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Recovery of transgenic trees after electroporation of poplar protoplasts

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Protoplasts from leaflets of *in vitro* cuttings were electroporated in osmotically adjusted and buffered solutions containing plasmid DNA: pABD1, carrying the *nptII* gene for resistance to neomycin; pGH1, carrying a mutant acetolactate synthase gene, *als*, for resistance to sulfonylurea; and pGSFR781A, carrying a synthetic phosphinothricin acetyltransferase (*pat*) for resistance to phosphinothricin (Basta). Gene transfer was repeatedly efficient, without use of carrier DNA, in the range of one transformant for 10^5 to 10^4 protoplast-derived cell colonies. This was probably due to the high plating efficiency (30%) of protoplasts in our culture process. Selection for expression of foreign genes was applied in liquid medium and repeatedly achieved with 30 μM paromomycin for NPTII, 200 nM chlorsulfuron for the mutant ALS of *Arabidopsis* and 25 μM phosphinothricin for PAT expression. Integration of foreign genes into genomic DNA of resistant poplar trees was demonstrated by Southern blot hybridizations, which revealed that for some transformants practically no other part of the vector plasmid than the selected gene was integrated.

Effective processes for protoplast culture, efficient selection at the cell colony stage and gene transfer will provide new possibilities in poplar breeding.

Keywords: gene transfer; electroporation; stable transformation; protoplasts; transgenic trees; *Populus tremula* × *P. alba*

Introduction

As a result of their rapid growth rate and world distribution, the aspens and the poplars have been studied for years (Ahuja, 1987). For some species, sexual maturity can be reached in less than ten years (Lemoine, 1973) and as a consequence poplar genetics has advanced faster than for other forest trees. Poplars display a natural predisposition to hybridize, so that interspecific crosses have been carried out in many breeding programmes and very interesting hybrids with superior growth rates have already been selected. Some hybrids have gained such sylvicultural and economic importance that they are now exploited as clones, owing to their easy vegetative reproduction for production of paper pulp, packing material, plywood and hardboards. Nowadays, breeding objectives can be roughly arranged in three classes: (1) further improvement of growth rates to accelerate rotation cycles for renewable energy and chemical production; (2) improvement of wood characteristics towards better structure for timber quality, as well as control of lignification; (3) improvement of resistance against viruses, bacteria, fungi and pests to secure industrial plantations from eventual explosions of existing pathogen populations. The diversification of uses for poplars, as well as the increase in area under culture, has thus reinforced the interest in many countries in the development of innovative and powerful breeding schemes for poplars.

In more recent years, besides this reappraisal of breeding programmes, poplars have also emerged as scientific model trees. In addition to simplicity of their sexual and vegetative reproduction, poplars have a small genome size (Dhillon *et al.*, 1984). Maternal inheritance has been recently characterized for mitochondria (Radetsky, 1990) as well as for chloroplasts (Mejnartowicz, 1991). Finally, the general ease of plant regeneration through *in vitro* tissue culture, has recently made genetic transformation a reality (Fillati *et al.*, 1987; De Block, 1990; Klopfenstein *et al.*, 1991; McCown *et al.*, 1991; Brasileiro *et al.*, 1992; Nilsson *et al.*, 1992), so that a

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variety of markers and desirable traits are already available or easy to create.

All these experimental possibilities, coupled with an ever increasing economic importance, make poplars good candidates for being a preferential experimental subject for tree physiology and genetics.

However, the use of protoplasts for *in vitro* selection and somatic hybridization for hybrid or cybrid production has not been possible. Although poplar protoplasts have been extensively studied (Kirby *et al.*, 1984), successes in regenerating trees from protoplasts have been limited by the accumulation of an exudate considered to be toxic (Russell and McCown, 1986, 1988; Park and Son, 1992).

Recently, procedures for healthy protoplast development to efficient tree regeneration have been established (Wang *et al.*, 1991; Chupeau *et al.*, 1993). Making use of the rapidity of response of protoplasts, we optimized each step of culture by varying the nature and concentration of practically all the components of the liquid culture medium. Dilution of protoplast derived cell colonies, by simple pipetting in a second liquid medium, ultimately led 3 to 5% of initially plated protoplasts to regenerate trees (Chupeau *et al.*, 1993). However to be really useful, such media have to permit clonal growth. To test the ability of clonal growth for poplar protoplast-derived cell colonies, we verified the possibility of selecting for various biochemical markers. We chose to adapt the electroporation process (Guerche *et al.*, 1987a) for poplar protoplasts, thus providing an alternative procedure for poplar transformation in addition to the gene transfer technique already developed.

