An Efficient Procedure to Stably Introduce Genes into an Economically Important Pulp Tree (Eucalyptus grandis × Eucalyptus urophylla)

Article *in* Transgenic Research · September 2003

DOI: 10.1023/A:1024217910354 · Source: PubMed

CITATIONS
79

READS
253

8 authors, including:

Vincent Tournier
13 PUBLICATIONS **526** CITATIONS
SEE PROFILE

Walid El Kayal
University of Alberta
38 PUBLICATIONS **650** CITATIONS
SEE PROFILE

Ricardo Penchel
FIBRIA
40 PUBLICATIONS **752** CITATIONS
SEE PROFILE

Transgenic Research **12:** 403–411, 2003.

An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* × *Eucalyptus urophylla*)

Vincent Tournier[1], Sabine Grat[1], Christiane Marque[1], Walid El Kayal[1], Ricardo Penchel[2], Gisele de Andrade[2,**], Alain-Michel Boudet[1] & Chantal Teulières[1,*]

[1]*UMR 5546, Pôle de Biotechnologie Végétale, 24 Chemin de Borde Rouge, BP17 Auzeville 31326 Castanet-Tolosan, France*

[2]*Aracruz Celulose S.A. Centro de Pesquisa e Tecnologia, Rodovia ES-257, km 25, Aracruz, ES 29197-000, Brazil*

Received 11 March 2002; revised 11 November 2002; accepted 11 November 2002

Key words: antisense, cinnamyl alcohol dehydrogenase, eucalyptus, genetic engineering, juvenile clones

Abstract

Regeneration problems are one of the main limitations preventing the wider application of genetic engineering strategies to the genus *Eucalyptus*. Seedlings from *Eucalyptus grandis* × *Eucalyptus urophylla* were selected according to their regeneration (adventitious organogenesis) and transformation capacity. After *in vitro* cloning, the best genotype of 250 tested was transformed via *Agrobacterium tumefaciens*. A cinnamyl alcohol dehydrogenase (CAD) antisense cDNA from *Eucalyptus gunnii* was transferred, under the control of the 35S CaMV promoter with a double enhancer sequence, into a selected genotype. According to kanamycin resistance and PCR verification, 120 transformants were generated. 58% were significantly inhibited for CAD activity, and nine exhibited the highest down-regulation, ranging from 69 to 78% (22% residual activity). Southern blot hybridisation showed a low transgene copy number, ranging from 1 to 4, depending on the transgenic line. Northern analyses on the 5–16 and 3–23 lines (respectively one and two insertion sites) demonstrated the antisense origin of CAD gene inhibition. With respectively 26 and 22% of residual CAD activity, these two lines were considered as the most interesting and transferred to the greenhouse for further analyses.

Abbreviations: BAP – Benzyl amino purine; BIP – Bud induction pre-treatment; BIT – Bud induction treatment; CAD – Cinnamyl alcohol dehydrogenase; CaMV – Cauliflower mosaic virus; CTAB – Hexadecyltrimethylammonium bromide; 2-4D – 2-4 Dichloro phenoxyacetic acid; GUS – Glucuronidase; MYA – Malt yeast agar medium; NAA – Naphthalene acetic acid; *nptII* – Neomycin phosphotransferase; PVP – Poly vinyl pyrrolidone; SDM – Shoot development medium; TDZ – Thidiazuron.

Introduction

Globally, broadleaves make up 40% of planted forest area with eucalyptus species being the main fast-growing, short-rotation crop, accounting for 17.8 million ha. About half of the forest plantation estate is for industrial end-use. The fast growing tree eucalyptus, originally from Australia, is used worldwide for pulp and paper, timber and fuel wood. South America has plantations covering 4.9 million ha of, mainly, eucalyptus hybrids such as *Eucalyptus grandis* × *Eucalyptus urophylla* (FAO, 2001).

Pulp and paper production in particular requires treatment to separate lignins from cellulose, a procedure that is costly, energy consuming and polluting. The isolation of genes encoding enzymes involved in lignification has made it possible to envisage controlled modulation of lignins through the down-regulation of gene expression (e.g., by the antisense RNA strategy). Significant results in changing lignin

*Author for correspondence
E-mail: teuliere@smcv.ups-tlse.fr
**Present address: University of Georgia, School of Forest Resources, Athens, GA 30602, USA

quantity and/or quality, which both influence wood processing, have already been obtained in different species (for review, see Boudet, 2000).

