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Transformation of elite white poplar (*Populus alba* L.) cv. 'Villafranca' and evaluation of herbicide resistance

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Abstract Transgenic white poplar plants (*Populus alba* L.) expressing the *nptII* gene and the *bar* gene from *Streptomyces hygroscopicus* have been produced using *Agrobacterium tumefaciens*-mediated gene transfer. Eleven kanamycin-resistant plant lines were obtained with a transformation frequency of 7%. Successful genetic transformation was confirmed by Southern and northern analyses. The level of resistance to the commercial preparation of phosphinothricin (Basta; Roussel-Hoechst Agrovet) was evaluated by in vitro and in vivo assays. Using in vitro selective conditions for phosphinothricin, only plantlets from four kanamycin-resistant independent lines remained green and continued to grow and root. After transfer to the growth chamber, all selected transgenic lines were shown to be completely resistant to the herbicide Basta with doses equivalent to 6 l ha⁻¹ (normal field dosage) and were tolerant at concentration of 12 l ha⁻¹. This is the first report describing the genetic transformation of a *P. alba* clonal cultivar of commercial interest with a gene of agronomic value.

Key words *Agrobacterium tumefaciens* · *bar* gene · Herbicide resistance · *Populus alba* L. · Transgenic plants

Introduction

White poplars (*Populus alba* L.) cover a natural range which includes Central and southern Europe, North Africa, western Asia and Central Asia (FAO 1980). Several cultivars are traditionally cultivated in the Near and Middle East ('Roumi'; 'Ankara AT'; 'Kabudeh Schirazih'; 'Kabudeh Bumi') and many more were selected and introduced to commercial culture in the second half of the twentieth century. One of these cultivars, a clone named 'Villafranca', was obtained at the Poplar Research Institute, Casale Monferrato (Italy) in 1954 by crossing a female *P. alba* from Villafranca Piemonte (Piedmont, Italy) and a male specimen of the same species from Lucca (Tuscany, Italy). This clone was registered for commercial use in Italy (1989) and Hungary (1987). 'Villafranca' is used for reforestation in the plains along rivers and in specialized stands for the production of sawlogs (furniture, pallets, etc.).

Recently, 'Villafranca' was tested in a research and demonstration program undertaken by ENEL (Italian Electric Company) with the aim of setting up suitable culture models for large-scale production of wood for energy by means of short rotation forestry systems (Schenone et al. 1997). 'Villafranca' showed good production of biomass (about 9 oven dry t ha⁻¹ year⁻¹) and a remarkable resprouting ability after coppicing. However, this clone requires careful tending in the first few months following planting and after every coppicing because weed competition drastically damages the young poplar plants during this period (Facciotto and Schenone 1998). Moreover, the use of selective herbicides is almost impossible since poplars are very sensitive to most herbicides. Beside this, the application of high doses of toxic herbicides has a negative environmental impact. Therefore, the production

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of 'Villafranca' plants resistant to a single, highly efficient non-selective herbicide without toxicity to animals and rapid degradation in the soil would be welcome. Recent developments in gene transfer techniques provide an opportunity to modify poplars by producing plants that are herbicide-resistant. The strategies adopted with poplars are based on transformation with the *bar* gene, encoding the phosphinothricin acetyltransferase enzyme which directly inactivates the herbicide phosphinothricin or by insertion of mutant genes (*aroA*; *csr1-1*) that exhibit lower sensitivity or complete insensitivity to glyphosate and chlorsulfuron herbicides, respectively. Transgenic poplar plants tolerant to phosphinothricin (De Block 1990; Devillard 1992), glyphosate (Fillatti et al. 1987; Donahue et al. 1994) and resistant to chlorsulfuron (Brasileiro et al. 1992) have been reported. In this paper, we describe the successful regeneration of transgenic white poplar plants from the cultivar 'Villafranca' expressing the *bar* gene, a foreign gene of agronomic value, using *Agrobacterium*-mediated gene transfer. Transgenic poplars were resistant to high levels of phosphinothricin.

Materials and methods

Plant material

Aseptic shoot cultures of 'Villafranca' were maintained in vitro on woody plant medium (WPM; Lloyd and McCown 1991) supplemented with 2% (w/v) sucrose and 0.15% (w/v) activated charcoal (Sigma). The cultures were incubated in a growth chamber at 22–25 °C, with a 16-h photoperiod at a light intensity of 27–33 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Six- to 7-week-old plant material was used for genetic transformation.

