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ORIGINAL PAPER

Evaluation of MAT-vector system in white poplar (*Populus alba* L.) and production of *ipt* marker-free transgenic plants by 'single-step transformation'

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Abstract Genetic transformation of an elite white poplar genotype (*Populus alba* L., cv. 'Villafranca') was performed with MAT vectors carrying the *ipt* and *rol* genes from *Agrobacterium* spp. as morphological markers. The effects associated with the use of different gene promoters and distinct in vitro regeneration protocols were evaluated. Poplar plantlets showing abnormal *ipt* and *rol* phenotypes were produced only in the presence of exogenous growth regulators. The occurrence of abnormal *ipt* and *rol* phenotypes allowed the visual selection of transformants. The *ipt*-type MAT vector pEXM2 was used to monitor the activity of the yeast site-specific recombination R/RS system in the transformed white poplar cells. Results from these experiments demonstrated that recombinase-mediated excision events occurred during the early stages of in vitro culture, thus causing the direct production of *ipt* marker-free transgenic plants with normal phenotype at an estimated frequency of 36.4%. Beside this unexpected finding, transgenic *ipt*-shooty plants were obtained at a frequency of 63.6% and normal shoots were subsequently recovered after a prolonged period of in vitro culture. Although the transformation efficiency observed in this study, using both *ipt* and *nptII* genes as selection markers, was similar to that previously reported with standard vectors carrying only the *nptII* gene, the easy identification of *ipt* transformants, the early recombinase-mediated excision events and finally the relatively short time period required to produce *ipt* marker-free transgenic plants support for the choice of MAT vectors as a reliable strategy for the future production of marker-free GM poplars.

Keywords *ipt* · Marker-free transgenic plant · Morphological marker · *Populus alba* L. · Recombinase · *rol* · Single-step transformation

Abbreviations

BAP	Benzyl-aminopurine
NAA	α-Naphthaleneacetic acid
TDZ	Thidiazuron

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Introduction

Crop improvement based on genetic transformation has led to the production of genetically modified (GM) plants expressing genes of interest associated with antibiotic or herbicide resistance genes, used as selection markers. The latter, necessary for the isolation of transgenic plants, are no longer needed in mature plants. The persistence of resistance genes in the GM plants is undesirable (Hohn et al. 2001; Goldstein et al. 2005).

Different approaches for the removal of selectable marker genes from GM plants have been described and sexual crosses have been traditionally used to segregate out the marker gene from the gene of interest (Hohn et al. 2001). In the MAT (Multi-Auto-Transformation) system, developed by Ebinuma and coworkers (Ebinuma et al. 1997; Sugita et al. 1999; Ebinuma and Komamine 2001), the *ipt* and *rol* oncogenes from *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively, are used as morphological markers due to their ability to induce abnormal phenotypes. The oncogenes are then removed from GM plants, using the yeast site-specific recombination system R/RS (Araki et al. 1987) and consequently marker-free transgenic plants can be easily regenerated. To date, the MAT vectors have been utilized to transform a limited number of annual species, such as *Nicotiana tabacum*, *Antirrhinum majus*, *Oryza sativa* and *Nierembergia caerulea* (Sugita et al. 1999; Cui et al. 2000, 2001; Sugita et al. 2000; Ebinuma and Komamine 2001; Endo et al. 2002; Khan et al. 2006). Among perennial plants, only the hybrid aspen (*Populus sieboldii* × *P. grandidentata*) and sweet orange (*Citrus sinensis* L.) have been tested with the *ipt*-type MAT system (Ebinuma and Komamine 2001; Matsunaga et al. 2002; Ballester et al. 2006). For all these systems, transformation/regeneration protocols were carried out in the absence of both selective chemical agents and hormones and resulted in the production of *ipt* or *rol* marker-free transgenic plants, which still harboured the *nptII*, *hpt*, *uidA* or *GFP* sequences located on the T-DNA region, outside the excised cassette. Except for rice, in all other species engineered with the MAT vector system, transformants were firstly selected based on the presence of the *ipt*-shooty or *rol* phenotype and, subsequently, only the phenotypically normal transgenic plants emerging from abnormal shoots were isolated (two-step transformation). However, the two-step procedure is laborious since it requires several in vitro subcultures and a prolonged period for the production of marker-free transgenic plants. For this reason, any further improvement of the methodology, saving time and work efforts, is strongly recommended. In this context, the direct regeneration of transgenic plants lacking their morphological marker, *ipt* or *rol*, as a consequence of early excision events (single-step transformation) could represent a reliable strategy for a more effective use of MAT vectors. Using an *ipt*-type MAT vector, the direct regeneration of *ipt* marker-free transgenic rice plants with normal phenotype from scutellum tissues was described by Endo et al. (2002). This single-step transformation method represents a useful transformation approach for those plant species that rely on somatic embryogenesis for in vitro regeneration. In the case of rice cells, it has been suggested that the overproduction of cytokinin associated with the overexpression of *ipt* gene causes the induction of embryogenic tissues and that, following excision, a change in the cytokinin level could determine the direct regeneration of shoots from embryogenic tissues (Endo et al. 2002).

Among woody species, white poplar (*Populus alba* L.) is considered a good biomass producer (about 9 oven dry t ha^{-1} $year^{-1}$), due to its remarkable resprouting ability after coppicing (Confalonieri et al. 2000). GM white poplar plants from the elite clonal cultivar 'Villafranca', expressing selectable marker genes (Balestrazzi et al. 2000) and relevant traits such as herbicide tolerance (Confalonieri et al. 2000), insect pest resistance (Delledonne et al. 2001; Balestrazzi et al. 2006), production of antioxidant compounds (Giorcelli et al. 2004), phytoremediation (Zelasco et al. 2004) and biomass production (Zelasco et al. 2006) have been obtained.

