Plant Cell Reports (1994) 13:256–261

Plant Cell Reports

Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*

M. Confalonieri [1], A. Balestrazzi [2], and S. Bisoffi [1]

[1] Istituto di Sperimentazione per la Pioppicoltura SAF/ENCC, Via die Frassineto 35, I-15033 Casale Monferrato (AL), Italy
[2] Dipartimento di Genetica e Microbiologia "A. Buzzati Traverso", Via Abbiategrasso 207, I-27100 Pavia, Italy

Received 8 March 1993/Revised version received 9 September 1993 – Communicated by G. Pelletier

Abstract

Two clones of *Populus nigra* L. were tested *in vivo* and *in vitro* for their susceptibility to three different *Agrobacterium tumefaciens* wild-type strains evaluating number and size of resulting calluses. Strain C58 proved to be the most virulent.

Various parameters affecting *Agrobacterium*-mediated transformation of *P. nigra* clones were further analyzed using ß-glucuronidase gene transient expression. The clone Jean Pourtet proved to be more susceptible than the clone San Giorgio. *A. tumefaciens* strain A281 pKIWI105 proved to be the most virulent. The optimal procedure involved dipping of leaf discs into a bacterial suspension ($7x10^8$ cells/ml) for 20 min, followed by a 48 h co-cultivation period on semi-solid regeneration medium.

Leaf explants were co-cultivated with two disarmed *A. tumefaciens* strains. Plantlets of San Giorgio were regenerated, tested for ß-glucuronidase activity and rooted on selective medium containing kanamycin. Polymerase chain reaction analysis and Southern blot hybridization confirmed the integration of the neomycin phosphotransferase II gene into the poplar genome.

Key words: *Populus nigra* L., *Agrobacterium tumefaciens*, Genetic transformation

Abbreviations. BAP: 6-benzyl-aminopurine, CaMV: Cauliflower Mosaic Virus, 2,4-D: 2,4-dichlorophenoxyacetic acid, GUS and *gus*: ß-glucuronidase, *hpt*: hygromycin phosphotransferase, IBA: indole-3-butyric acid, KIN: kinetin, LB: Luria Bertani, MS: Murashige and Skoog, NAA: ß-naphthaleneacetic acid, NOS: Nopaline synthase, NPTII and *nptII*: neomycin phosphotransferase II, PCR: Polymerase chain reaction, PVC: poly-vinyl-cloride, SDS: sodium dodecyl sulfate, SSC: sodium cloride-sodium citrate, Tris: tris(hydroxymethyl)amino-methane, WPM: Woody Plant Medium.

Correspondence to: M. Confalonieri

Introduction

Conventional breeding of forest trees proceeds at a rather slow pace due to great dimensions of the plants, long juvenile period, seasonal dormancy cycles and the scanty information on the heritability of important characters. Although displaying faster growth rates than most tree species, poplars and aspens (*Populus spp.*) are no exception.

In the temperate regions of the world poplars represent a primary source for the production of wood. These trees represent the majority of all artificial forests (FAO 1980); due to fast growth and short rotations poplars are the only solution to the growing wood demand in many countries.

The black poplar (*P. nigra* L.) is widely employed worldwide for wind-breaks and landscaping. It is also used as male parent in crosses with *P. deltoides* Bartr. for the production of *P. euramericana* hybrids.

The genus *Populus* shows some genetic resistance to pests and pathogens, but classical breeding/selection is not yet in the position to rapidly incorporate these traits into the genetic background of commercially appreciated clones, because of both heterozygosity and incompatibility.

Given the quite high susceptibility to *A. tumefaciens* and to the amenability to *in vitro* manipulation, poplar can profit from the application of advanced molecular techniques.

Although gene transfer by disarmed strains of the soil bacterium (*A. tumefaciens*) is now routinely applied to several dicotyledonous species, this is not yet the case for poplars, even if transformation of aspens and poplars has been already described (Fillatti *et al.* 1987; De Block 1990; Klopfenstein *et al.* 1991; Brasileiro *et al.* 1991; McCown *et al.* 1991; Leplé *et al.* 1992; Brasileiro *et al.* 1992; Devillard 1992).