Cinnamyl alcohol dehydrogenase (CAD), the enzyme involved in the last step of monolignol synthesis, is considered as a good target for modulating lignification. Its down-regulation by genetic engineering in poplar led to a reduced kappa number during the bleaching process, indicative of more efficient lignin removal, both for young and adult trees, without modifying growth (Baucher et al., 1996).

We have isolated the CAD gene from *Eucalyptus gunnii* (Grima-Pettenati et al., 1993) and fused it, in an antisense orientation, to a constitutive promoter to generate a transformation vector. The extension of lignin engineering to eucalyptus is usually limited by the general recalcitrance of the species to genetic transformation, combined with a low regeneration capacity. As the extent of recalcitrance is genotype-dependent and is particularly crucial for adult material, we started with seedlings (*E. grandis* × *E. urophylla*) originating from a clonal seed orchard. The best genotypes for transformation were selected according to both their regeneration and transformation capacities. These were multiplied by micropropagation and transformed with the eucalyptus CAD antisense construct. This paper presents the molecular analysis of the transformants, and assesses the degree of CAD enzyme down-regulation, and discusses their potential application for the paper industry.

Materials and methods

Plant material and explant screening

Eucalypt half-sib seeds were derived from *E. grandis* × *E. urophylla*, and about 250–300 mature seeds were collected in a clonal seed orchard, in Aracruz, Brazil, at 19°48′S latitude, 40°17′W longitude and 30 m altitude. Larger seeds were selected for this work and were surface-sterilised and sown on a standard medium for *in vitro* germination at 23°C, 16 h day-length. After this period (30–40 days), the best plantlets were selected on the basis of their viability, germination vigour (<4 days) and relative stem growth rate ($>0.7\,\mathrm{cm\,day^{-1}}$). In order to obtain juvenile clones, we selected the 50 best seedling clones (based on shoot growth rate: shoot height), which were micropropagated on a basal MS medium (Murashige & Skoog, 1962) supplemented with vitamins and hormones (medium M, Table 1). Under these conditions the microcuttings did not develop roots. Stem nodes (0.5 cm long) containing a leaf pair were excised from the selected shoot culture. This material was cultured in micropropagation medium (medium M, Table 1) aiming at proliferation of axillary buds. During subsequent subcultures, clumps of multiple shoots were established as stock clonal plants, in large wide-mouth glass jars and subcultured to fresh medium M every 3 weeks.

Regeneration procedure

In vitro shoot cultures of a number of seedling clones were used as a source of explants for regeneration and transformation studies. Explants consisting of small growing leaves (5 mm long) with the petiole removed were grown on a cellular reactivation medium (BIP medium, Table 1) for 5 days, and then transferred to bud induction medium (BIT medium, Table 1) for 5 days in the dark, followed by 14 days in low light ($1.5\,\mathrm{W\,m^{-2}}$), 16 h photoperiod, 24°C, with subculture every 5 days. After this initial 20 day-period, cultures were transferred to shoot development medium (SDM medium, Table 1) for 60 days, with subculture every 14 days.

Genetic transformation and selection of transformants

Genetic transformation was achieved via *Agrobacterium tumefaciens* (strain AGL1 pTiBo542) containing the pBin19 plasmid. The transferred sequences consisted of:

- 1/‘p35S GUS INT’ (Vancanneyt et al., 1990) containing p35SCaMV-GUS Intron-35SCaMVt and pNos-nptII-Nost, for optimization of transformation and genotype screening.
- 2/‘p35S-DE-AS EuCAD’ (Yahiaoui et al., 1998) containing the full length CAD cDNA (Grima-Pettenati et al., 1993) in antisense orientation under the control of the CaMV 35S promoter with double enhancer, and *nptII* as the selectable gene. This construct was used for generating CAD antisense transformants from the selected genotype (Figure 1). Three independent transformation experiments were performed on leaves (200 leaves for each experiment) cut crosswise into two halves in antioxidant mixture ($250\,\mathrm{mg\,l^{-1}}$ ascorbic acid, $25\,\mathrm{mg\,l^{-1}}$ citric acid, $1\,\mathrm{g\,l^{-1}}$ PVP40 at pH 5) under low light intensity and cultured for 2 days at

Table 1. Composition of nutrient media used in eucalyptus transformation and regeneration