Materials and methods

From protoplasts to trees

A female hybrid of *Populus tremula* × *P. alba* (clone 717 1 B 4), originally developed at INRA Orléans (Lemoine, 1988), was used in this work. Plantlets were propagated *in vitro* in glass tubes. All *in vitro* cultures were conducted in the same culture room at 25 ± 2°C and 16 h of lighting from fluorescent tubes providing about 60 μE $m^{-2}s^{-1}$ on the glass benches, but only 10 μE $m^{-2}s^{-1}$ in the tubes at leaflet level. Relative humidity in the growth room was maintained at 70% ± 5% day and night. Plantlets were propagated in the rooting medium B (Bourgin *et al.*, 1979), except that FeEDTA was replaced by 50 mg l^{-1} of ferric ammonium citrate complex. The best protoplast preparations were obtained with 6-week-old plantlets. Our MOD cocktail of enzymes in GSG maceration medium (Chupeau *et al.*, 1989) was used throughout this work. Before electroporation, protoplasts were washed in MKM buffer: 0.5 M mannitol, 5 mM KCl, 0.2 mM MOPS, pH 7, to ensure a low and reproducible conductivity. A detailed description of the protocol for liquid culture of protoplasts for tree regeneration has already been published (Chupeau *et al.*, 1993). Briefly, the liquid medium composition was the following in mg l^{-1}: 202 KNO_3; 160 NH_4NO_3; 328 $Ca(NO_3)_2$; 440 $CaCl_2$, $2H_2O$; 370 $MgSO_4$, $7H_2O$; 170 KH_2PO_4; 50 Fecitrate NH_4; 0.8 KI; 3 H_3BO_3; 30 $MnCl_2$, $2H_2O$; 12 $ZnSO_4$, $7H_2O$; 0.9 Na_2MoO_4, $2H_2O$; 0.09 $CoCl_2$, $6H_2O$; 0.09 $CuSO_4$, H_2O; 100 inositol; 1 panthotenate Ca; 0.01 biotin; 1 niacin; 1 pyridoxin; 1 thiamin; 3 2,4-D; 0.0011 thidiazuron; 0.7 MES; 8 Bromocresol purple; and glucose at 100 g l^{-1}. After three weeks in culture, cell colonies were diluted in the same overall liquid medium except that 2,4-D was omitted, thidiazuron augmented to 0.022 mg l^{-1} and nitrogen level increased with 1010 mg l^{-1} KNO_3. About one month later, microcalluses were transferred to a gelosed medium with only 0.022 mg l^{-1} thidiazuron which induced bud formation for all transplanted calluses in one month. Buds were then easily rooted on B medium (Bourgin *et al.*, 1979).

Electroporation conditions

A simple device (Guerche *et al.*, 1987a) was used in this work. In short, various capacitors (15, 30 and 63 μF) were charged at the desired voltages (150, 200, 250, 300 and 350 V) and discharged in plexiglass electroporation chambers of 1 ml (1 × 1 × 1 cm) loaded with 2×10^6 protoplasts mixed with 50 μg of plasmid in MKM buffer. Plasmids pABD1 (Paszkowski *et al.*, 1984), pGH1 (Haughn *et al.*, 1988) and pGSFR781A (De Block, 1990), were extracted according to Holmes (1982).

Selection of transformants

Selective levels of the three metabolic inhibitors were evaluated with control cultures. The same timing was applied in each case: protoplast-derived cell colonies were diluted three-fold after three weeks in culture, and three weeks later inhibitors were added directly to the diluted culture. In this scheme, to kill poplar colonies completely, paromomycin was used at 20 mg l^{-1}, phosphinothricin at 5 mg l^{-1}, and chlorsulfuron at 72 μg l^{-1}. The selective pressure was maintained during the bud formation step as well as in the rooting medium.

Plant DNA isolation and Southern analysis

DNA was extracted from leaves of greenhouse trees as reported (Dellaporta *et al.*, 1983) and further purified in a CsCl-ethidium bromide gradient. 10 μg of genomic DNA were digested with restriction enzymes and separated on 0.8% agarose gels. DNAs were transferred and UV-cross linked to Hybond-N filters (Amersham) (Khandjian, 1987). DNA blots were hybridized and washed according to standard procedures (Sambrook *et al.*, 1989). Probes (^{32}P-labelled) were synthesized using random oligonucleotide primers and ^{32}P-dCTP (New England Nuclear) according to Feinburg and Volgestein (1983).