In this study we evaluate the efficacy of the MAT vector system to transform the white poplar (*Populus alba* L.) elite clonal cultivar 'Villafranca'. Both *ipt* and *rol* genes were used as morphological markers for the visual selection of transformants. We also investigated the effects of different gene promoters and regeneration protocols on the efficiency of white poplar transformation. Moreover, we tested the effectiveness of the yeast R/RS system in removing the morphological marker *ipt* gene from the

transgenic white poplars, using an *ipt*-type MAT vector. Finally, an efficient short-term and cost-effective transformation/regeneration procedure producing *ipt* marker-free transgenic plants is reported. Our results clearly demonstrate that MAT vectors can be considered a useful tool for the production of marker free transgenic plants in those agronomically relevant species which can be reproduced only by vegetative propagation

Materials and methods

Plant material, plasmids and *A. tumefaciens* strain

The clonal cultivar 'Villafranca' (*Populus alba* L.) was obtained by controlled crossing in 1954 at the Poplar Research Institute (Casale Monferrato, Italy) and registered for commercial use in Italy and Hungary. Aseptic shoot cultures of 'Villafranca' were maintained and propagated in vitro as previously described (Confalonieri et al. 2000). All the constructs used in this work, prepared and supplied by the Nippon Papers Industries (Tokyo, Japan), are derivatives of the pBI121 binary vector (Ebinuma and Komamine 2001). A schematic representation of these constructs is described in Fig. 1. The binary control vectors pIPT5, pIPT10 and pIPT20 contain the *ipt* gene associated with the 35SCaMV, native ipt and rbcS3B promoters, respectively (Fig. 1A). Plasmid pROL20 is a *rol*-type vector carrying the *rolABC* genes and their native promoters (Fig. 1A). The excision monitor control vector pEXM2 contains a 'hit and run' cassette, composed of the native *ipt* gene and the *R* gene, flanked by two recognition sites (RS) (Fig. 1B). In this construct the coding region of the reporter gene *uidA* and the associated 35SCaMV promoter are separated by one of the recognition sites. The recombinase-mediated excision of the 'hit and run' cassette leads to the inactivation of the *uidA* gene. In this model system, the *nptII* marker gene has been inserted into the multiple cloning site located outside the 'hit and run' cassette (Fig. 1B). All the above described constructs were transferred to the *A. tumefaciens* strain EHA105, a non oncogenic derivative of strain A281 which harbours the hypervirulent helper Ti plasmid pTiBo542 (Hood et al. 1993).

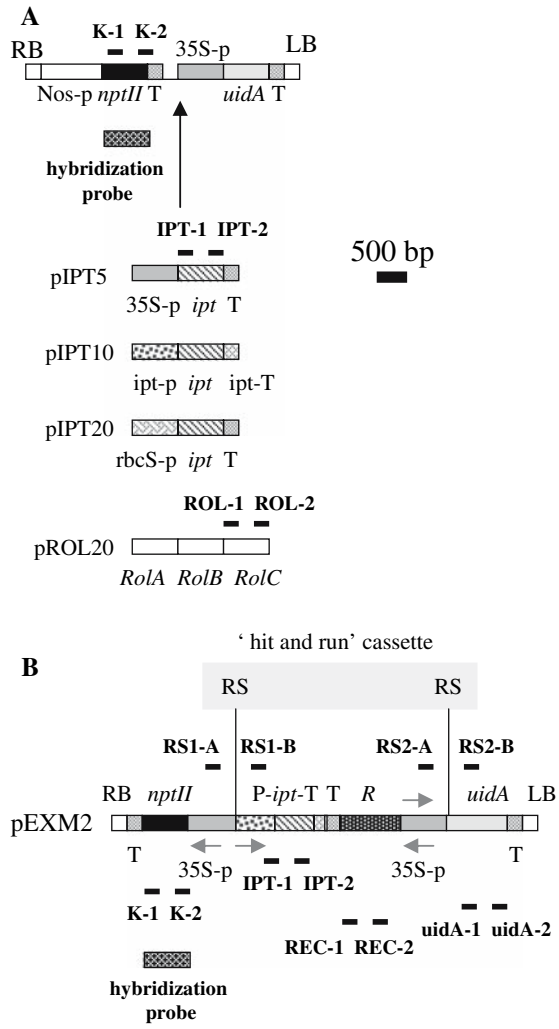

Fig. 1 Schematic representation of the binary control plasmids pIPT5, pIPT10, pIPT20 and pROL20 (**A**) and the *ipt*-type MAT vector pEXM2 (**B**) RB, right border; LB, left border; 35S-p, cauliflower mosaic virus 35S promoter; *nptII*, neomycin phosphotransferase-coding sequence; T, nopaline synthase gene terminator; p, promoter; *uidA*, *β*-glucuronidase-coding sequence; RS, recognition site; R, recombinase-coding sequence; *ipt*, isopentenyl transferase gene; ipt-p and ipt-T, promoter and terminator regions of the *ipt* gene; *rolA*, *rolB* and *rolC*, *Agrobacterium rhizogenes* genes from Ri plasmid with their associated promoter and terminator regions; RbcS, ribulose-1,5-biphosphate carboxylase small subunit; EXM, excision monitor. Gene-specific oligonucleotide primers are indicated by bars. The black arrow indicates the region of the pBI121 vector in which the different cassettes represented as 35S-p-*ipt*-T, ipt-p-*ipt*-ipt-T, rbcS-p-*ipt*-T and *RolA-RolB-RolC* have been inserted to produce the pIPT5, pIPT10, pIPT20 and pROL20 constructs. Transcription orientation for each cassette of the pEXM2 vector is indicated by grey arrows. The position of the *nptII* hybridization probe is also indicated