Constituents	Bud induction				Shoot development		
	BIP	C	A2	BIT	SDM	M	E
Inorganic substances ($mg\,l^{-1}$)							
$CaCl_2 \cdot 2H_2O$	440	220	440	441.9	441.9	440	440
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	–	–	0.025	0.025
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.25	0.25	0.025	0.025
$FeSO_4 \cdot 7H_2O$	27.85	27.85	27.85	27.85	27.85	27.85	27.85
H_3BO_3	6.2	6.2	6.2	6.2	6.2	6.2	6.2
K_2SO_4	990	–	–	990	990	–	–
KCl	1401	–	–	–	–	–	–
KH_2PO_4	170	85	170	170	170	170	170
KI	0.83	0.83	0.83	–	–	0.83	0.83
KNO_3	–	950	1900	–	–	1900	1900
$MgSO_4 \cdot 7\,H_2O$	370	185	370	370	370	370	370
$MnSO_4 \cdot H_2O$	16.9	16.9	16.9	16.9	16.9	16.9	16.9
Na_2EDTA	37.25	37.25	37.25	37.25	37.25	37.25	37.25
$Na_2MoO_4, 2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25
NH_4NO_3	2344	825	1650	391.8	391.8	1650	1650
$(NH_4)_2SO_4$	–	–	–	323.3	323.3	–	–
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8.6	8.6	8.6	8.6	8.6
Organic substances ($mg\,l^{-1}$)							
Arginine[a]	–	–	–	59.23	–	–	–
Myo–Inositol	200	200	200	200	200	200	100
Nicotinic acid	5	3	5	5	5	5	1
Calcium pantothenate	–	3	–	–	–	–	–
Pyridoxine–HCl	1.2	3	1.2	1.2	1.2	1.2	1
Thiamine–HCl	10	3	10	10	10	10	10
Agar ($g\,l^{-1}$)	6	–	7	6	6	7	7
Charcoal (%)	–	–	–	–	–	–	0.1
Coconut water (%)[a]	–	–	–	10	–	–	–
Sucrose ($g\,l^{-1}$)	30	20	30	30	30	30	30
Growth regulators (μM)							
2.4-D[a]	0.5	–	–	–	–	–	–
BAP[a]	1	–	1	–	2	1	–
NAA[a]	–	–	2	0.1	0.5	0.5	–
Putrescine[a]	–	–	–	500	100	–	–
Spermidine[a]	–	–	–	100	10	–	–
TDZ[a]	–	–	–	3	–	–	–
pH	5.6	5.7	5.6	5.6	5.6	5.6	5.6

[a] Compounds were added after autoclaving.

23°C in the dark on a medium for cell reactivation (BIP medium, Table 1), supplemented with 50 μM acetosyringone. Agrobacteria were cultured until OD = 1 in liquid medium MYA (Tepfer & Casse-Delbart, 1987). Inoculation was then initiated by immersing the leaf pieces in the Agrobacteria suspension (OD = 0.5) in liquid co-culture medium (medium C, Table 1), supplemented with 1 mM proline. After 15 s sonication and 5 min infiltration under vacuum, the plant tissues were transferred

Figure 1. Construct used in a pBin19 vector for introducing antisense CAD by *A. tumefaciens* (AGL1 pTiBo542) leaf transfection. *Eco*RI restriction site, *nptII* probe positions and restriction fragment length are indicated.

for 5 days, in the dark at 23°C, to a solid co-culture medium (medium A2, Table 1) supplemented with 50 μM acetosyringone.

For selection of the transformants, leaf tissues were grown (immediately after infection), on a regeneration medium (BIT medium, Table 1), containing kanamycin (50 mg l^{-1}) and augmentin (300 mg l^{-1}) and transferred every 2 weeks to fresh medium. The kanamycin resistant buds were propagated on a kanamycin (50 mg l^{-1}) and augmentin (300 mg l^{-1}) containing SDM medium until they developed into shoots; the individual shoots were transferred to elongation medium (medium E, Table 1) again supplemented with kanamycin. All further steps of plant recovery were carried out according to the regeneration procedure by adding 50 mg l^{-1} kanamycin to different media.

Individual transformed shoots about 4 cm tall were selected for *in vitro* and *ex vitro* rooting studies and rooted plants were transferred to soil in the greenhouse. The plants were potted in plastic containers (15 l) containing sand and drip irrigated five times a day with a completely water soluble fertilizer with all main and trace elements. Ambient conditions were controlled to maintain 90 ± 5% relative humidity, 27 ± 3°C temperature, 14 h photoperiod, in natural light conditions of 600–900 μmol m^{-2} s^{-1}.

