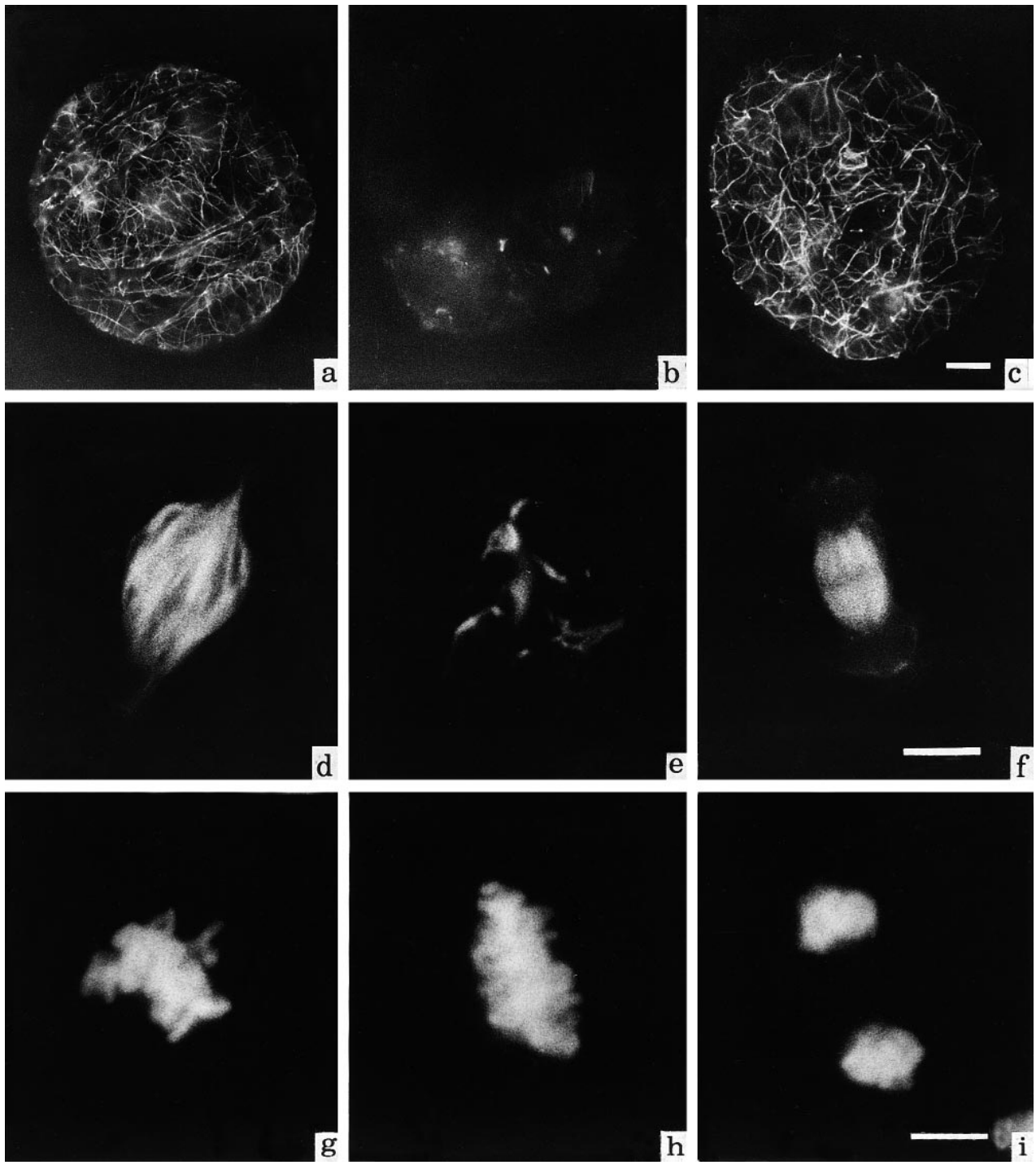

Fig. 4 Organization of cortical microtubules (**a, b, c**) and mitotic spindles (**d, e, f**) with the corresponding chromosome images (**g, h, i**) of untreated protoplasts (**a, d, g**), protoplasts isolated from control plants and treated with 5 μM of APM for 3 h (**b, e, h**), and protoplasts isolated from apm5^r plants and treated with 5 μM of APM for 3 h (**c, f, i**): **a, b, c, d, e, f**–immunostaining with anti-tubulin antibodies TU-01; **g, h, i**–staining of DNA with Hoechst 33258

276 individuals were resistant to APM and 136 were sensitive to it. The results of the segregation in F_2 progeny (3 : 1), as well as the results of the backcrossing (1 : 1), are consistent with Mendelian laws. The results obtained testify that apm5^r plants were homozygous for a single dominant nuclear mutation conferring resistance to APM.

Analysis of microtubular stability to APM action

Protoplasts were isolated from wild type or apm5^r lines and after 3 days of regeneration were used for the examination of APM effects on microtubular organization in these cells. The protoplast suspensions were incubated in the presence of 0.1, 1, 3 and 5 μM of APM for 3 h, and the microtubules present were visualised by indirect immunofluorescence microscopy. Figure 4 shows the organization of cortical microtubules and the