The aim of this study was to establish a transformation procedure for a *P. nigra* clonal variety of commercial interest, obtaining transformants of this species for the first time.

Materials and methods

Plant material, tissue culture and regeneration. Two clones of *P. nigra* were used in this study: San Giorgio (cv. *italica*) and Jean Pourtet. Stabilized shoot cultures of these two clones were maintained *in vitro* on hormone-free WPM medium (Lloyd and McCown 1981) and cultured *in vitro* in sterile GA7 boxes (Magenta Corp.) under controlled environmental conditions (25°C, 16 h, 2500 lux; 22°C, 8 h, darkness). Stem nodal segments, with two or three axillary buds, were propagated on WPM supplemented with 0.5 mg/l IBA.

To obtain shoot regeneration, leaf discs were cultivated in Petri dishes on MS medium (Murashige and Skoog 1962) with 0.5 mg/l BAP and 0.2 mg/l NAA. For callus induction, stem segments longitudinally split into two halves were cultured on modified MS medium (macrosalts 3/4 MS) containing 0.5 mg/l 2,4-D and 0.5 mg/l KIN. These media were solidified with 0.4% (w/v) agar (Merck) and 0.2% (w/v) gerlite (Schweizerhall).

Leaf discs from San Giorgio and Jean Pourtet were cultured on regeneration medium in the presence of increasing concentrations of kanamycin (0,2,10,50,100 mg/l, Sigma) and hygromycin (0,0.5,2,5,10 mg/l, Sigma) in order to establish selective conditions.

Assessement of *in vitro* and *in vivo* susceptibility of *P. nigra* clones to wild type *A. tumefaciens* strains. Three wild type strains were used: C58, Ach5 (Phabagene Collection, University of Utrecht, NL); A281 (An G., Washington State University, Pullman USA). These were of different opine types: nopaline (C58), octopine (Ach5) and agropine (A281). C58 strain has been previously used to study susceptibility of intra and inter-specific hybrid poplars (Riemenschneider 1990). Ach5 and A281 strains are used for the same purpose in other species (Mackay *et al.* 1988; Clapham *et al.* 1990). For each strain a Petri dish, containing 25 ml of LB solid medium (Sambrook *et al.* 1989), was inoculated with a single bacterial colony the day before stem inoculation took place.

The *in vivo* infection was performed using twenty plants for each clone/strain combination. Inoculation was carried out in a greenhouse at temperatures of 20-25°C, and with a high relative humidity (70-90%). Plants of San Giorgio and Jean Pourtet were wounded on the stem (two wounds/plant) with a surgical blade. Immediately after wounding, bacteria were applied to the wounds and the inoculation sites were covered with a PVC-foil, for ten-twenty days, to prevent desiccation.

In vitro cultures of two clones were grown in test tubes on hormone-free medium in a controlled environment chamber (22°C, 16/8 h light/darkness period, 2500 lux). Ten plantlets of each clone were infected at one point on the stem with wild type strains.

In all tests, wounded but not inoculated plants were used as controls. The percentage of inoculated sites showing the growth of undifferentiated tissue was investigated two-three weeks after inoculation.

Transient GUS expression in leaf discs and stem segments co-cultivated with *A. tumefaciens* strains. Evaluation of optimal conditions was as follows.

Leaf discs and stem segments were co-cultivated with a disarmed strain of *A. tumefaciens* GV2260 p35S GUS (Vancanneyt *et al.* 1990), which is a derivative of the C58 strain; the binary vector contains both the *nptII* and *gus* gene coding sequences (the latter one expressed only in eukariotic cells). Several factors were studied:

- pre-cultivation period; explants were cut either 24 h or just before co-cultivation. Leaf discs were cultured in Petri dishes in the dark on regeneration medium while stem segments were cultivated on callus induction medium.
- concentration of bacterial suspension; bacteria were grown overnight in LB medium with appropriate antibiotics on a rotary shaker (120 rpm) at 25°C. The bacterial suspension was centrifuged (2500 rpm, 15 min) and resuspended in MS medium before inoculation. Three different concentrations were tested ($2x10^8$, $7x10^8$, $1.2x10^9$ cells/ml; OD_{550nm}).
- co-cultivation; explants were placed into the *A. tumefaciens* suspension for 20 min, blotted dry and co-cultivated for 24 h or 48 h in the dark either on liquid MS or semi-solid (regeneration/callus induction) medium.