Agrobacterium strain and plasmid

Agrobacterium tumefaciens strain EHA105 containing the disarmed hypervirulent plasmid pTiBo542 in the C58 chromosomal background (Hood et al. 1993) was used for transformation. The pBI-BAR vector plasmid (11 kb) contains a 550 bp fragment that was obtained by PCR amplification of the *bar* gene from *Streptomyces hygroscopicus* strain ATCC 21705 (Thompson et al. 1987) using two specific primers carrying a *Bam*HI site (Bar₁: GGATCCATGAGCCCGAACGACGCGCCG) and a *Sac*I site (Bar₂: GAGCTCTCAGATCTCGGTGACGGGCAG). The amplified fragment containing the coding region of the *bar* gene was purified from agarose gel and inserted into pGEM-T vector (Promega). The resulting pGEM-BAR plasmid was *Bam*HI/*Sac*I digested in order to release the *bar* fragment that was subsequently used to replace the *UidA* gene in the binary vector pBI121 (Clontech). The binary plasmid pBI-BAR (Fig. 1), carrying the *bar* gene coding region under the control of the cauliflower mosaic virus (CaMV) 35S promoter and the poly-adenylation region from the nopaline synthase gene (*nos-ter*) was subsequently transferred to *A. tumefaciens* by electroporation at 2500 volts an EHA105 suspension culture grown overnight and washed with 10% glycerol. Bacterial cultures were grown overnight in LB liquid medium (Sambrook et al. 1989) containing 150 mg l⁻¹ kanamycin (Sigma) and 150 mg l⁻¹ rifampicin (Lepetit) and collected by centrifugation at 3000 rpm for 15 min. For transformation of internodal explants, bacteria were resuspended in liquid WPM to a final density of 0.6 (OD_{550nm}).

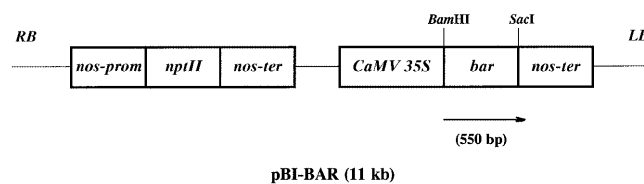


Fig. 1 Schematic representation of the binary plasmid pBI-BAR. *RB* right border, *nos-prom* nopaline synthase gene promoter, *nptII* neomycin phosphotransferase-coding sequence, *nos-ter* nopaline synthase gene terminator, *CaMV 35S* cauliflower mosaic virus 35S promoter, *bar* phosphinothricin resistance gene, *LB* left border

Plant transformation, selection and regeneration

Internodal stem segments (5–10 mm long) were excised from growing micropropagated plantlets and pre-cultured for 24 h in the dark on a callus-inducing medium (CIM) [modified (3/4 macrosalts) Murashige and Skoog (1962) medium supplemented with 1 mg l⁻¹ indole-3-butyric acid and 0.5 mg l⁻¹ kinetin]. Subsequently, they were immersed for 2 h in the bacterial suspension containing acetosyringone (200 μM), then blotted dry with sterile filter paper to remove the excess bacteria and placed horizontally for 48 h on the same medium. After this period, the infected explants were washed for 15 min with sterile water containing 1 g l⁻¹ cefotaxime and 1 g l⁻¹ carbenicillin, blotted dry and transferred to CIM containing 300 mg l⁻¹ cefotaxime, in the dark. Twenty days later, the growing calli were isolated from the explants, transferred to light and cultured on WPM containing 100 mg l⁻¹ kanamycin and 1 μM thidiazuron, to induce shoot regeneration. After 3 weeks, they were subcultured on the same regeneration medium, except that thidiazuron was decreased to 0.01 μM . Twenty days later, the regenerating calli were cultured on selective Driver and Kuniyuki (1984) medium containing 0.25 mg l⁻¹ 6-benzyl-aminopurine and 0.1 mg l⁻¹ of α -naphthaleneacetic acid. The shoots emerging from the selected calli were excised and elongated on WPM medium lacking growth regulators. After 3–4 weeks, the regenerated plantlets were rooted on the same medium containing kanamycin. Only those plantlets which produced roots were considered kanamycin-resistant and were further propagated in vitro in order to test for in vitro herbicide resistance. All regenerating cultures were maintained at 22–25 °C, with a 16:8-h light:dark cycle. Fifty stem segments were used for each Petri dish with three replicate plates. Transformation frequency was defined as the percentage of inoculated stem segments that produced kanamycin-resistant plantlets.