A. tumefaciens-mediated transformation and in vitro selection/regeneration

Agrobacterium tumefaciens-mediated transformation and in vitro shoot regeneration of *Populus alba* L. cv. 'Villafranca' was performed using two different protocols. The first procedure was described by Confalonieri et al. (2000). After co-cultivation and a callus induction step, shoot regeneration was achieved in the presence of the cytokinin analog thidiazuron (TDZ) and benzyl-aminopurine (BAP). In the second procedure, internodal explants were co-cultivated as described (Confalonieri et al. 2000) but placed and subcultured on a modified (3/4 macrosalts) MS medium (Murashige and Skoog 1962) without growth regulators. Three replications of 50 internodal stem explants each were used for all the tested vectors. Transformation efficiency was defined as the frequency of stem explants which produced kanamycin-resistant (Km^R) calli or shoots. Data were $\arcsin\sqrt{x}$ transformed before statistical analysis.

Molecular analyses

Plant DNA for PCR analysis was extracted as described by Rogers and Bendich (1988) and subsequently purified using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences). PCR was carried out as described by Confalonieri et al. (1994) in a Perkin-Elmer Thermo-Cycler 480 and amplification products were analysed on 1.4% (w/v) agarose gels (Duchefa Biochemicals). The following conditions were used to amplify the 599 bp fragment region of *nptII* gene: 35 cycles at 94°C, 2 min, 65°C, 1 min, 72°C, 1 min. The gene-specific oligonucleotide primers were K-1 (5′-AGGCTATTCGGCTATGACTGG-3′) and K-2 (5′-GCGGTCCGCCACACCCAGCCG-3′), respectively. Oligonucleotide primers IPT-1 (5′-CTTGCACAGGAAAGACGTCG-3′) and IPT-2 (5′-AATGAAGACAGGTGTGACGC-3′) were used to amplify the 800 bp fragment corresponding to the *ipt* gene with the following conditions: 35 cycles at 94°C, 50 s, 56°C, 50 s, 72°C, 1 min. Oligonucleotide primers REC-1 (5′-TCTGGGACGCCTCGGGAACTTC-3′) and REC-2 (5′-TGTCCCAGAGACTAAGACTGGTAC-3′) were used to detect the 666 bp fragment corresponding to the coding region of the *R* gene at the following conditions: 35 cycles at 94°C, 50 s, 68°C, 50 s, 72°C, 1 min. Primers RS1-A (5′-CAGCGCATCGCCTTCTATCG-3′) and RS1-B (5′-GATGTCTTTCCTGTGCAAG-3′) were used to amplify the 1.08 kb fragment spanning 42 bp of the *nptII* region, 255 bp of the *Nos* terminator, the RS site (58 bp) and 736 bp of the native ipt promoter. Primers RS2-A (5′-GTGGTCCCAAAGATGGACC-3′) and RS2-B (5′-TGCATCGGCGAACTGATC-3′) were then used to amplify the 422 bp fragment spanning 188 bp of the 35SCaMV region, the RS site (58 bp) and 176 bp of the *uidA* gene. In each case amplification was performed at the following conditions: 94°C, 50 s, 58°C, 50 s, 72°C, 1 min (RS1-A and RS1-B), 94°C, 50 s, 56°C, 50 s, 72°C, 50 s (RS2-A and RS2-B). When the R/RS system is activated, the absence of these amplification products is expected while, in the presence of the 'hit and run' cassette, both the 1.08 kb and the 422 bp fragments are produced. For the detection of the *rolC* gene sequence, primers ROL-1 (5′-AAATGCGAAGTAGGCGCTCCG-3′) and ROL-2 (5′-TACGTCGACTGCCCGACGATGATG-3′) were used and amplification of the predicted 0.2 kb fragment was obtained at 94°C, 50 s, 60°C, 50 s, 72°C 1 min. Oligonucleotide primers uidA-1 (5′-TATGCGGGCAACGTCTGG-3′) and uidA-2 (5′-TCACGCGGCTATCAGCTCTT-3′) were designed to amplify the 1.0 kb fragment from the *uidA* coding region at the following conditions: 94°C, 50 s, 54°C, 50 s, 72°C 2 min. For Southern blot hybridization analysis, plant genomic DNAs (10 μg) were digested with the indicated restriction enzymes (M-Medical S.r.l.), separated on 0.4% (w/v) agarose gels and transferred to a nylon membrane (Hybond™ N^+, Amersham Biosciences) according to manufacturer's instructions. The *nptII* fragment (599 bp) was used as probe and labelled with [α-^{32}P]-dCTP using the Hexa Label Plus™ DNA labelling kit (M-Medical S.r.l.). Filters were hybridized as described by Sambrook et al. (1989).

Results

Role of *ipt* and *rol* oncogenes as morphological markers for the selection of transformation events in *Populus alba* L. cv. 'Villafranca'.

Transformation experiments were carried out to evaluate the role of the *ipt* and *rolABC* oncogenes as morphological markers in *P. alba* L. cv.