Ten leaf discs and ten stem segments were used for each combination of the analyzed factors.

Subsequently leaf discs taken from the same clones of *P. nigra* were co-cultivated with four different strains of *A. tumefaciens* (GV2260 p35S GUS; C58 pKIWI105, A281 pKIWI105 (oncogenics) and LBA4404 pKIWI105 (Janssen and Gardner 1989)). The characteristics of these strains are summarized in Table 1. The plasmid pKIWI105 contains the coding sequence of the *nptII* gene driven by the NOS promoter and the coding sequence of the *gus* gene driven by the CAMV 35S promoter. The *gus* gene gives negligible expression in *A. tumefaciens*.

Table 1 Characteristics of the *A. tumefaciens* strains used.

Strain	Chromosomal background	Type of plasmid	Characteristics
GV2260 p35S GUS	C58	binary	pNOS-*NPTII*; p35S-*GUS*
A281 pKIWI105	C58	binary, oncogenic	pNOS-*NPTII*; p35S-*GUS*
C58 pKIWI105	C58	binary, oncogenic	pNOS-*NPTII*; p35S-*GUS*
LBA4404 pKIWI105	Ach5	binary	pNOS-*NPTII*; p35S-*GUS*
GV3850 HPT pKU2	C58	cointegrate	pOCS-*NPTII*; p35S-*HPT*

Two different concentrations ($7x10^8$; $1.2x10^9$ cells/ml) of bacterial suspension were tested. Fifteen leaf discs were used

for each combination clone/strain/concentration. Leaf discs were pre-incubated in the dark for 24 h on regeneration medium and subsequently placed in bacterial suspension for 20 min, blotted dry and co-cultivated for 48 h in the dark on the same culture medium.

In both experiments, after co-cultivation explants were rinsed with double-distilled sterile water containing 1.5 g/l of carbenicillin, blotted dry and transferred in the dark on selective regeneration (or callus induction) medium containing 300 mg/l carbenicillin (Pfizer), 300 mg/l cefotaxime (Hoechst) and 100 mg/l kanamycin. All the cultures were kept in a controlled environment chamber (25°C, 16 h; 22°C, 8 h). Three days after co-cultivation the GUS activity was assayed histochemically as described by Jefferson (1987). For each combination the number of single GUS^+ spots per explant was counted. Data were analyzed with analysis of variance following logarithmic transformation.

Genetic transformation. Two tests were carried out using leaf discs of *P. nigra* (San Giorgio, Jean Pourtet) and two different strains of *A. tumefaciens* GV2260 p35S GUS and GV3850HPT pKU2 (Baker *et al.* 1987). The characteristics of these strains are reported in Table 1. The pGV3850HPT::pKU2 cointegrate plasmid carries the hygromycin resistance gene (*hpt*) and the *nptII* gene. For each strain, two different concentrations of bacterial suspension ($2x10^8$; $6x10^8$ cells/ml) were tested. Moreover, the effect of the presence or absence of light during the first seven days after the co-cultivation period was assessed.

Leaf discs were pre-incubated on regeneration medium, in the dark for 24 h, and subsequently placed in bacterial suspension for 20 min, blotted dry and transferred to regeneration medium for 48 h in the dark. After co-cultivation, they were rinsed for 30 min with sterile double-distilled water containing 1 g/l cefotaxime and 1.5 g/l carbenicillin, blotted dry using sterile filter paper and transferred on selective (300 mg/l cefotaxime, 300 mg/l carbenicillin, 100 mg/l kanamycin, 10 mg/l hygromycin) regeneration medium in a growth chamber (25°C, 16 h, 2500-3000 lux; 22°C, 8 h, darkness). Fifty leaf discs were used for each combination of analyzed factors and levels. Control leaf explants were cultivated on regeneration medium either with or without 100 mg/l of kanamycin.