Screening for transformation and regeneration capacity

The stock shoot cultures originating from the multiplication of the 50 best seedlings were first screened for *A. tumefaciens* mediated transformation by evaluating transient glucuronidase expression 6 days after co-culture. GUS activity was visualised by staining the leaves overnight at 37°C in a substrate solution containing 2 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide according to the procedure of Jefferson et al. (1987). The clones were classified according to the number and the size of the GUS positive surface: localised spots, areas between 5 and 39%, 40–69%, or 70–100%, of plant tissues.

The transformation capacity of these genotypes was subsequently checked over the long-term at 2 weeks, 1 month and 2 months after co-culture. Viability after transformation was evaluated by counting, under a stereo-microscope, the number of leaves exhibiting green or white growing areas. Screening for regeneration capacity was performed after transformation of the most transformable clones which were transferred to a selective regeneration medium. This parameter was evaluated by counting, every 2 weeks, the percentage of buds appearing on the leaf surface, the average number of buds per leaf, and the percentage of shoot-developing buds per leaf.

PCR screening of transformants

DNA was extracted from leaves of selected plantlets using a CTAB procedure adapted from Gawel and Jarret (1991). The presence of the *nptII* gene was confirmed by PCR (data not shown) using specific primers (5′-GAGGCTATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGCGATACCGTA-3′) generating a 700 bp amplification fragment. In order to check that positive PCR signals were not due to the presence of Agrobacteria in the leaves, we performed the same experiments using v*irG* specific primers as a control (5′-CGATGTCGCTATGCGGCATCT-3′ and 5′-CAATGAGAAGTTGCTCGCGCG-3′).

CAD activity measurements

CAD assays were performed on *in vitro* transformed and 13 control microcuttings (leaves and stems, 2–3 cm in size). Proteins were extracted from 200 to 400 mg frozen microcuttings, ground in liquid nitrogen for 3 min and treated as described in previous studies (Goffner et al., 1992). Extractions were done in replicate samples. CAD activity was assayed, following the conversion of coniferyl alcohol into coniferyl aldehyde. The reaction was monitored by the change of absorbance at 400 nm due to product formation. The assay was carried out in 0.5 ml of reaction mixture containing Tris–HCl 100 mM (pH 8.8), 100 μM coniferyl alcohol, 200 μM β-NADP

and 50 μL of protein sample. For each extract, two activity assays were performed, providing a total of four measurements per plant. Protein concentration was determined by the method of Bradford using the BIO-RAD dye binding reagent. The mean CAD value of four measurements from each plant was compared to the mean value of the control population (based on 13 individual plants). The residual CAD activity of each transgenic line, representing the level of inhibition, was expressed as a % of the mean value of control plants.

Molecular analyses

Genomic DNA was prepared from leaf tissues using Plant DNA Easy kit (from Qiagen). The extract (10 μg) was digested with *Eco*RI (5 U μg^{-1} DNA) (one restriction site within the T-DNA). After electrophoresis, the gels were blotted onto Hybond N^+ membrane (Amersham), according to the manufacturer's instructions. A [α ^{32}P]dCTP labelled 700 bp sequence of the *nptII* gene was used as a probe for hybridisation, as described in Sambrook et al. (1989).

Total RNA was isolated from 400 mg of frozen *in vitro* microcuttings using the Extract-All Kit (Eurobio). RNA samples (15 μg RNA, 40% formamide, 5% formaldehyde, 0.4 × MOPS and 10 μg ml^{-1} BET) were denatured for 10–15 min at 60°C and 10% 6 × Ficoll Blue was added. The samples were then separated on 1% agarose, 1 × MOPS, 6% formaldehyde gels and blotted to positively charged nylon filters (Hybond N^+, Amersham). Pre-hybridisation and hybridisation were performed as described in Sambrook et al. (1989) using a partial length (1081 bp *Hin*dIII restriction fragment) double-stranded CAD cDNA [α ^{32}P]dCTP labelled probe capable of detecting both sense and antisense RNA. Another hybridisation with 18S rRNA probe was performed for an evaluation of signal intensity.

Results

Selection of genotypes exhibiting the best transformation capacity

Among the mature seeds (250), we selected the best 50 genotypes on the basis of their germination capacity and growth rate (shoot height). These clones were tested for *in vitro* regeneration capacity and for transformation competence using the *gusA* reporter gene. Six days after co-cultivation, we observed transgene expression in 39 clones. Among them, eight exhibited a very high level of GUS expression: all the assayed leaves were positive up to 100% of the leaf surface. Clone 251 gave one of the best results with 44.4% of the leaves expressing the GUS gene on 5–69% of the surface and 55.6% of leaves showing GUS-positive areas covering more than 70% of their surface. In addition, GUS expression was observed with a similar range 2 and 6 weeks after transformation strongly suggesting stable gene transfer.

