INTRODUCTION OF THE RS-AFP2 GENE INTO *EUCALYPTUS UROPHYLLA* FOR RESISTANCE TO *PHYTOPHTHORA CAPSICI*

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OUYANG LJ, HE WH, HUANG ZC, ZHAO LY, PENG SH, SHA YE, ZENG FH & LU XY. 2012. Introduction of the RS-AFP2 gene into *Eucalyptus urophylla* for resistance to *Phytophthora capsici*. We developed an *Agrobacterium tumefaciens*-mediated transformation system for *Eucalyptus urophylla* using hypocotyl explants. Antibiotic concentrations, pre-culture times, pH of the inoculation medium and co-culture times were optimised. Pre-cultured hypocotyl explants were co-cultured with *A. tumefaciens* strain EHA105 harbouring the binary vector pPBR-2 containing the Rs-AFP2 gene, which encodes an antifungal protein, under the control of the *prp1-1* promoter, for six days and were then transferred to selective callogenesis-inducing medium containing kanamycin and cefotaxime. Calluses developed shoots and were cultured in an elongation medium and finally multiplied. The integration of T-DNA into the genome of transgenic *E. urophylla* was confirmed by polymerase chain reaction (PCR). The reverse transcription (RT)-PCR results showed that Rs-AFP2 gene expression could be detected only after the transformed plants were inoculated with *Phytophthora capsici* 60 hours after inoculation. These results indicated that the *prp1-1* promoter was inducible and Rs-AFP2 could enhance the resistance of *E. urophylla* to *P. capsici*. This protocol enabled effective transformation and regeneration of *E. urophylla*.

Keywords: *prp1-1* promoter, genetic transformation, fungus resistance

OUYANG LJ, HE WH, HUANG ZC, ZHAO LY, PENG SH, SHA YE, ZENG FH & LU XY. 2012. Kemasukan gen RS-AFP2 dalam *Eucalyptus urophylla* bagi kerintangan terhadap *Phytophora capsici*. Kami membangunkan sistem pengubahan berantarakan *Agrobacterium tumefaciens* bagi *Eucalyptus urophylla* menggunakan eksplan hipokotil. Kepekatan antibiotik, masa prakultur, pH medium inokulasi dan masa pengkulturan bersama dioptimumkan. Eksplan hipokotil dikultur bersama-sama dengan *A. tumefaciens* jenis EHA105 selama enam hari dan kemudiannya dipindahkan ke medium pengaruh pengkalusan terpilih yang mengandungi kanamisin dan sefotaksim. Baka ini menyimpan vektor perduaan pPBR-2 yang mengandungi gen Rs-AFP2 yang mengekod protein antikulat di bawah kawalan penggalak *prp1-1*. Kalus menghasilkan pucuk dan dikultur dalam medium pemanjangan dan kemudiannya digandakan. Integrasi T-DNA ke dalam genom *E. urophylla* yang transgenik disahkan oleh reaksi rantai polimerase (PCR). Keputusan trankripsi (RT)-PCR yang bertentangan menunjukkan yang gen Rs-AFP2 terperi boleh dikesan hanya selepas tumbuhan terubah telah diinokulasi dengan *Phytophthora capsici* (60 jam selepas inokulasi). Keputusan menunjukkan yang penggalak *prp1-1* boleh diaruh dan Rs-AFP2 boleh meningkatkan kerintangan *E. urophylla* terhadap *P. capsici*. Protokol ini membolehkan pengubahan dan pertumbuhan semula *E. urophylla* yang berkesan.

INTRODUCTION

Eucalyptus, belonging to the Myrtaceae family, is the most widely planted hardwood crop in tropical and subtropical regions. Its growth is superior to that of other hardwood crops; it adapts well to the environment and its wood can be used for multiple purposes (Doughty 2000, Potts & Dungey 2004). *Eucalyptus* is the most important forest tree in China, covering more than three million hectares of commercial plantations (FAO 2001). *Eucalyptus urophylla* can reach a height of up to 50 m and its wood is widely used as timber for heavy structures and bridges. It is highly prized in China for its superior wood properties, rooting ability and disease resistance. Plantation forestry of *E. urophylla* supplies high-quality raw material for pulp, paper, wood and energy while reducing the pressure on native forests and associated biodiversity (Lu et al. 2010). However, diseases such as bacterial wilt, fungal infection and grey mould have seriously endangered the *Eucalyptus* crop in China, especially *E. urophylla* and the hybrid *E. grandis* × *E. urophylla* (Wu et al. 2007).