mitotic spindle in control and apm5^r lines treated with 5 μM of APM. The microtubules of protoplasts isolated from the mesophyll of wild-type plants were highly sensitive to APM. Although a majority of microtubules in these cells at all cell-cycle stages remained intact after treatment with APM at concentrations of 0.1 μM or lower, higher concentrations (>1 μM) induced a dramatic disruption of the cortical network (Fig. 4b), the mitotic spindle (Fig. 4 e), and preprophase bands and phragmoplasts (data not shown). Interestingly, some of the kinetochore microtubules were still present at 5 μM of APM (Fig. 4 e). In contrast, the cortical microtubules of protoplasts isolated from apm5^r plants remained unaffected after 3 h of 5 μM-APM treatment (Fig. 4 c). Likewise, the mitotic spindle (Fig. 4 f) and the pre-prophase band and phragmoplast (data not shown) remained unaffected, confirming the insensitivity of the mutant line apm5^r to APM.

Analysis of tubulin constitution

Tubulin isoforms in control and apm5^r plants were compared by two-dimensional polyacrilamide gel electrophoresis (Fig. 5 a,b) and by immunoblotting using the monoclonal antibodies TU-01 and TU-06 directed against α- and β-tubulin respectively (Fig. 5 c,d,e,f). Although single spots were identified for α- and β-tubulins in both the control (Fig. 5 c,e) and the apm5^r plants