The 10 most responsive genotypes including clone 251, were kept for further studies and tested after transformation for viability and regeneration on a selective kanamycin containing medium. Clone 251 was again one of the most responsive genotypes, with 42.3% of the leaves exhibiting alive areas for 3 months and more than twice the number of leaves with shoots compared to other genotypes. This result suggested for this genotype a better regeneration capacity which was also associated with a very high micropropagation efficiency.

Finally, clone 251, exhibiting the best compromise between short- and long-term GUS expression level, regeneration and micropropagation efficiency under selection after transformation was selected for transformation with the CAD antisense construct.

Recovery and analysis of CAD antisense transformants

From three independent transformation experiments (in total 1200 half leaves), 152 individual shoots were regenerated on kanamycin containing medium. After screening for *nptII* gene presence by PCR, (data not shown), 120 positive lines were selected indicating a high relative efficiency of the selection pressure (21% of escapes). The absence of remaining Agrobacteria on the regenerated shoots was also demonstrated by the lack of amplification with the primers corresponding to the *virG* gene.

Therefore, the integrative and regenerative transformation rate was about 10%, a very high efficiency considering the plant species. None of the 120 lines had any particular phenotype at this stage. 114 were micropropagated in order to evaluate the efficiency of the down-regulation of the CAD gene expression. CAD activity was measured on this population and compared to the control plants of the same clone at the same stage of development. For the 13 measured wild type microcuttings, CAD activity ranged between

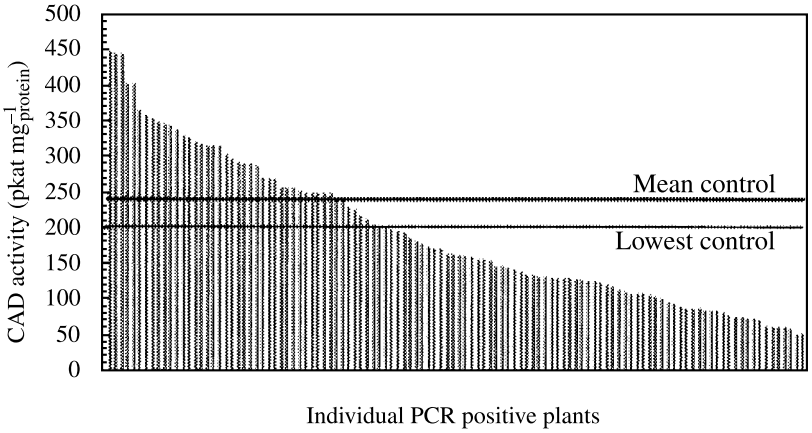

Figure 2. Specific activity of CAD for antisense transgenic *E. grandis* × *E. urophylla in vitro* grown plantlets. Each vertical line represents one transgenic plant. The mean value is calculated from four measurements for each plant extract. The 240 pkat $mg^{-1}_{protein}$ line corresponds to the average of 13 control cuttings and the 201.5 pkat $mg^{-1}_{protein}$ corresponds to the lowest control: wild type.

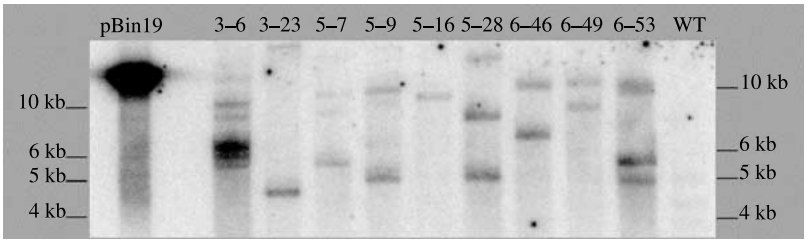

Figure 3. Southern analysis of selected plants. Hybridisation was performed with an *nptII* gene probe. Plants were transformed using a pBin 19 vector housing the full length CAD cDNA in antisense orientation under the control of CaMV 35S promoter (Figure 1). Plant DNA was digested by *Eco*RI. Negative control is the wild type 251 clone (WT), and linearised pBin19 plasmid is the positive control (pBin19). Nine down-regulated transformants from three independent experiments (the first number being 3, 5 or 6) were tested: 3–6, 3–23, 5–7, 5–9, 5–16, 5–28, 6–46, 6–49 and 6–53.