'Villafranca'. The effects of *ipt* gene expression controlled by different promoters and regeneration protocols were also investigated. Km^R calli were obtained only in the presence of the cytokinin analogs TDZ and BAP, using the procedure described by Confalonieri et al. (2000). The frequency of stem internodes that produced Km^R calli was 2%, 8% and 14%, respectively for the pIPT5, pIPT10 and pIPT20 constructs (data not shown). As expected, the frequency of stem internodes from the untransformed control line that produced calli was 100%. After several subcultures, a reduced number of Km^R calli developed shoots with either normal or abnormal phenotype and the plant transformation efficiency ranged from 1 to 5% (data not shown). Analysis of variance revealed no significant differences ($P = 0.54$) in plant transformation efficiency among different constructs. All the *ipt*-shooty lines showed a reduced apical dominance, did not root on a hormone-free medium and new abnormal shoots and calli were continuously produced at contact sites with the agar medium (Fig. 2A). Only the abnormal *ipt*-shooty phenotype was observed among plants regenerated following transformation with the pIPT5 and pIPT10 constructs. Using the pIPT20 construct, both Km^R plants with a typical *ipt*-shooty phenotype and Km^R plants with normal phenotype were regenerated. The phenotypically normal shoots grew and rooted on kanamycin-containing medium lacking growth regulators. The frequency of stem explants producing phenotypically normal plants was 1% while the pIPT20 *ipt*-shooty lines were regenerated at a higher frequency (5%). Stem internodal explants co-cultivated with EHA105 carrying pROL20 produced Km^R plants with normal and *rol* phenotype with a similar frequency (2–3%). The presence of hairy roots is a typical feature of the *rol* phenotype (Fig. 2B). In this study we demonstrated that the *ipt* and *rolABC* genes can be used as visually selectable markers for the transformation of white poplar, and produced abnormal phenotypes clearly distinguishable from the untransformed control plant lines (Fig. 2C).

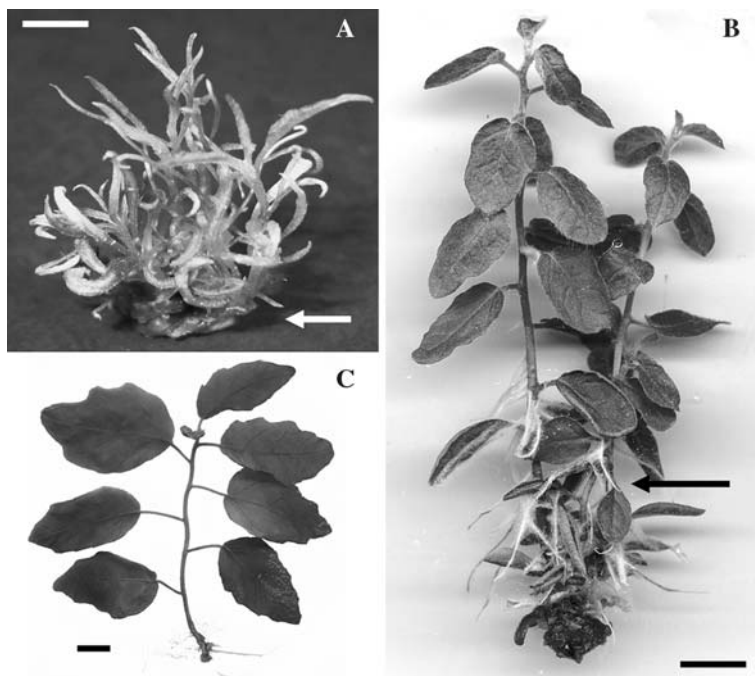

Fig. 2 Phenotypes deriving from the overexpression of the *ipt* and *rolABC* oncogenes in *P. alba* cv. 'Villafranca'. (**A**) Poplar shoot regenerated following transformation by *A. tumefaciens* EHA105 carrying pIPT20 and showing a typical *ipt*-shooty phenotype with absence of both apical dominance and rooting ability and callus production at the contact sites with the agar medium (indicated by arrow). Bar: 0.5 cm. (**B**) Poplar plantlet regenerated following transformation by EHA105 carrying pROL20 and showing a typical *rol* phenotype. Root proliferation is evident. Hairy roots are indicated by arrow. Bar: 0.5 cm. (**C**) Non transformed poplar plantlet with normal phenotype. Bar: 1 cm

Molecular analysis of the *ipt* and *rol* poplar lines

In order to confirm the presence of transgene sequences, four different putative transgenic lines, regenerated following co-cultivation with EHA105 carrying pIPT20, and two lines obtained following co-cultivation with EHA105 carrying pROL20, were analysed by PCR. Results from these experiments are summarized in Fig. 3. The predicted *nptII* fragment (599 bp) was amplified in all the tested pIPT20 lines (Fig. 3A, lanes 3–6), except for the non transformed control line (Fig. 3A, lane 2). Oligonucleotide primers IPT-1 and IPT-2 revealed the presence of the predicted *ipt* fragment (800 bp) (Fig. 3B, lanes 3–6). No amplified products were evidenced using DNA extracted from the non transformed plant (Fig. 3B, lane 2). The positive controls obtained using pIPT20 plasmid as template showed the expected amplification products (Fig. 3A, lane 1 and Fig. 3B, lane 1, respectively). In the case of pROL20, the presence of the *nptII* sequence was demonstrated in both the tested lines (Fig. 3C, lanes 3 and 4) while the same fragment was not amplified in the non transformed control line (Fig. 3C, lane 2). The *rolC* sequence was also detected in the same poplar lines (Fig. 3D, lanes 3 and 4) while no amplification products were observed in the non transformed control line (Fig. 3 D, lane 2). The positive controls obtained using pROL20 as template are shown in Fig. 3C (lane 1) and Fig. 3D (lane 1), respectively. The same