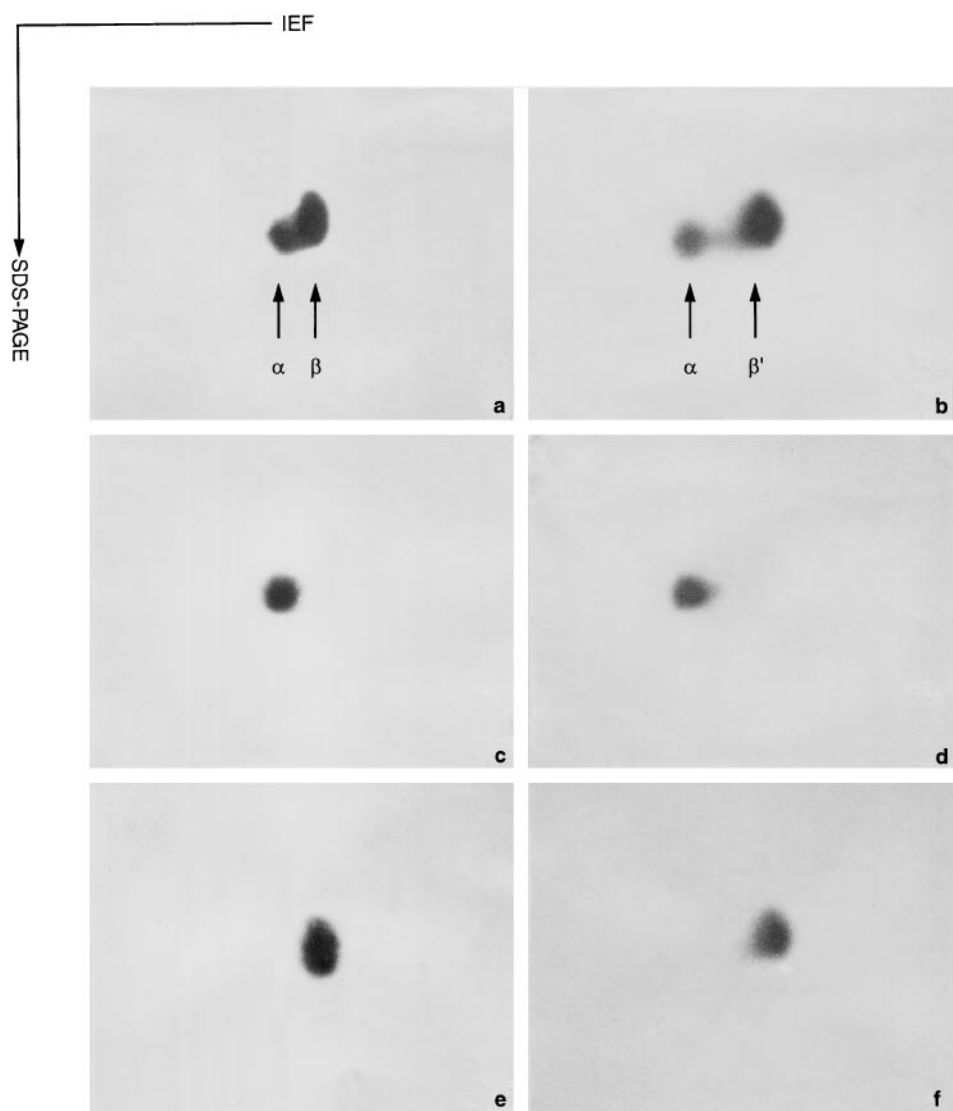

Fig. 5 2D-SDS-PAGE and immunodetection of α- and β-tubulin from wild type (**a, c, e**) and 5apm^r (**b, d, f**) plants: **a, b** – Coomassie Brilliant Blue stained gels; **c, d** – immunostaining with antibody TU-01 against α-tubulin; **e, f** – immunostaining with antibody TU-06 against β-tubulin. Only areas of electrophoregrams containing tubulins are shown