In vitro assay for herbicide resistance

In vitro selected kanamycin-resistant plantlets and untransformed controls were cultured on WPM containing 100 mg l⁻¹ kanamycin with or without 5 mg l⁻¹ phosphinothricin (PPT; from the commercial product Basta, Roussel-Hoechst Agrovet) to evaluate their ability to grow and form roots. Two to five in vitro plantlets were used for each independent poplar line. Plants were observed every week for 1 month, after treatments. At this time, only the putative transgenic plantlets which survived the kanamycin and herbicide applications were propagated and transplanted to plastic pots and then kept in a growth chamber in order to collect the amounts of tissues required to perform molecular analyses and in vivo herbicide resistance assays.

DNA isolation and Southern hybridization analysis

Total genomic DNA was isolated from leaves of selected PPT-resistant plants regenerated from independent transformation

events and from untransformed control plants. Tissues were ground to a fine powder in liquid nitrogen and DNA was extracted as described by Rogers and Bendich (1988). DNA (10 µg) was digested with *Bam*HI/*Sac*I (Promega) following the supplier's instructions. The restriction fragments were separated on 0.8% (w/v) agarose gels (Boehringer, Mannheim) and then transferred onto nylon membranes (Appligene) according to the manufacturer's instructions. Prehybridization and hybridization were performed as previously described (Confalonieri et al. 1998). The 0.6 kb *bar* fragment was obtained from the pGEM-BAR plasmid by *Bam*HI/*Sac*I digestion and labelled with [³²P]-dCTP by the Prime-a-Gene System kit (Promega).

Northern blot hybridization analysis

Total RNA was extracted from control and transgenic poplar lines using the small-scale procedure described by Verwoerd et al. (1989). For northern blot analysis, RNA samples (5 µg) were separated on 1.5% (w/v) agarose denaturing formaldehyde gels which were subsequently stained with ethidium bromide (1 mg ml⁻¹). RNAs were blotted onto nylon membranes (Appligene) according to the manufacturer's instructions. Prehybridization, hybridization and probe labelling were performed as for the Southern blot analysis.

In vivo herbicide resistance assay

Four selected independent transgenic lines expressing the *bar* gene and a control untransformed line were analysed for PPT resistance. Two to eight plants (about 45–60 cm high) were used for each tested poplar line. They were sprayed with a 1% (normal field dosage) and 2% aqueous solution of the commercial product Basta (Roussel-Hoechst Agrovet, containing 120 g l⁻¹ PPT) at 15-day intervals, using a microspray applicator (Wagner). The application rate of Basta was respectively 6 and 12 l ha⁻¹. Plants were observed for symptoms on leaves, stems and shoot tips every 2–3 days for two weeks, after treatments.