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Prevention and treatment of these diseases involve chemical pesticides, which often cause environmental pollution and ecological damage. In contrast, the process of producing disease-resistant trees through genetic engineering can potentially be faster, more controllable, predictable and cheaper than traditional breeding (Giri et al. 2004). Powell et al. (2005) provided a thorough review of the techniques used for enhancing fungal and bacterial resistance in transgenic trees. Transgenic technology can provide alternative options through the development of new varieties with resistance to fungal diseases (Quoirin & Quisen 2006, Grattapaglia & Kirst 2008).

Agrobacterium tumefaciens-mediated transformation can be used to introduce genes from other species into the target plant genome. This transformation, which is characterised by integration of intact DNA and genetic stability, is not limited to short DNA fragments (Gustavo et al. 1998). These features make *A. tumefaciens* a widely used agent for stable genetic transformation of *Eucalyptus* (Shao et al. 2002, Tournier et al. 2003, Dibax et al. 2005, Lai et al. 2007, Dibax et al. 2010a).

The *Raphanus sativus*-antifungal protein 2 (Rs-AFP2) was first identified in carrots (Terras et al. 1993). This protein has broad-spectrum antifungal activity against plant pathogen filamentous fungi; it causes hyperbranching and inhibits growth at the hyphal tips (Samblanx et al. 1996). There are few publications on genetic transformation of *E. urophylla* and no study has been published on the introduction of the Rs-AFP2 gene into *E. urophylla* tissues. The conditions required for effective genetic transformation of *E. urophylla* were examined to improve the success rate of genetic transformation in this plant. By introducing Rs-AFP2, we aim to enhance the resistance of *E. urophylla* to *Phytophora capsici,* which causes devastating disease on many crop species, including *Eucalyptus* (Liang et al. 2004).

MATERIALS AND METHODS

Plant materials

Viable seeds of *E. urophylla* were provided by the China Eucalyptus Research Centre, Zhanjiang, China. The seeds were surface-disinfected with 70% ethanol for 2 min and 0.1% (w/v) aqueous mercuric chloride solution for 10 min and then washed three times with sterilised distilled water. They were sown on Murashige and Skoog (MS) (Murashige & Skoog 1962) basal medium devoid of growth regulators but supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. Seeds were incubated at 25 ± 2 °C in darkness for 10 days and then for another 2 days under 16/8 hours light/dark regime with photosynthetic photon flux density of approximately 50 µmol m^{-2} s^{-1} emitted from cool fluorescent tubes (standard culture conditions). Hypocotyls healed until the cotyledons (8–10 mm) were excised and inoculated on MS medium supplemented with 13.2 µM N-phenyl-N'-[6-(2-chlorobenzothiazol)-yl] urea (PBU) and 0.285 µM indole-3-acetic acid (IAA) for pre-culturing. PBU is a di-substituted urea. It was first synthesised and purified in the laboratory and is a useful growth regulator (Li & Luo 2001). The cultures were maintained at 25 ± 2 °C in the dark for 0–8 days and the hypocotyls were used as explants for transformation experiments.

Strains and plasmids

The *A. tumefaciens* strain used for the genetic transformation was EHA105 (Hood et al. 1993), harbouring a binary vector pPBR-2 (Hong et al. 2000) developed in the laboratory (Luo et al. 2004) containing the Rs-AFP2 gene under the control of the *prp*1-1 (Martini et al. 1993) promoter. The neomycin phosphotransferase II (*nptII*) selection marker gene was inserted under the control of the CaMV35S constitutive promoter.