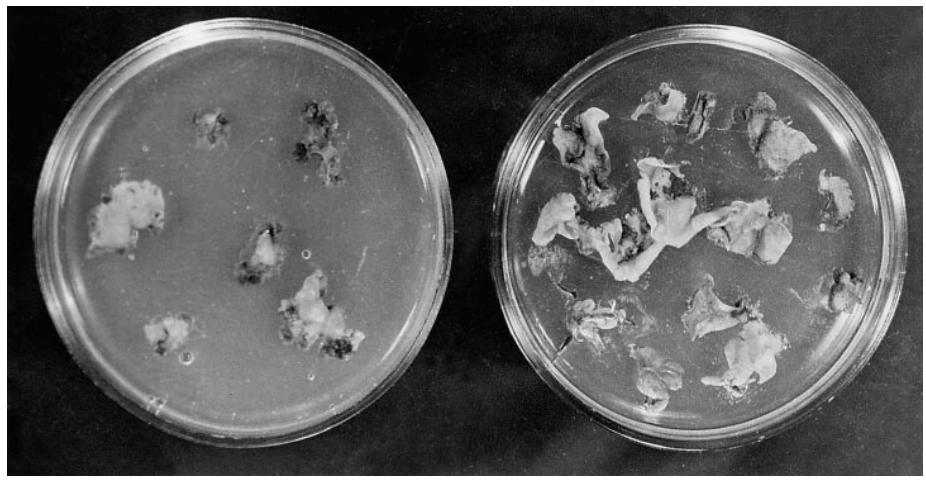

Fig. 6 Regeneration of leaf explants from *N. plumbaginifolia* wild type (left) and apm5^r (right) plants on the RMOP medium containing 12 μM of triflurain

(Fig. 5 d,f), the irregular shape and size of the spots suggests that several improperly separated α- and β-tubulin isoforms are present in both cases. Despite the fact that no apparent alterations in the molecular weight of apm5^r α- and β-tubulin were observed, some of the β-tubulin isoforms of the APM-resistant line possessed a more acidic pİ than those of control plants (Fig. 5).

Resistance of apm5^r plants to trifluralin

Trifluralin is a dinitroaniline herbicide which, like APM, is an effective inhibitor of tubulin polymerization (rewieved in Morejohn and Fosket 1991; Blume and Strashnyuk 1993), and which apparently shares with APM a common binding site on the tubulin molecule (Ellis et al. 1994). To determine whether apm5^r plants also show resistance to trifluralin, leaf explants from apm5^r plants were cultivated on RMOP medium containing trifluralin at a concentration of between 3 and 12 μM. Figure 6 shows that leaf explants from apm5^r plants were able to regenerate in the presence of 12 μM of trifluralin, whereas regeneration of leaf explants from control plants was almost completely inhibited under this condition.

Discussion

APM-resistant mutants were obtained by gamma irradiation and direct selection following a method described by Sidorov (1990). Cell lines resistant to 10 μM of APM were identified after the first round of selection. However, those resistant lines selected at, or below, 5 μM of APM retained the ability to regenerate into mature fertile plants. Such a phenomenon was observed both in lines selected from protoplasts as well as those selected from callus tissue. However, we noted that resistant lines derived from protoplasts such as apm5^r possessed a higher regeneration and growth potential in the presence of APM than those derived from callus and selected under similar conditions. Callus tissue is genetically heterogeneous and, as a result, some selected lines may continue to be chimaeric in nature (Sidorov 1990). The use of protoplasts for mutant selection allows the cloning of independent plant cells and the regeneration of normal and fertile plants, so reducing the frequency of chimaeras (Gleba et al. 1974).

Resistance to APM in the apm5^r line remained even after several passages *in vitro* in the absence of the selective agent, suggesting a genetic, rather than an adaptive, character for the mutation. The seedling growth assay used in scoring crosses gave the first indication that mutation, selected at the cellular level for resistance to APM, was expressed by the whole plant.The results of genetic crosses showed that the resistant trait was transmitted to the F_1 and F_2 populations confirming the genetic character of the mutation. Table 3 demonstrates the resistant trait to APM to be

Table 3 Inheritance of APM resistance in F_1 and F_2 progenies in crosses of control and apm5^r plants. Plants regenerated from the APM-resistant cell line are designated as apm5^r. Wild type plants are designated as K

Cross	Number of individuals			
	Resistant		Sensitive	
	Observed	(Expected)	Observed	(Expected)
K selfed	0	0	356	(356)
apm5^r selfed	306	(306)	0	(0)
apm5^r × K	338	(338)	0	(0)
K × apm5^r	320	(320)	0	(0)
(apm5^r × K) × K	140	(138)	136	(138)
(apm5^r × K) Selfed	208	(211.75)	73	(70.25)