201.5 and 297.4 pkat $mg^{-1}_{protein}$ corresponding to an average of 240 pkat $mg^{-1}_{protein}$ and a standard deviation of 27.86 pkat $mg^{-1}_{protein}$. Taking into account this natural variability, classically observed for this enzyme, it was therefore decided to consider the lowest control activity, rather than the average, as a reference for identifying significantly inhibited lines. Under these conditions, 58% of the PCR positive transformants were considered down-regulated for CAD activity (Figure 2). Statistical analysis (Student's *T*-test) showed that the CAD activity of 66 transgenic plants was significantly different from that of the control, at a confidence level of 99.95%.

The number of transgene copies was then investigated through Southern hybridisation for the nine plants exhibiting the strongest inhibition (corresponding to a residual activity ranging from 22 to 31% of the mean control value). According to the restriction map (Figure 1), after the digestion of genomic DNA with *Eco*RI (one restriction site located between left border and CAD antisense sequence), we expected bands larger than 4.96 kb for transgenic lines housing a complete cassette. After hybridisation with the radiolabelled *nptII* probe, under stringent conditions, the absence of signal for the wild type was verified and up to four integration loci were detected in transgenic lines (Figure 3). All of the tested lines had at least one T-DNA copy, and, except for line 3–23, the band size larger than 5 kb indicated the integration of the complete sequence (CAD antisense and *nptII* genes). The single band observed for line 3–23, shorter than the expected size, could result from a small deletion in the T-DNA, outside sequences of interest. Following PCR screening of the *nptII* gene, Southern analysis (using the *Eco*RI site on the left border and the *nptII*

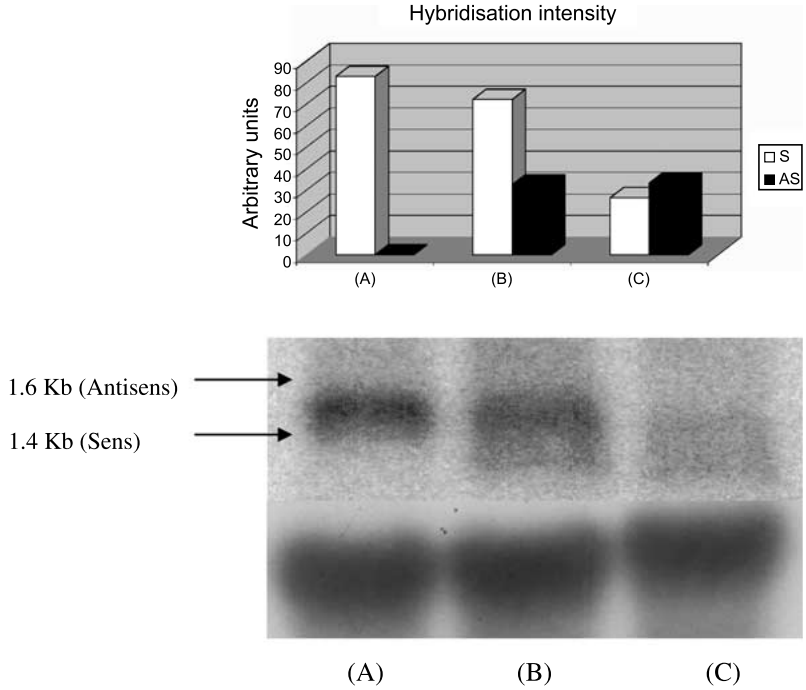

Figure 4. Northern analyses of the two most down-regulated plants (5–16 and 3–23). Total RNA extracted from control or transformed lines was separated by electrophoresis before hybridisation with partial length CAD cDNA probe. (A) Endogenous CAD transcript signal (1.6 kb) in wild type plant; (B) and (C) transgene signal (1.4 kb) and endogenous CAD transcript signal, respectively in transgenic lines 5.16 and 3.23. Hybridisation with 18S rRNA probe was used to evaluate RNA loading of the individual lanes.

probe on the right border), led us to select lines with at least one copy of the complete cassette.

According to these data, clone 3–23 has two copies while clone 5–16 has a single copy. Finally, northern analyses were performed on these two lines in order to demonstrate the antisense origin of CAD gene inhibition. For the two transgenic lines, we observed a band (1.6 kb) absent from the control line and corresponding to the antisense signal demonstrating transgene transcription. A quantitative evaluation of the signal intensity indicates a similar amount of antisense RNA in both transgenic lines, associated to a reduced sense transcript amount, particularly in the case of the 3–23 line (Figure 4).

In conclusion, with respectively 26 and 22% of residual CAD activity, in agreement to the RNA amount and the transgene copy number, these transgenic lines appear to be the most interesting candidates for further analyses.