Results

Preparation and culture of protoplasts

Standard procedures initially developed for preparing protoplasts from various *Nicotiana* species and recently adapted to lettuce (Chupeau *et al.*, 1989) proved effective for poplar protoplasts. However, as for lettuce protoplasts, replacement of mannitol in the maceration medium by an iso-osmotic mixture of glucose-sorbitol-glycine improved the viability of poplar protoplasts. Culture media for the hybrid poplar used here have been described in detail (Chupeau *et al.*, 1993). In short, culture in liquid media without exudate accumulation was possible as a result of four essential modifications: (1) use of 550 mM glucose as the only source of carbon and energy, (2) lowering the nitrogen supply to 2 mM KNO_3 + 2 mM NH_4NO_3, (3) use of 0.1 μM thidiazuron as the only cytokinin throughout the culture process, in conjunction with 14 μM 2,4-D for the three first weeks, (4) modification of the oligoelement formulation. In this liquid medium, cell colony yields were repeatedly between 30 and 50% of plated protoplasts. Poplar cell colonies were diluted into fresh liquid medium with only 0.1 μM thidiazuron, and subsequently 12% formed microcalluses. As tree regeneration was about 100% from calluses, the overall regeneration efficiency was up to 6% of initially plated protoplasts.

Electroporation conditions

We chose to assess directly electroporation conditions for stable transformation, since there was still no convincing correlation between poration conditions for optimal transient activity and those for high rates of stable transformation. For this purpose, we applied various conditions to poplar protoplasts mixed with 20 μg pABD1. The first successful selection for resistance to paromomycin was obtained with protoplasts submitted to three discharges of a 15 μF capacitor charged at 250 V. It was a surprise since the same procedure was effective for transformation of tobacco (Guerche *et al.*, 1987a), rape (Guerche *et al.*, 1987b), potato (Masson *et al.*, 1989) and lettuce (Chupeau *et al.*, 1989). As poplar protoplasts are smaller (15 μm in diameter) than the protoplasts of the other species (about 60 μm), the theory (Neumann *et al.*, 1982) predicted that poplar protoplasts require stronger discharges to be effectively permeabilized. A more precise and consistent analysis revealed that the conductivity of the electroporation buffer (MKM) stayed at 425 μS even after addition of 2×10^6 protoplasts, and even after three 250 V discharges of the 15 μF capacitor. This stable low conductivity made the discharges more efficient as they lasted longer, about 175 ms. Furthermore, this stable low conductivity revealed the resistance of poplar protoplasts to electroporation. In comparison, the conductivity of the same buffer with 2×10^6 lettuce protoplasts was up to 1000 μS, owing to leakages of solutes and protoplast instability, and was about 1500 μS after three 250 V discharges of the 15 μF capacitor, which lasted 50 ms (Chupeau *et al.*, 1989).

Selection for transformed cell colonies

The above electroporation conditions were not too detrimental to protoplasts. After electroporation, 20% of the poplar protoplasts were still able to form cell colonies, versus 30% for control cultures. Given this reduction of 10% in plating efficiency, we adapted the optimal process of dilution used for standard cultures. Three weeks after starting cultures of electroporated protoplasts, colonies were diluted by three, and selection was applied three weeks afterwards. At first we used 20 mg (30 μM) paromomycin, which had already proved to be more efficient than other neomycins for selecting tobacco (Guerche *et al.*, 1987a), and lettuce (Chupeau *et al.*, 1989) cell colonies expressing NPTII at selectable levels.

In these conditions, resistant poplar cell colonies were repeatedly selected, in variable number according to the various batches of protoplasts, but each time in the range of 1 resistant colony for 10^5 to 10^4 growing colonies in control cultures. Resistant calluses formed green buds on regeneration medium with 100 mg l^{-1} kanamycin, and these buds rooted normally on rooting medium plus 100 mg l^{-1} kanamycin. This constituted a first confirmation of their resistance as rooting is especially sensitive to the presence of neomycins. Twelve independent resistant trees grown in a greenhouse exhibited normal appearance and growth rates. Southern blots with DNA extracted for some trees confirmed further that the *nptII* gene was integrated in the genome of neomycin resistant trees (Fig. 1).