For all calculations $P > 0.05$

transmitted to all F_1 progeny. The phenotype segregation of F_2 progeny into resistant and sensitive plants is observed in the ratio of 3 : 1, which is consistent with Mendelian laws. The data obtained permit one to presuppose that apm5^r plants were homozygous for a single dominant nuclear mutation conferring resistance to APM. Apparently, the mutation conferring the resistance of the apm5^r line to APM occurred in a haploid cell that subsequently diploidized under the influence of in vitro cultivation conditions in the presence of the antimicrotubular agent. Similar results were obtained by Chaleff and Ray (1984) in the genetic analysis of *Nicotiana tabacum*, resistant to chlorsulfuron and sulfometuron. The regenerated plants were homozygous for a single dominant nuclear mutation conferring resistance to sulfometuron methyl and chlorsulfuron. Plants regenerated from only one isolate (C3) were heterozygous. Apparently, the mutation conferring resistance to isolate C3 arose after the diploidization of a haploid cell of the parental H1 cell line (Chaleff and Ray 1984).

The microtubules of protoplasts isolated from the mesophyll of wild type *N. plumbaginifolia* plants were highly sensitive to APM treatment (Fig. 4). Other studies have shown that 1–3 μM of APM can within 1 h of treatment induce the complete depolymerization of both mitotic and interphase microtubules (Falconer and Seagull 1987; Murata and Vada 1989; Murthy et al. 1994), thus suggesting that depolymerization of microtubules is one of main targets of APM in plant cells. In contrast, the majority of the interphase and mitotic microtubules in the apm5^r line remained intact in the presence of APM (Fig. 4). This result confirms earlier work by Vaughn and co-authors who examined interphase and mitotic microtubules in dinitroaniline-resistant carrot and goosegrass (Vaughn et al. 1987; Vaughan and Vaughn 1988). Thus, it appears that the mutation introduced into the *N. plumbaginifolia* genome confers resistance to the herbicide by altering the microtubules and allowing mutant cells to go through normal cell cycles.

It has been reported that APM binds specifically to plant tubulin (Morejohn and Fosket 1984). Therefore, one would expect alterations in the tubulin dimer in APM-resistant plants. Routinely, only 10% of animal cell lines showing resistance to antimicrotubular compounds contained altered tubulins (reviewed in Oakley 1985). However, no changes in the tubulin genes of goosegrass plants were found by RFLP analysis (Mysore and Baird 1995). This may suggest site-mutations, as was shown for *Chlamydomonas reinhardtii* resistant to antimicrotubular drugs by Lee and Huang (1990). Although we found no changes in the molecular weight of α- and β-tubulin subunits, the isoelectric points of part of the β′-tubulin isoforms of the APM-resistant line, but not those of the α-tubulins, were shifted to the more acidic area (Fig. 5). This result implies that the introduced mutation in the apm5^r line might have affected a β-tubulin gene resulting in one or several amino acid substitutions in the β-tubulins derived from this gene. It is also possible, however, that the mutation may have altered β-tubulin post-translational modification processes which could have resulted in the alteration of their isoelectric points.

Apm5^r plants showed cross-resistance to lethal concentrations of trifluralin, a dinitroaniline herbicide. Analogously, earlier reports have shown that dinitroaniline-resistant plants can show cross-resistance to APM (Vaughn et al. 1987), supporting the hypothesis that the nature of the mutation is tubulin related. Resistance to dinitroaniline and phosphoroamide herbicides may be similar in nature and may reflect the common binding site for these herbicides on the tubulin molecule (Ellis et al. 1994).

In conclusion, we have selected a mutant line of *N. plumbaginifolia* resistant to APM. This resistant line, apm5^r, also showed cross-resistance to trifluralin, another antimicrotubular compound with a similar mechanism of action. The resistance is most likely the result of a dominant nuclear mutation, suggesting a β-tubulin gene; the end result of such a mutation being a slight alteration in the pl of the β′-tubulin polypeptides. Further studies directed to the elucidation of the molecular mechanisms for APM resistance in the selected plants are in progress.

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