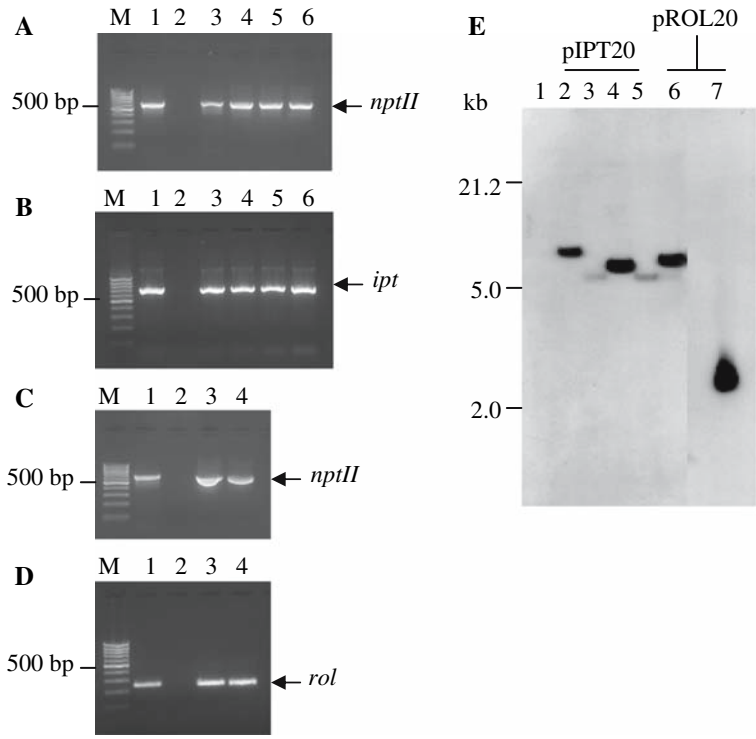

Fig. 3 Molecular analyses of the pIPT20 and pROL20 lines. (**A**) Detection of *nptII* transgene sequence in the pIPT20 lines. Control plasmid (pIPT20) and non transformed line are shown (lane 1 and 2). PCR analysis of DNAs extracted from four putative transgenic lines of *P. alba* cv. 'Villafranca' regenerated following co-cultivation with EHA105 carrying pIPT20 (lanes 3–6). (**B**) Detection of *ipt* transgene sequence in the pIPT20 lines. Control plasmid (pIPT20) and non transformed line are shown (lane 1 and 2). PCR analysis of the putative transgenic pIPT20 poplar lines (lanes 3–6). (**C**) Detection of *nptII* transgene sequence in the pROL20 lines. Control plasmid (pROL20) and non transformed line are shown (lane 1 and 2). PCR analysis of two putative transgenic lines obtained following co-cultivation with EHA105 carrying pROL20 (lanes 3 and 4). (**D**) Detection of the *rolC* transgene sequence in the pROL20 lines. Control plasmid (pROL20) and non transformed line are shown (lane 1 and 2). PCR analysis of the putative transgenic pROL20 poplar lines (lanes 3 and 4). M, Gene Ruler^{TM} 100 bp DNA Ladder (M-Medical S.r.l.). (**E**) Southern blot analysis of the pIPT20 and pROL20 lines. Lane 1, non transformed control line; lanes 2–5, pIPT20; lanes 6 and 7, pROL20. DNAs were digested with *Hind*III and probed using the [α-^{32}P]-dCTP-labelled 599 bp *nptII* fragment

transgenic lines were further analysed by Southern hybridization to confirm the stable integration of the *nptII* sequence into the poplar genome (Fig. 3E). Restriction analysis of poplar DNA performed with *Hind*III was expected to produce a DNA fragment (2.0 kb) spanning NosP-*nptII*-NosT region of the T-DNA and a variable portion corresponding to the flanking plant chromosomal region. Hence each hybridization band should represent a single copy of the transgene sequence. As expected, no hybridization signal was evident in the non transformed control line (Fig. 3E, lane 1). The *nptII* probe recognized a single fragment of approximately 10.0 kb in one of the tested pIPT20 lines (Fig. 3E, lane 2). Single hybridization signals ranging between 7.0 and 8.0 kb were evident in the other poplar lines (Fig. 3E, lanes 3–5). An hybridization signal of approximately 9.0 kb was observed for one of the pROL20 lines (Fig. 3E, lane 6). A smaller band of approximately 3.0 kb was observed for the second pROL20 line (Fig. 3E, lane 7).

Activation of the site-specific recombination system R/RS in white poplar cells transformed with the pEXM2 vector

To evaluate the efficacy in white poplar of the R/RS system for site-specific recombination associated with the expression of the *ipt* gene as morphological marker, a transformation experiment was performed with EHA105 carrying pEXM2. Eighteen independent pEXM2 Km^R callus lines were recovered. Approximately three-four months after co-cultivation, both normal and abnormal shoots were regenerated. Eleven Km^R plant lines, corresponding to a transformation frequency of 7.3%, continued to grow on kanamycin-containing medium lacking growth regulators. Four of them (2.6%) were characterized by the presence of shoots with normal phenotype and rooting (single-step transformation) while the remaining seven (4.6%) exhibited the typical *ipt*-shooty phenotype. The *ipt*-shooty lines were, as expected, unable to form roots (Fig. 4A). Four months later three *ipt*-shooty lines were able to develop normal shoots. The occurrence of new shoots with normal morphology from an *ipt*-shooty line is represented in Fig. 4B. The other *ipt*-shooty lines continued showing their abnormal phenotype.