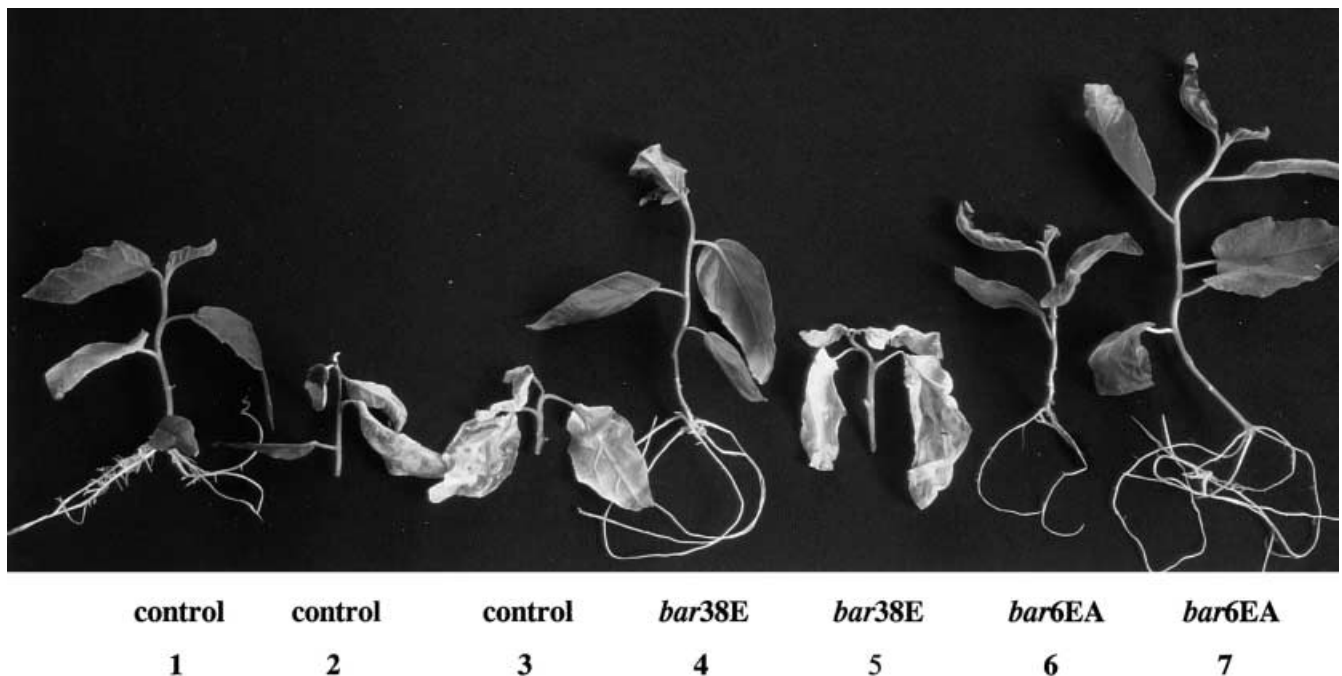
Results and discussion

Transformation, plant regeneration and in vitro herbicide selection

Eleven kanamycin-resistant plant lines were obtained from 150 co-cultivated stem internodes with a transformation frequency of 7%. Compared with the untransformed control plants, transformants showed normal development and plant morphology, except in a transformed plant line (*bar*5E) which exhibited reduced apical dominance and internode length, and continued to produce new shoots from axillary buds. No shoot organogenesis was observed in control calli cultured on selective regeneration media.

Kanamycin-resistant plant lines were further tested for resistance to the herbicide PPT in vitro. After 1 month of selection on WPM with 5 mg l⁻¹ PPT and 100 mg l⁻¹ kanamycin, only plantlets from four independent transformants survived the treatment and continued to grow and root. In contrast, all kanamycin-resistant plant lines continued their normal growth on WPM containing kanamycin (Fig. 2). Untransformed control plants were very sensitive to the kanamycin and herbicide treatments. They showed signs of necrosis

Fig. 2 Evaluation of herbicide resistance in control and transgenic poplar plant lines under in vitro conditions. A control poplar plant line (*left*) cultured for 1 month on woody plant medium without phosphinothricin and kanamycin (1), with 100 mg l⁻¹ kanamycin (2) and with 100 mg l⁻¹ kanamycin + 5 mg l⁻¹ phosphinothricin (3), compared with two transformed poplar plant lines (*bar*38E and *bar*6EA, respectively) cultured on the same basic medium containing 100 mg l⁻¹ kanamycin (4 and 6, respectively) or with 100 mg l⁻¹ kanamycin + 5 mg l⁻¹ phosphinothricin (5 and 7, respectively)



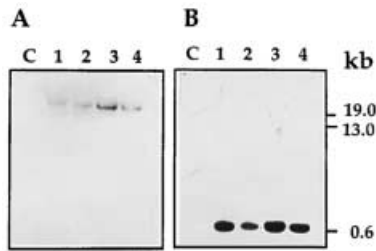


Fig. 3 Southern blot analysis of genomic DNAs extracted from four different transgenic poplar lines (lanes 1–4: *bar27E*, *bar5E*, *bar6EA*, *bar31EC*, respectively) following transformation with EHA105 pBI-BAR *Agrobacterium tumefaciens* strain. The 0.6 kb *bar* gene coding sequence was used as a probe for Southern blot hybridization. Lane C Untransformed control line. **A** High molecular weight undigested DNA; **B** *Bam*HI/*Sac*I-digested DNA