After one-two months, the regenerating calluses were excised from the leaf discs and cultured separately on the same medium for one month. They were subsequently transferred on modified selective regeneration medium (macrosalts WPM) containing 0.5 mg/l BAP and 0.05 mg/l NAA and then subcultured every four weeks. Shoots were isolated when they were 1-2 cm long, transferred to hormone-free selection medium (100 mg/l kanamycin) for rooting and assayed for GUS activity.

The transformation frequency was calculated as the ratio between the number of leaf discs which regenerated kanamycin resistant plantlets and the number of leaf discs co-cultivated.

DNA isolation and PCR analysis. DNA was extracted from plant tissues according to Rogers and Bendich (1988) by freezing and grinding the plant tissue in liquid N_2.

Each PCR reaction was carried out in 10 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 µM of dATP, dTTP, dCTP and dGTP, 0.25 u of Taq Polymerase (Perkin Elmer, Cetus), poplar DNA (100 ng) and oligonucleotide primers (21 bases each, 50 ng). The denaturation temperature was 90°C (duration 2 min), the annealing temperature was 65°C (duration 1 min) and the extension temperature was 72°C (duration 2 min).

The cycle number was 23 using a DNA Thermal Cycler 480 (Perkin Elmer, Cetus). DNA was detected after running on a 1% (w/v) agarose gel.

For Southern blot analysis, PCR products were subsequently transferred to Zetabind membranes (Cuno Inc.) according to the manufacturer's instructions. Filters were prehybridized for 1-2 h at 42°C in 50% (v/v) formamide, 5x SSC, 0.5% SDS, 5x Denhardt solution, 100 µg/ml sonicated salmon sperm DNA.

pABDI plasmid (kindly supplied by Prof. I. Potrykus, ETH, Zürich) was EcoRV digested and the resulting 1.2 kbp fragment (containing only the translated part of the *nptII* gene) was recovered from 1% (w/v) low melting temperature agarose (Sigma) as described (Sambrook *et al.* 1989). Fifty ng of the fragment were labelled with the "Ready to go" DNA labelling kit (Pharmacia), according to manufacturer's instructions.

Hybridization was carried out for 16 h in the same conditions described for prehybridization. Filters were rinsed twice in 2x SSC, 0.1% SDS (10 min) at room temperature, once in 1x SSC, 0.1% SDS (15 min) at 65°C and subsequently exposed to Hyperfilm™-MP (Amersham) with intensifying screens for ten-twenty days.

Results and discussion

Tolerance to selective agents. After sixty days of culture on regeneration medium containing 5 mg/l hygromycin, no shoot or callus formation was observed for any of the leaf discs of either clone. The concentration of kanamycin inhibiting shoot formation was 50 mg/l for Jean Pourtet and 100 mg/l for San Giorgio.

Hygromycin and kanamycin concentrations used in transformation experiments were 10 and 100 mg/l, respectively.

Assessement of *in vivo* and *in vitro* susceptibility of *P. nigra* clones to wild-type *A. tumefaciens* strains. The *in vivo* tests demonstrated that, from a morphological point of view, tumours caused by *A. tumefaciens* and wound healing callus are difficult to distinguish.

The response of two clones of *P. nigra* to *in vitro* infection with several wild-type *A. tumefaciens* strains is reported in Table 2. Growth of undifferentiated tissue was observed in all the combinations used. For both clones, 20% of uninoculated sites (control) displayed a limited growth of undifferentiated tissue. As regards susceptibility to C58 and A281 strains respectively, no differences were noticed between the two clones. For both clones, macroscopic growth of undifferentiated tissue was detected in almost all sites inoculated with C58 strain, which proved to be the most virulent.

Table 2 Absolute frequencies of sites producing undifferentiated tissue growth (B) out of the total number (A) of sites inoculated with wild-type *A. tumefaciens* (*A.t.*) strains in the *P. nigra* clones.