Molecular analysis of the pEXM2 lines

All the *ipt*-shooty and the phenotypically normal pEXM2 lines were tested by PCR. Results from this analysis are summarized in Figs. 4 and 5, respectively. When PCR analysis was performed on five selected *ipt*-shooty pEXM2 lines, the predicted *nptII* and *ipt* fragments were amplified. Results from molecular analyses performed on three representative *ipt*-shooty pEXM2 lines are shown in Fig. 4C, D, E and F. The presence of both *nptII* and *ipt* fragments was evidenced in the pEXM2 lines (Fig. 4C, *nptII*, lanes 5–7; *ipt*, lanes 8–10). No amplification products were obtained with the non transformed control (Fig. 4C, lanes 3 and 4). The pEXM2 plasmid was used as positive control (Fig. 4C, lanes 1 and 2). The same pEXM2 lines were tested for the presence of the *R* gene sequence encoding the yeast recombinase. As shown in Fig. 4D (lanes 3–5), PCR performed on genomic DNAs with gene-specific oligonucleotide primers allowed amplification of the 666 bp fragment corresponding to the *R* gene coding region. Positive and negative controls are also reported (Fig. 4D, lanes 1 and 2, respectively). In addition, the two different amplification products corresponding to the flanking regions of the RS sites were detected using specific oligonucleotide sets (Fig. 4E, RS1, lanes 5–7; RS2, lanes 8–10). Positive and negative controls are visible in Fig. 4E (lanes 1–4). Southern hybridization was carried out on the same *ipt*-shooty pEXM2 lines (Fig. 4F). In this case DNA samples underwent the double digestion *Hind*III/*Eco*RI since the use of the single *Hind*III digestion revealed identical hybridization patterns for all the tested lines (not shown). As expected no hybridization signal was evident in the non transformed control line (Fig. 4F, lane 1). The *nptII* probe recognized three different DNA fragments ranging between 8.0 and 20 kb in one of the tested pEXM2 lines (Fig. 4F, lanes 2 and 3). Two hybridization signals of approximately 9.0 and 18.0 kb were evident in the second poplar line (Fig. 4F, lane 3). An hybridization band of approximately 8.0 kb was detected in the third line (Fig. 4F, lane 4). Similarly, all the tested pEXM2 lines with normal shoots were found to contain the predicted 599 bp *nptII* fragment (Fig. 5B, *nptII*, lanes 3–6). The expected *nptII* fragment was not amplified in the non transformed control line (Fig. 5B, *nptII*, lane 1). A positive control (pEXM2 vector) was also tested

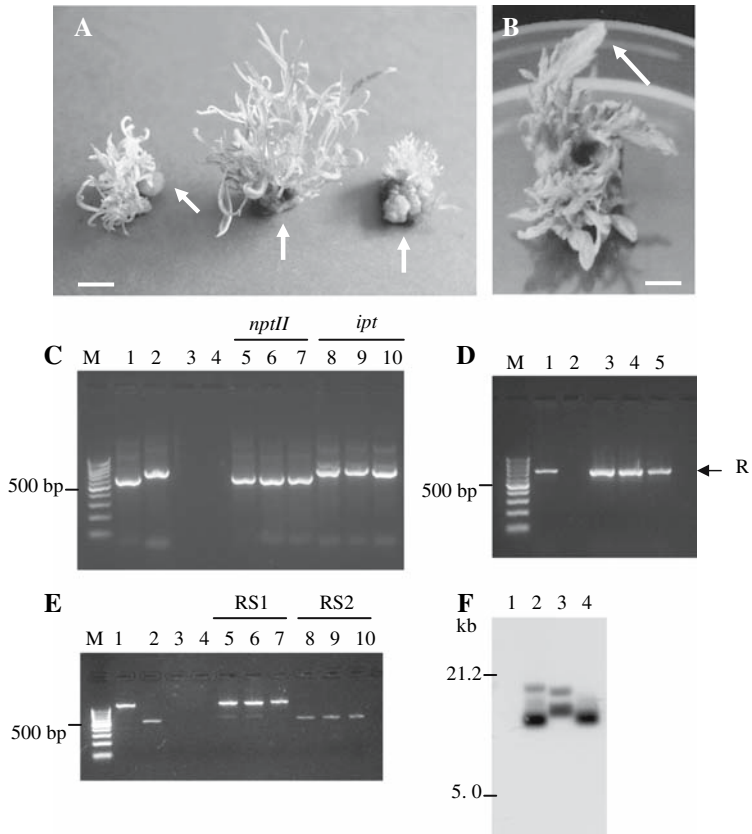

Fig. 4 (**A**) Three different pEXM2 lines showing the typical *ipt*-shooty phenotype. The presence of callus tissue at the contact sites with the agar medium is indicated by arrows. Bar: 1 cm. (**B**) New normal shoots elongating from an *ipt*-shooty plantlet are indicated by arrows. Bar: 0.5 cm. (**C**) Molecular analysis of genomic DNA from three different *ipt*-shooty lines regenerated following transformation of *P. alba* cv. 'Villafranca' with EHA105 carrying pEXM2. The *nptII* transgene sequence was evidenced by PCR analysis using K-1 and K-2 oligonucleotide primers (*nptII*, lanes 5–7). The *ipt* transgene sequence was revealed using IPT-1 and IPT-2 oligonucleotide primers (*ipt*, lanes 8–10). (**D**) The sequence corresponding to the coding region of the *R* gene from yeast was evidenced using the oligonucleotide primers REC-1 and REC-2 (lanes 3–5). (**E**) The presence of the "hit and run" cassette was demonstrated using oligonucleotide primers RS1-A and RS1-B (RS1) and oligonucleotide primers RS2-A and RS2-B (RS2) (lanes 5–7 and 8–10, respectively). All the oligonucleotide sets were used with the pEXM2 plasmid (positive control, **C–E**, lane 1) and with the non transformed line (**C–E**, lane 2). M, Gene Ruler^{TM} 100 bp DNA Ladder (M-Medical S.r.l.). (**F**) Southern blot analysis of the three different *ipt*-shooty lines regenerated following transformation of *P. alba* cv. 'Villafranca' with EHA105 carrying pEXM2. Lane 1, non transformed control line; lanes 2–4, *ipt*-shooty. DNAs were digested with *Hind*III/*Eco*RI and probed using the [α-^{32}P]-dCTP-labelled 599 bp *nptII* fragment