Establishment of plants in the greenhouse

Transformed regenerants were transferred to potting mix without significant loss during acclimatisation of *in vitro* grown plantlets in the greenhouse. A survival rate of over 95% was obtained by controlling ambient relative humidity, temperature and light *ex vitro*, which contributed to a higher percentage of plantlet survival after *in vitro* culture.

About 120 whole plants were established successfully in the greenhouse from 30 transformed lines

Figure 5. General view of 6-month-old actively growing plants in the greenhouse. All plants in the greenhouse were derived from *in vitro* shoot cultures. At this stage they were 1–1.2 m high and the stems were 1.5–3 cm in diameter at the base.

and all of them possessed juvenile characteristics and appeared indistinguishable from micropropagated plantlets of similar age (Figure 5).

Discussion

The first objective of this work was to design tools (transformation and regeneration procedures) for the hybrid *E. grandis* × *E. urophylla* in order to introduce genes of interest with priority on antisense lignification genes. An efficient system is necessary for a good probability of recovering interesting transformed lines.

Despite the clear potential of genetic engineering for improving woody plants during the last 10 years, progress has been slow on eucalyptus which is still considered recalcitrant. In particular, regeneration in tissue culture is often very poor, probably due to the high concentration of phenolic compounds in the cells or to a low endogenous cytokinin content as shown by Azmi (1999). Organogenesis or embryogenesis capacity is even lower on a selective antibiotic-containing medium making it often impossible to recover transgenic shoots even when stable transformation is achieved (e.g., Serrano et al., 1996). Nevertheless, transgenic eucalyptus have been recovered in a few species (for review, see MacRae & van Staden, 1999), mainly from juvenile material that showed better transformation and regeneration capacity than adult clones. Among these species, *E. grandis* appeared as one of the most amenable for transformation. For the hybrid, *E. grandis* × *E. urophylla*, one of the best materials for the paper industry in Brazil, Machado et al. (1997) only reported the recovery of shooty tumours after transformation of seedlings with *A. tumefaciens* wild type strains.

Our strategy consisted of choosing from a batch of seeds from superior parents, the genotype exhibiting the best transformation capacity when submitted to a procedure optimised in our lab for adult material from the same hybrid. As usually observed, also in eucalyptus (Mullins et al., 1995), gene transfer and integration were very dependent on genotype. This integrative transformation capacity obviously influenced the regeneration of transformants on the selective medium, as confirmed by the good regeneration data for the genotypes with the best transformation response. However, the two capacities did not appear to be strictly correlated since the most regeneration-capable clone was not the best one for transient or stable transformation. Considering that this ability to regenerate transformants on a selective medium is the limiting step in developing transgenics, juvenile clone 251 was chosen as the most amenable for transformation.

When the CAD gene-harbouring T-DNA was introduced into this clone, 10% of the starting explants produced kanamycin resistant and PCR-positive shoots (about 40 transgenic plantlets per experiment). This efficiency looks quite good considering the transformation rates usually reported for woody plants, particularly, for eucalyptus: between 10 and 50% of the seedlings produced shooty tumours but without a selection step for *E. grandis* × *E. urophylla* (Machado et al., 1997), 4% of the hypocotyls or cotyledons regenerated shoots on a selective medium for *E. camaldulensis* (Kawasu, personal communication) and 2.6% of the seedlings exhibited kanamycin resistant shoots for *Eucalyptus globulus* (Moralejo et al., 1998). More importantly, according to previous data on tobacco and poplar, our transformation efficiency is high enough to obtain interesting transformants through an RNA antisense strategy.

In previous reports aiming to modulate lignification (Halpin et al., 1994; Baucher et al., 1996; Yahiaoui et al., 1998), 20–100 transgenic plantlets were studied. Therefore, the transformation system defined in this paper seems convenient to test the effect of gene transfer and suitable for recovering economically interesting transgenic lines.

The CAD_2 gene was chosen as the first target to down-regulate since in model plants, including poplar, the same strategy improved the extractability of lignin without significantly modifying its quantity nor the growth and development of the plant (Halpin et al., 1994; Baucher et al., 1996; Bernard Vailhé et al., 1996). In our hands through the use of the antisense strategy, 13 transgenic plants were generated, with CAD residual activities ranging between 26 and 39% of the wild type level.

According to the data of Baucher et al. (1996), on poplar, this inhibition could be sufficient to observe an increase in lignin extractability.