To make sure that the selection scheme developed for paromomycin could be of general use, we also selected for phosphinothricin and chlorsulfuron resistances after electroporation, in the above conditions, with the corresponding plasmids respectively pGSFR781A and pGH1. In both cases resistant colonies were selected with relative frequencies between 10^{-5} to 10^{-4} (Table 1).

A selecting dose of 5 mg l^{-1} (25 μM) of phosphinothricin was applied three weeks after dilution of protoplast-derived cell colonies. This dose consistently killed all the cell colonies over a week in control cultures. Chlorsulfuron (DPX 4189) was not so rapidly lethal and had to be added at the rather high dose of 72 μg l^{-1} (200 nM) to effectively select resistant calluses without any escapes.

In both cases, the majority of selected colonies (56 out of 73) regenerated tress on selective media. Again resistance was biologically confirmed by root formation for all selected trees on rooting medium with 25 μM phosphinothricin or 50 nM chlorsulfuron, which appeared to be the lowest doses which killed non-transformed *in vitro* cuttings in the rooting medium. Low numbers of nonregenerating calli, is a good indication that the selection schemes were quite efficient (Table 1).

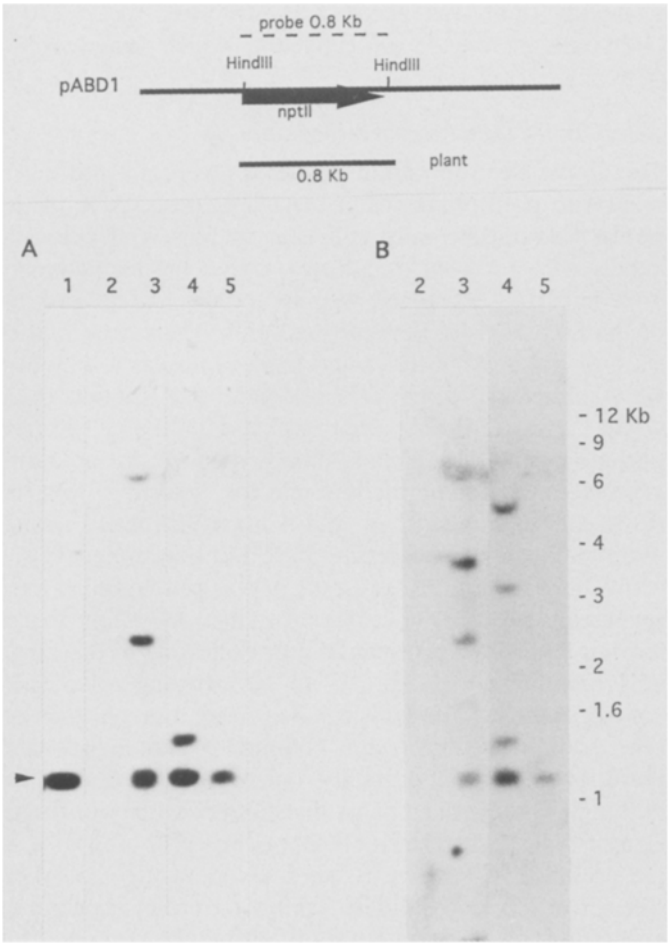

Fig. 1. Southern blot analysis of three independent neomycin resistant trees and diagram of pABD1. DNA was restricted by *Hin* dIII and hybridized with a probe corresponding to the 0.8 kb *Hin* dIII fragment of pABD1 (panel A) and with an entire pABD1 probe (panel B). Panel: lane 1: 90 pg of pABD1 restricted with *Eco* RI representing two copies per haploid poplar genome. For panel A and B lane 2: DNA of a control tree. Lane 3: DNA of tree 1a. Lane 4: DNA of tree 8a. Lane 5: DNA of tree 23a.

Some transformed trees were able to root and grow *in vitro* on rooting media with higher doses of inhibitors (Table 2). Again, transgenic trees developed normally as compared to those from normal cuttings arising at the same time in the greenhouse.