(Fig. 5B, *nptII*, lane 2). The 800 bp *ipt* fragment was not amplified in the same lines, thus indicating the excision of the 'hit and run' cassette and the negative results obtained using the RS2-A and RS2-B oligonucleotide primer sets seem to further confirm that the excision had occurred (data not shown). When PCR was carried out using oligonucleotide primers designed on the *uidA* coding sequence, the expected 1.0 kb fragment located outside the 'hit and run' cassette was detected (Fig. 5D, lanes 3–6). Positive and negative controls are also shown (Fig. 5B *nptII*, lanes 1 and 2; *uidA*, lanes 1 and 2). Results from PCR analysis confirmed that all the phenotypically normal pEXM2 lines regenerated in this transformation experiment were *ipt* marker-free transgenic poplar plants deriving from the early excision of the 'hit and run' cassette and the consequent removal of the morphological marker *ipt* gene.

The presence and stable integration of the *nptII* sequence was subsequently confirmed by Southern

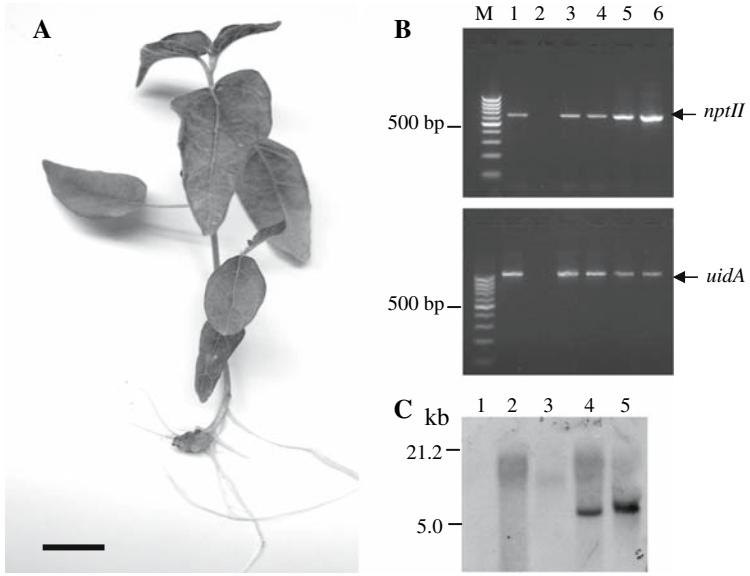

Fig. 5 Production of *ipt* marker-free transgenic poplar plants by 'single-step' transformation. **(A)** Phenotypically normal poplar plantlet resulting from the early excision of the *ipt* gene caused by the 35SCaMV-driven recombinase activity. Bar: 1 cm. **(B)** Molecular analysis of genomic DNA from phenotypically normal shoots regenerated following transformation of *P. alba* cv. 'Villafranca' with EHA105 carrying pEXM2. The *nptII* transgene sequence was evidenced by PCR analysis using K-1 and K-2 oligonucleotide primers (*nptII*, lanes 3–6). The *uidA* sequence was detected using the gene-specific oligonucleotides uidA-1 and uidA-2 (*uidA*, lanes 3–6). All the oligonucleotide primer sets were used with the pEXM2 plasmid (positive control, lane 1) and with the non transformed control line (lane 2). M, Gene Ruler^{TM} 100 bp DNA Ladder (M-Medical S.r.l.). **(C)** Southern blot analysis of the four different pEXM2 lines with normal phenotype (lanes 2–5). Non transformed control line (lane 1). DNAs were digested with *Hind*III and probed using the [α-^{32}P]-dCTP-labelled 599 bp *nptII* fragment

blot hybridization analysis using the gene-specific probe (Fig. 5C). *Hind*III digestion of genomic DNAs from the non transformed control line and the four marker-free transgenic pEXM2 lines was carried out. As expected, no hybridization bands were found in the genomic DNA from the non transformed control. Hybridization fragments of approximately 15–18 kb were detected in two different lines (Fig. 5C, lanes 2 and 3) while a stronger signal of approximately 6.0 kb was detected in the other two lines (Fig. 5C, lane 4 and 5).

Discussion

The efficacy of the morphological marker genes *ipt* and *rolABC* from *Agrobacterium* spp. and the activity of the yeast site-specific recombination R/RS system carried by an *ipt*-type MAT vector were evaluated using an elite *Populus alba* genotype. The typical morphological changes (lack of apical dominance and rooting ability) induced by the overexpression of the *ipt* oncogene were observed in the transgenic white poplar lines obtained following transformation with the pIPT5, pIPT10 and pIPT20 constructs. These constructs were already tested in hybrid aspen by Ebinuma and Komamine (2001). These authors reported that the use of pIPT20 construct resulted into regeneration of transgenic shoots at the highest frequency. Differently from this report, no statistically significant differences in transformation efficiency were observed in our experimental conditions. The *ipt* oncogene from *A. tumefaciens* is considered a regeneration-promoting factor due to its ability to stimulate plant regeneration from explants by the organogenic pathway (Zuo et al. 2002). Overexpression of the *ipt* gene produces high cytokinin levels and consequently the direct formation of shoots from the organogenic cells. In our experimental conditions, the co-cultivated poplar internodal explants regenerated shoots only in the presence of growth regulators. These results suggest that the