However, our activity measurements performed on *in vitro* plantlets will have to be confirmed on greenhouse grown plants, in parallel to the chemical and physical analyses.

Finally, molecular analysis of the transgenic lines showed a general low transgene copy number for the integration. Several lines exhibiting one or two copies coupled with significant down-regulation appear to be

the most interesting for further analysis with a reduced risk of transgene instability.

For the eucalyptus hybrid studied, an efficient transformation system was defined by optimising gene transfer and regeneration protocols and choosing a very responsive genotype within an economically important variety. The system allowed the generation of CAD antisense lines down-regulated to levels compatible with positive alterations of wood quality. The shoots obtained are now being allowed to grow in order to be analysed for lignin quantity and quality and wood characteristics for pulping. Physiological and agronomic alterations and biochemical changes that could be associated with this inhibition, in particular for the metabolism of phenolics, should also be evaluated.

References

Azmi A (1999) Bases cellulaires et physiologiques de la régénération et de la transformation génétique chez *Eucalyptus globulus* Labill. PhD Thesis, Pierre et Marie Curie University, Paris, France.

Bernard Vailhé MA, Cornu A, Robert D, Maillot MP and Besle JM (1996) Cell wall degradability of transgenic tobacco stems in relation to their chemical extraction and lignin quality. *J Agric Food Chem* **44**: 1164–1169.

Baucher M, Chabbert B, Pilate G, Van Doorsselaere J, Tollier M-T, Petit-Conil M et al. (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar (*Populus tremula* × *Populus alba*). *Plant Physiol* **112**: 1479–1490.

Boudet A-M (2000) Lignins and lignification: selected issues. *Plant Physiol Biochem* **38**(1): 1–16.

FAO (2001) Global Forest Resources Assessment 2000 – FRA 2000 Main Report. FAO Forestry Paper 140, Rome, 479 pp.

Gawel NJ and Jarret RL (1991) A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea*. *Plant Mol Biol Rep* **9**: 262–266.

Goffner D, Joffroy I, Grima-Pettenati J, Halpin C, Knight M, Schuch W et al. (1992) Purification and characterization of isoforms of cinnamyl alcohol dehydrogenase from *Eucalyptus* xylem. *Planta* **188**: 48–53.

Grima-Pettenati J, Feuillet C, Goffner D, Borderies G and Boudet A-M (1993) Molecular cloning and expression of a *Eucalyptus gunnii* cDNA clone encoding cinnamyl alcohol dehydrogenase. *Plant Mol Biol* **21**: 1085–1095.

Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet A-M, Boon JJ et al. (1994) Manipulation of lignin quality by down-regulation of cinnamyl alcohol dehydrogenase. *Plant J* **6**(3): 339–350.

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusion: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* **6**(13): 3901–3907.

Machado LO, de Andrade GM, Cid LPB, Penchel RM and Brasileiro ACM (1997) *Agrobacterium* strain specificity and shooty tumour formation in eucalypt (*Eucalyptus grandis* × *E. urophylla*). *Plant Cell Rep* **16**: 299–303.

MacRae S and van Staden J (1999) Transgenic eucalyptus. In: Bajaj YPS (ed.), Biotechnology in Agriculture and Forestry. Vol. 44 (pp. 88–114) Springer, Heidelberg.

Moralejo M, Rochange F, Boudet A-M and Teulières C (1998) Generation of transgenic *Eucalyptus globulus* plantlets through *Agrobacterium tumefaciens* mediated transformation. *Aust J Plant Physiol* **25**: 207–212.

Mullins KV, Hartney VJ, Llewellyn DJL, Strauss S and Dennis ES (1995) Regeneration and transformation of *Eucalyptus camaldulensis*. In: CRCTHF-IUFRO Conference Eucalypt Plantations: Improving Fibre Yield and Quality. (pp. 413–415) Hobart, Australia.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**: 473–497.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning: A laboratory Manual. Cold Spring Harbor, New York.

Serrano L, Rochange F, Semblat JP, Marque C, Teulières C and Boudet AM (1996) Genetic transformation of *Eucalyptus globulus* through biolistics: parallel development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue. *J Exp Bot* **47**(295): 285–290.

Tepfer M and Casse-Delbart F (1987) *Agrobacterium rhizogenes* as a vector for transforming higher plants. *Microbiol Sci* **4**: 24–28.

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L and Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in Agrobacterium-mediated plant transformation. *Mol Gen Genet* **220**: 245–250.

Yahiaoui N, Marque C, Myton KE, Negrel J and Boudet A-M (1998) Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco plants. *Planta* **204**: 8–15.