DNA analysis

Southern blot analysis confirmed the integration of the coding sequences as well as rearranged gene fragments. Three independent kanamycin-resistant trees contained the *nptII* gene in the range of 1 to 3 copies (Fig. 1, panel A), and limited numbers of fragments that did not comigrate with the 1.2 kb *Eco* RV *nptII* insert. For the resistant tree no. 23 (Fig. 1, panel A, lane 5), blots indicated that a single copy was integrated along with a single additional fragment showing such a faint signal that it suggested a large deletion of the *nptII* sequence. Hybridization with the entire pABDI plasmid (Fig. 1, panel B) revealed a limited number of additional fragments. No additional fragment was detected in tree no. 23, thus confirming a simple insertion event.

The various patterns of hybridization indicated an independent origin for each selected colony. We also analysed several regenerated trees arising from the same selected callus. They exhibited the same hybridization profile (data not shown) which suggested that our culture scheme effectively selected independent and stable transformation events and, that culture, selection and regeneration steps did not induce any instability or variation.

The same conclusions were reached after Southern blots performed with genomic DNA from leaves of chlorsulfuron resistant trees. Profiles appear a little more complicated because we chose to digest DNA with *Eco* RI thus generating two fragments in the *als* gene in the hope of gaining a more detailed analysis of insertions in relation with the various classes of resistance as revealed by rooting tests (Table 2). Chlorsulfuron-resistant trees did contain the *als* gene without modification since the two expected *Eco* RI fragments of 2.1 and 2.4 kb are detected

Table 1. Transformation frequencies generated through electroporation of poplar protoplasts

Selection for	*Plasmid amounts μg*	*Total porated protoplasts*	*Selected with*	*Number of selected colonies*	*Frequency*	*Independent bud-forming colonies*	*Number of independent trees*	*Relative frequency*[a]
PAT	50	2×10^6	phosphinothricin (25 μg)	13	7.5×10^6	9	9	2.10^{-4}
PAT	150	6×10^6	phosphinothricin (25 μg)	8	1.3×10^6	6	6	5.10^{-5}
ALS	100	4×10^6	DPX4189 (200 nM)	52	1.3×10^5	42	41	5.10^{-4}

[a]Calculated on the basis of 2% electroporated protoplasts ultimately regenerating trees.

Table 2. Range of resistance of regenerated poplars

Chlorsulfuron nM	100	500	1000	2000	5000
Number of resistant trees	41	30	23	8	2
Phosphinothricin μM	45	150	500	2500	5000
Number of resistant trees	9	9	9	8*	6[a]

Independent trees from resistant colonies were rooted on media with 50 nM chlorsulfuron or 15 μM phosphinothricin. Cuttings of each tree were transferred to rooting medium with 100 nM chlorsulfuron or 45 μM phosphinothricin. Cuttings of each tree were then transferred to the higher dose and again to the next one.
[a]Although cuttings were rooting and growing, development was slow and modified as leaves were shortened, except for one tree growing quite normally at least *in vitro*.

by the 5.8 kb *Xba* I portion of pGH1 used as probe (Fig. 2, panel A). Here again, the variable additional fragments of rearranged copies of *als* revealed the independent origin of the 6 trees chosen for analysis (Fig. 2, panel A). Tree Y (Fig. 2, panel A, lane 6), which rooted *in vitro* on 1000 nM chlorsulfuron, showed quite a simple transformation event without any significant integration other than the *als* gene as revealed by hybridization with the entire pGH1 as a probe (Fig. 2, panel B, lane 6). Hybridization with the entire plasmid (Fig. 2, panel B) proved again that the selection scheme did select effectively for the desired gene and not for other parts of the plasmid.