A.t. strains	San Giorgio			Jean Pourtet		
	A	B	C [a]	A	B	C
Ach5	10	9	++	10	5	+
A281	10	9	+	10	9	+
C58	10	10	+++	10	10	+++
Control	10	2	+	10	2	+

[a] size of undifferentiated tissue growth: small +, medium ++, large +++

Transient GUS expression in leaf discs and stem segments co-cultivated with *A. tumefaciens* strains. The results of co-cultivation of leaf discs and stem segments of two *P. nigra* clones with a disarmed strain GV2260 p35S GUS are shown in Table 3. Differences in susceptibility between the two clones were observed: Jean Pourtet proved to be more susceptible than San Giorgio. The average number of GUS^+ spots/explant was significantly higher for leaf discs both with and without a pre-co-cultivation period. Moreover, significant differences were noticed when the three bacterial suspension concentrations were tested: $2x10^8$ and $7x10^8$ cells/ml gave, respectively, the lowest and the highest average number of GUS^+ spots/explant. Differences were also noticed among the various co-cultivation systems used: immersion of explants in the bacterial suspension for 20 min, followed by co-cultivation on solid medium for 48 h, gave the best results. Table 4 shows the results of co-culture of leaf discs with different strains of *A. tumefaciens*. A higher number of GUS^+ spots/explant was obtained with Jean Pourtet (38) compared with San Giorgio (26). A significant variability was found when different strains of *A. tumefaciens* were used. A281 pKIWI105 gave the highest average number of GUS^+ spots/explant (85); LBA4404 pKIWI105 always showed little or no virulence (0.7). No differences were noticed when the two different concentrations of bacterial suspension were tested. The analysis of variance did not reveal any interactions between the various factors tested in both experiment.

GUS activity analysis showed monitoring gene transfer events and promptly assessed the importance of analyzed factors. Results obtained made it possible to compare the transformation efficiency observed when different *A. tumefaciens* strains were used with the two clones of *P. nigra*, and to improve factors affecting the co-cultivation system.

Table 3 Co-cultivation of leaf discs and stem segments with GV2260 p35S GUS. Average number of GUS^+ spots/explant and F value in the analysis of variance for each factor.

A: with pre-co-cultivation

Factors	Levels	Average no. of GUS^+ spots/explant [a]	F
Clone	San Giorgio	5.68	5.8*
	Jean Pourtet	7.89	
Explant	leaf discs	11.07	77.9**
	stem segments	2.5	
Concentration bacterial suspension	$2x10^8$ cells/ml	4.41	3.7*
	$7x10^8$ cells/ml	9.06	
	$1.2x10^9$ cells/ml	6.88	
Co-cultivation	24h Solid	10.07	15.9**
	48h Solid	12.09	
	24h Liquid	0.98	
	48h Liquid	3.19	

B: no pre-co-cultivation

Clone	San Giorgio	3.91	9.3**
	Jean Pourtet	7.19	
Explant	leaf discs	9.93	52.2**
	stem segments	1.17	
Concentration bacterial suspension	$2x10^8$ cells/ml	1.42	21.1**
	$7x10^8$ cells/ml	10.03	
	$1.2x10^9$ cells/ml	5.2	
Co-cultivation	24h Solid	0.1	68.7**
	48h Solid	14.6	
	24h Liquid	1.4	
	48h Liquid	6.09	

Table 4 Co-cultivation of leaf discs with different *A. tumefaciens* (*A.t.*) strains. Average number of GUS^+ spots/explant and F value in the analysis of variance for each factor.

Factors	Levels	Average no. of GUS^+ spots/explant [a]	F
Clone	San Giorgio	26.3	8.8**
	Jean pourtet	38.9	
A.t. strains	GV2260 p35S GUS	23.73	54.7**
	C58 pKIWI105	20.35	
	A281 pKIWI105	85.58	
	LBA4404 pKIWI105	0.73	
Concentration bacterial suspension	$7x10^8$ cells/ml	27.46	0.16
	$1.2x10^9$ cells/ml	37.73	

*,** significant at the 0.05 and 0.01 probability levels, respectively.
[a] average values of each level of main effects.