amount of cytokinins produced by the activity of the bacterial isopentenyltransferase could not be sufficient for stimulating the organogenesis pathway in the white poplar cells. Another possible explanation might be related to the response of 'Villafranca' to genetic transformation with bacterial oncogenes. Balestrazzi and coworkers (2000) reported the occurrence of tumors when the same genotype was inoculated with A281 and 82.139 wild type *A. tumefaciens* strains. When excised tumors were cultivated in the absence of growth regulators, shoots with normal phenotype were regenerated. Based on this evidence, it might be hypothesized that additional functions, provided by other oncogene sequences contained in the T-DNA, such as the *iaaH* and *iaaM* genes involved in auxin biosynthesis, may be required to promote shoot differentiation in 'Villafranca'. Von Schwartzemberg et al. (1994) reported on poplar transformation with the *ipt* gene, using the clone 717-1B4 (*Populus tremula* × *P. alba*) with the aim to provide a model system for the study of cytokinin metabolism. Although the poplar genotype and the callus induction protocol differed from our experimental conditions, similarly, regeneration of *ipt*-shooty plant lines was achieved in the presence of the cytokinin TDZ.

The *rolABC* genes from the Ri T-DNA of *A. rhizogenes* have been also used as morphological markers due to their ability to induce hairy roots. The *rol* genes have been already proved to modify the architecture of transgenic poplars as reported by Nilsson et al. (1996). Transgenic aspen engineered with the CaMV35S-*rolC* construct and showing both reduced size and small leaves were described by Fladung et al. (1997). The expression of the *rolABC* coding sequences in transgenic aspen was associated with reduced apical dominance and breaking of axillary shoot buds (Tzfira et al. 1998). Moreover, since the expression of *rolC* in aspen produced light-green coloured leaves, this phenotypic feature was used to test in forest trees the recombination events mediated by the transposable element *Ac* from maize (Fladung et al. 1997).

The *rol*-type MAT vector system has been tested in *N. tabacum* (Ebinuma and Komamine 2001) and *A. majus* (Cui et al. 2000, 2001). These studies proved that the *rolABC* genes can be used as a selection marker for *Agrobacterium*-mediated transformation. The *rolABC* genes, introduced for the first time into white poplar genotype using the pROL20 construct, were able to induce the typical abnormal phenotype usually associated with their expression in planta (Fig. 2B). Plantlets were characterized by the presence of abundant hairy roots on stems. Moreover, transgenic plants with bushy phenotype were obtained (data not shown). Similar observations were already reported by Nilsson et al. (1996) who transformed *P. tremula* × *P. tremuloides* with a different construct carrying only the *rolC* sequence. Although kanamycin was used in our experiments as selection agent during callus induction and regeneration phases, the morphological changes induced in white poplar by *ipt* and *rolABC* oncogenes proved the feasibility of the MAT vector system as a tool for the gene transfer and the visual detection of transformants.

The efficacy of the yeast site-specific recombination R/RS system in removing the marker gene *ipt* in white poplar cells was evaluated using the control vector pEXM2. When the pEXM2 construct was used to transform the 'Villafranca' cultivar, the frequency of co-cultivated explants that produced Km^R plants, was 7.2%. This value was similar to the average transformation efficiency (7–11%) already reported for 'Villafranca' (Balestrazzi et al. 2000; Delledonne et al. 2001), achieved using only the *nptII* gene as selectable marker. Endo et al. (2002) has shown that the effectiveness of the *nptII* gene as selection marker was enhanced when it was co-transformed with the *ipt* gene. Recent work from Ballester et al. (2006) showed that *ipt* overexpression in *Citrus sinensis* greatly enhanced transformation efficiency, although they used a different transformation MAT vector. Our results did not confirm these findings. Among the eleven pEXM2 plant lines, a fraction corresponding to 63.6% showed the expected *ipt*-shooty phenotype. The latter was evident at approximately three-four months from the beginning of the experiment, while an additional four-month period was necessary to see the appearance of normal shoots from the *ipt*-shooty poplar plantlets (Fig. 4B). Our data demonstrated that the yeast recombinase system R/RS was active in removal of *ipt* marker gene from transgenic poplar cells and produced the intended results. Similar observations have been recently reported by Khan et al. (2006) and Ballester et al. (2006) who produced *ipt* marker-free transgenic *Nierembergia caerulea* and *Citrus sinensis* plants, respectively. More

interestingly, the use of the pEXM2 construct generated Km^R lines with normal phenotype (36.4%) which were obtained only three-four months after the beginning of the transformation experiment. The production of *ipt* marker-free transgenic plants without the recovery of *ipt*-shooty intermediates (single-step transformation) was already reported in rice by Endo et al. (2002), using a different *ipt*-type MAT vector. Activation of the 35SCaMV promoter during the early developmental stage has been documented in plant tissues (Ohta et al. 1990; Vancanneyt et al. 1990) and this might explain the results obtained in the white poplar system. In this study we have used a pEXM2 control vector in which the *nptII* gene is located outside the 'hit and run' cassette and the regeneration of *ipt* marker-free shoots was achieved by means of exogenous cytokinins in the presence of kanamycin as selective agent. These experimental conditions could not represent the best way to generate marker-free transgenic poplars, however they resulted into useful information concerning the effectiveness of the in vitro procedure required when testing MAT vectors.

In conclusion, our study illustrates the feasibility of *ipt* and *rolABC* genes as efficient positive morphological markers for the visual selection of transformants in an elite white poplar genotype. Furthermore, this is the first demonstration of the efficacy of the R/RS system in promoting efficiently the early excision of the *ipt* marker gene in a woody plant species, with the consequent rapid production of *ipt* marker-free transgenic plants. The occurrence of single-step transformation events is also described, suggesting for possible future improvements in the production of marker-free GM poplars. However, further experimental work will be necessary to acquire detailed information on the temporal mechanism involved in the precocious activation of yeast recombinase and to assess the practicality and efficiency of the single-step phenomenon.

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