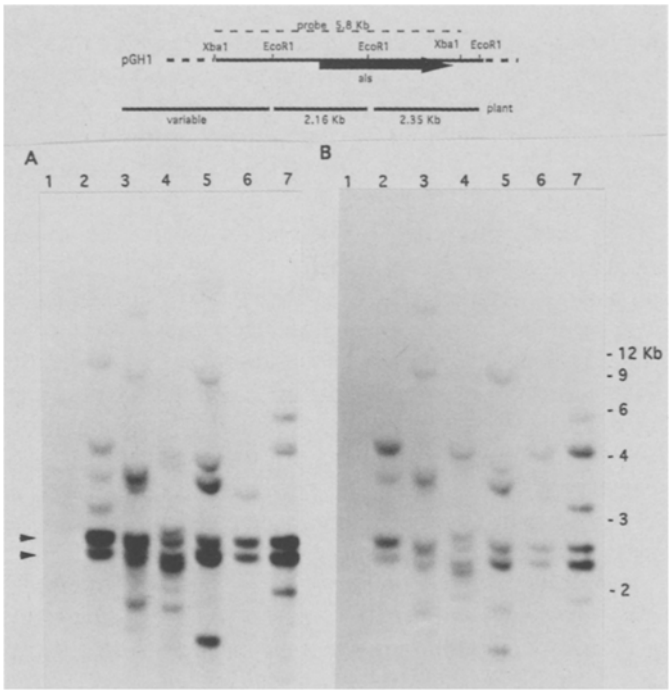

Fig. 2. Southern blot analysis of 6 independent chlorsulfuron-resistant trees and diagram of pGH1. DNA was restricted by *Eco* RI and hybridized with a probe corresponding to the 5.8 kb *Xba* I fragment of pGH1 (panel A) and with the entire pGH1 as a probe (panel B), for each panel: lane 1: DNA of a control tree. Lane 2: DNA of tree F resistant to 100 nM. Lane 3: DNA of tree L resistant to 100 nM. Lane 4: DNA of tree P resistant to 100 nM. Lane 5: DNA of tree S resistant to 2000 nM. Lane 6: DNA of tree Y resistant to 1000 nM. Lane 7: DNA of tree K resistant to 2000 nM.

Discussion

We verified that the 'en masse' culture procedure already established for protoplasts of this hybrid poplar also permitted early and effective biochemical selection for resistance to various metabolic inhibitors. This constitutes a further indication of the precise adaptation of the compositions of the culture media for the clonal growth of young protoplast-derived cell colonies of poplar. The demonstration of clonal growth was made accessible to experimentation through direct transfer of selectable markers to poplar protoplasts. This appears to be the first report of successful stable gene transfer to trees by means of protoplast electroporation.

The somewhat slow growth of poplar cell colonies did not complicate the definition of effective selective doses of paromomycin and phosphinothricin. However, non-transformed cell colonies often resumed growth after two or three months of selection with chlorsulfuron below 200 nM. In contrast to antibiotics, chlorsulfuron does not kill cells, as the inhibition of the acetolactate synthase generates a progressive starvation in valine and isoleucine (Haughn *et al.*, 1988). It is possible that this hybrid poplar has a naturally higher ALS activity than *Arabidopsis* and tobacco for which a 10 nM chlorsulfuron dose is suitable for selection (Haughn *et al.*, 1988).

The frequency of stable transformations, about the same for the three marker genes we selected for, in the order of 10^{-4}, proved to be convenient for furnishing adequate numbers of independent transformed trees. Transformation frequencies work out at higher rates when expressed as about 0.5 transformant per μg DNA but remain quite low (200 times lower) in comparison with the average efficiency of transformation of animal cells (Neumann *et al.*, 1982).

It was a surprise to us that our standard electroporation conditions proved effective with poplar protoplasts. At first, we expected to have to apply higher electric fields to permeate poplar protoplasts due to their small size. In fact, the low conductivity of the electroporation buffer made the electric discharge more effective. In addition, we had suspected that the rather long delay between electroporation and the onset of the first divisions five days later, would result in a very low frequency of transformation. The efficiency of transformation observed in this work might result from a high DNA repair activity associated with a low DNase level in poplar protoplasts.

Additionally, we would like to stress that direct transfer can be effective with low amounts of plasmid DNA, in contrast to the high amounts required for transient activity measures (Tautorus *et al.*, 1989) especially for tree protoplasts in which RNA and protein synthesis do not peak as early as in model system such as tobacco protoplasts.

The most interesting aspect of this work lies in the confirmation that direct transfer through electroporation can generate simple and clean integration of desired genes (Meyer *et al.*, 1992). Hence electroporation should no longer be compared unfavourably to the supposedly more defined process of transformation mediated by disarmed *Agrobacterium* Ti plasmids (Spielman and Simpson, 1986). *Agrobacterium* mediated transfer of the same genes (*nptII* and mutant *als*) to the very same hybrid poplar led to multicopy and multisite insertions at least for one construct (Brasileiro *et al.*, 1992).

Practically, the possibility of clonal growth for poplar cells provides additional experimental flexibility for transformation or mutant selection *in vitro*. Additionally, use of complementary traits to select for hybrid cells after fusion of protoplasts will secure an important step toward the use of somatic hybridization within the Salicacae family.

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