Genetic transformation. After co-cultivation of leaf discs from *P. nigra* (San Giorgio, Jean Pourtet) with disarmed *A. tumefaciens* strains (GV2260 p35S GUS, GV3850HPT pKU2) some calluses of San Giorgio were obtained on a selective medium which regenerated several shoots. These were characterized by the rooting test in the presence of kanamycin. The plantlets transformed with GV2260 p35S GUS were also analyzed for GUS enzyme activity (Table 5). Organogenesis or callus production from control explants (not co-cultivated) was completely inhibited by kanamycin at 100 mg/l.

The highest transformation frequency (10/50) was obtained with the GV2260 p35S GUS strain present at the concentration of $6x10^8$ cells/ml. Regardless of the *A. tumefaciens* strain used, best results were obtained with a bacterial suspension concentration of $6x10^8$ cells/ml.

The histochemical GUS assay showed that more than 73% of the San Giorgio plantlets, regenerated after co-cultivation with GV2260 p35S GUS, were transformed.

Data show that the absence of light during the first 7 days after co-cultivation helps keep explants in a good physiological condition (reduced necrosis and tissue desiccation). During the phase of callus differentiation, vitrification phenomena took place which delayed normal regeneration. The problem was overcome transferring vitrified callus on a modified regeneration medium (macrosalts WPM) containing a reduced amount of BAP (0.05 mg/l).

Significant differences in the transformation frequency were observed between the two clones tested. Explants of Jean Pourtet showed no resistance to kanamycin after co-cultivation, nor any callus formation or organogenesis on selective regeneration medium.

Molecular analysis. DNA extracted from leaves of five different lines of San Giorgio was tested by PCR.

PCR analysis was carried out using two primers which were designed in order to bind an internal part of the *nptII* gene, thus amplifying a 599 bp fragment.

Three of these lines were transformed with GV2260 p35S GUS (Fig. 1, lanes 1-2-3) and the other two lines were transformed with GV3850HPT pKU2 (Fig. 1, lanes 4-5).

PCR products were subsequently blotted and hybridized using the 1.2 kbp EcoRV fragment (a structural part of the *nptII* gene) of pABDI plasmid as a probe.

Conclusions

This study reports the successful *Agrobacterium*-mediated transformation of *P. nigra* cv. San Giorgio. The best results were obtained using either GV2260 p35S GUS or GV3850HPT pKU2 *A. tumefaciens* strains, dipping leaf discs into a bacterial suspension ($6x10^8$ cells/ml) for 20 min and co-cultivating them for 48 h on semi-solid regeneration medium. Moreover, reported results show that foreign DNA is transiently expressed in Jean Pourtet but in this case no stable integration was recovered. An obvious explanation of this fact is that, while transfer of T-DNA occurs at high efficiency, its integration into genomic DNA is a much rarer event, thus limiting the overall process.

The availability of an efficient and reproducible

Table 5 Results of genetic transformation experiments of *P. nigra* (San Giorgio) obtained using two different *A. tumefaciens* strains.

A. tumefaciens strain	Concentration of the bacterial suspension (cells/ml)	Presence (+)/ absence (-) of light	Leaf discs co-cultivated		Plantlets obtained		
			Total no.	No. of discs regenerating kanamycin resistant plantlets	Total no.	No. of kanamycin resistant	GUS+ no.
GV3850 HPT pKU2	$2x10^8$	+	50	5	40	32	
		-	50	5	45	18	
	$6x10^8$	+	50	-	-	-	
		-	50	9	105	83	
GV2260 p35S GUS	$2x10^8$	+	50	-	-	-	-
		-	50	5	23	19	17
	$6x10^8$	+	50	-	-	-	-
		-	50	10	88	85	75

transformation system gives us a chance for transferring genes of agronomic interest (such as those for insect pest and disease resistance) into *P. nigra* cultivars of considerable commercial value.