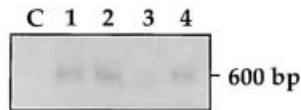


Fig. 4 Northern blot analysis of total RNAs extracted from four different transgenic poplar lines (lanes 1–4: *bar27E*, *bar5E*, *bar6EA*, *bar31EC*, respectively) regenerated following transformation with *A. tumefaciens* EHA105 pBI-BAR strain. The 0.6 kb *bar* fragment was used as a probe. Lane C Untransformed control line

and chlorosis on basal leaves 1 week after the beginning of the treatments and died 2 weeks later. The in vitro inhibition of shoot organogenesis in poplar transformation experiments by this PPT level has also been reported by De Block (1990). Selected putative transformants were propagated in vitro and then transferred to a growth chamber and used for further analyses.

Molecular analyses of transgenic plants

Southern blot hybridization analysis was performed on four selected different poplar lines (*bar27E*, *bar5E*, *bar6EA*, *bar31EC*, respectively) regenerated following transformation with EHA105 pBI-BAR *A. tumefaciens* strain. Undigested and *Bam*HI/*Sac*I-digested DNAs were tested using the 0.6 kb *bar* fragment as probe (Fig. 3). A hybridization band corresponding to the expected 0.6 kb *bar* gene was detected in the digested samples except for the negative untransformed control, while in the case of undigested DNAs the signal was associated only with the high molecular weight fraction.

Fig. 5 Evaluation of herbicide resistance in control and transgenic poplar plant lines under growth chamber conditions. The picture was taken 15 days after treatment with Basta herbicide at a dose of 6 l ha⁻¹. The untransformed control plant (left) showed extensive defoliation, while the transgenic poplar lines *bar27E*, *bar6EA* and *bar5E* displayed no damage and normal growth



Results of this analysis indicate the presence and stable integration of the *bar* gene into the poplar genome.

Northern blot hybridization analysis performed using the same probe showed the presence of a specific transcript of approximately 0.6 kb in all selected transgenic poplar lines. The steady-state level of the *bar* mRNA seemed to vary among independent transgenic poplar lines, as shown by differences in band intensity (Fig. 4). No hybridization signals were detected for the untransformed poplar line.

In vivo herbicide resistance

Untransformed control plants and rooted plantlets propagated from four selected transgenic poplar lines, which had been confirmed to have and express the *bar* gene, were tested for phosphinothricin resistance in a growth chamber by spraying them with Basta herbicide at doses equivalent to 6 and 12 l ha⁻¹. Two weeks after the first application, no symptoms were observed on any transgenic plant lines treated with 6 l ha⁻¹ and they continued their normal growth and development (Fig. 5). No visible effects or damage were further observed on the resistant transgenic plantlets. Subsequently, the same transformed plant lines were sprayed with a herbicide dose of 12 l ha⁻¹ and, 2 weeks later, most of them (three out of four) showed small browned and necrotized areas on some leaves which represented, on average, about 13% of the total number of treated transgenic leaves. However, all transformed plant lines survived, continued to grow, and produced new leaves. Relative to the untransformed control plants, 2 days after the application of different herbicide treatments, they showed complete necrosis and browning of leaves and most of them were wrinkled and detached from stems within 7 days following all herbicide applications. Untransformed poplar plants displayed atrophy of the apical meristems and inhibition of growth, and died about 30–40 days after the beginning of herbicide treatments.

In the present study, we used the CaMV promoter region to direct *bar* gene expression and we demonstrated that this sequence can support an expression level which allows the production of herbicide-resistant transgenic poplar plants. The resistance level observed in our transgenic plants differed from that reported for transgenic hybrid poplar and aspen, whose complete resistance was achieved using 20 l ha⁻¹ of the Basta herbicide (De Block 1990). Although the expression levels were different between independent transformants, all transformed poplar lines showed complete herbicide resistance at the normal field rate and therefore could have commercial potential. This resistance could permit control of weed competition during the establishment of short rotation forestry systems and also lead to a reduction of deleterious environmental effects due to the application of high doses of toxic herbicides. This work demonstrates that the heterolo-

gous expression of the *bar* gene in transgenic white poplar is associated with resistance to the herbicide phosphinothricin. However, a conclusive evaluation of the resistance level of transgenic poplars will require the completion of field trials.

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