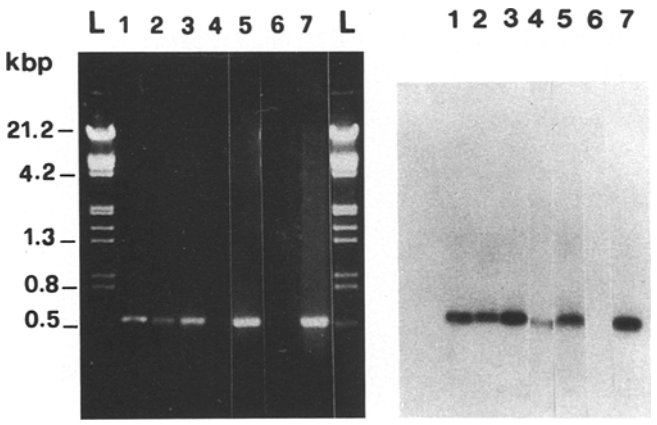

Figure 1 PCR analysis of five different lines of San Giorgio transformed with GV2260 p35S GUS (lanes 1-2-3) and with GV3850IIPT pKU2 (lanes 4-5). L = EcoRI/HindIII digested λ DNA; lane 6 = untransformed control; lane 7 = pABDI plasmid (positive control).
Southern blot hybridization of PCR products as shown on the right.

Acknowledgements

The Authors thank M. Rondanin and R. Bruschini for their technical assistance and Prof. R. Cella for helpful discussions. Acknowledgement is also made to the University of Utrecht for providing *Agrobacterium tumefaciens* strains C58 and Ach5, G. An for A281, L. Willmitzer for GV2260 p35S GUS INT, B. J. Janssen and R. Gardner for C58, LBA4404, A281 with plasmid pKIWI105 and B. Baker for GV3850HPT pKU2. Research was supported by the Italian Ministry of Agriculture in the framework of the project "Resistenze genetiche delle piante agrarie agli stress biotici e abiotici".

References

Baker B, Coupland G, Fedoroff N, Starlinger P, Schell J (1987) EMBO Journal 6: 1547-1554

Brasileiro ACM, Leplé JC, Muzzin J, Ounnoughi D, Michel MF, Jouanin L (1991) Plant Mol. Biol. 17: 441-452

Brasileiro ACM, Tourneur C, Leplé JC, Combes V, Jouanin L (1992) Transgenic Research 1:133-141

Clapham D, Ekberg I, Eriksson G, Hood EE, Norell L (1990) Theor. Appl. Genet. 79: 654-656

De Block M (1990) Plant Physiol. 93: 1110-1116

Devillard C (1992) C. R. Acad. Sci. 314:291-298

FAO (1980) Poplars and willows in wood production and land use. FAO Forestry Series no. 10, FAO, Rome

Fillatti JJ, Sellmer J, McCown B, Haissig B, Comai L (1987) Mol. Gen. Genet. 206:192-199

Janssen BJ, Gardner RC (1989) Plant Mol. Biol. 14: 61-72

Jefferson RA (1987) Plant Mol. Biol. Rep. 5: 387-405

Klopfenstein NB, Shi NQ, Kernan A, McNabb HS, Hall RB, Hart ER, Thornburg RW (1991) Can. J. Forest Res. 21:1321-1328

Leplé JC, Miranda Brasileiro AC, Michel MF, Delmotte F, Jouanin L (1992) Plant Cell Reports 11: 137-141

Lloyd G, McCown BH (1981) Proc. Int. Plant Prop. 30: 421-427

Mackay J, Séguin A, Lalonde M (1988) Plant Cell Reports 7: 229-232

McCown BH, McCabe DE, Russell DR, Robinson DJ, Barton KA, Raffa KF (1991) Plant Cell Reports 9: 590-594

Murashige T, Skoog F (1962) Physiol. Plant. 15:73-97

Riemenschneider DE (1990) Phytopathology 80: 1099-1102

Rogers SO, Bendich AJ (1988) Plant Molecular Biology Manual A6: 1-10

Sambrook J, Fritsch EF, Maniatis T (1989) In: Molecular Cloning, Book 1. Cold Spring Harbor Laboratory Press, New York, pp 6.30-6.31

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L., Rocha-Sosa M (1990) Mol. Gen. Genet. 220: 245-250