Antibiotic study

Kanamycin

To determine the optimal kanamycin (Kan) concentration, the adventitious bud induction medium [MS medium containing 0.25 µM 6-benzyladenine (BA) and 4.4 µM naphthalene acetic acid (NAA)] was supplemented with 5, 15, 25, 35, 45 and 55 mg L^{-1} Kan and the rooting medium [half-strength MS mineral salts medium (MS/2) supplemented with 2.46 µM IBA] was supplemented with 5, 15, 25, 35, 45, 55, 65 and 75 mg L^{-1} Kan. The rates of adventitious shoot induction and rooting induction were calculated after 30 days.

Cefotaxime

To determine the effective cefotaxime (Cef) concentration, two factors were taken into account:

(1) Effect of Cef on adventitious shoot induction in *E. urophylla* hypocotyls.
To assess this effect, the calluses were transferred to adventitious bud induction medium (MS medium containing 0.25 µM BA and 4.4 µM NAA) with 50, 150, 250, 350, 450 and 550 mg L^{-1} Cef, and the rate of adventitious shoot induction in each case was calculated after 30 days.
(2) Cef-induced growth inhibition of *A. tumefaciens*

After the hypocotyls were infected with *A. tumefaciens* cultures, they were transferred to adventitious bud induction medium (MS medium containing 0.25 µM BA and 4.4 µM NAA) with 50, 100, 150, 200, 250, 300, 350 and 400 mg L^{-1} Cef concentrations. Colony growth at 25 ± 2 °C was observed after two days.

Plant tissue culture and genetic transformation system

To determine the best transformation conditions, different factors were optimised separately, including pre-culture times, pH of the infection medium and co-culture times. The experimental design was totally randomised. Each treatment contained five replicates with 30 explants per flask. Hypocotyl segments were pre-cultured in MS medium for 0, 2, 4, 6 and 8 days, and then immersed in bacterial solution (the pH adjusted to 5.2, 5.4, 5.6, 5.8 and 6.0) of A_{600} = 0.5 for three hours. After infection, the explants were blotted dry on sterile filter paper and plated on MS medium supplemented with 13.2 µM PBU and 0.285 µM IAA to co-culture for 0, 2, 4, 6 and 8 days. After co-cultivation, *A. tumefaciens* was removed by swirling these explants in sterile water. Explants were blotted dry and transferred to the callus-induction medium (MS medium supplemented with 13.2 µM PBU and 0.285 µM IAA) and adventitious bud induction medium (MS medium containing 0.25 µM BA and 4.4 µM NAA) supplemented with 200 mg L^{-1} Cef for decontaminating residual *A. tumefaciens* and 45 mg L^{-1} Kan sulphate for selecting transformed resistant bud. After 30 days, the rate of adventitious bud induction in each case was calculated. The impacts of different pre-culture times, pH of the infection medium and co-culture times were tested on the rate of resistant bud induction in *E. urophylla* hypocotyls. The explants were subcultured onto fresh medium biweekly. After four subcultures, Cef was omitted from the selection medium to continue the screening for putative transformants in the presence of Kan. The Kan-resistant cultures were subcultured every two weeks until shoots regenerated from the callus clumps. Shoot elongation was then stimulated on half-strength MS mineral salts medium (MS/2) supplemented with 6.6 µM PBU and 0.285 µM IAA for 20 days. The shoots that regenerated from the Kan-resistant callus clumps were excised at approximately 1.5 cm in length and cultivated in modified 1/2 MS medium containing 2.46 µM IBA for rooting. Subsequently, the rooted plantlets grown in conical flasks covered with ventilate pellicle were transferred to a greenhouse (25 ± 2 °C and 80% relative humidity) for seven days, following which plantlets were transferred to a mixture of 2 soil:1 fine sand (by volume) in the greenhouse and allowed to develop into fully grown plants.

Plant genomic DNA extraction and polymerase chain reaction (PCR) analysis

Total genomic DNA was isolated from the transformed and non-transformed control plants grown in green house and processed according to the protocol described by Doyle (1990). The presence of transgenes was confirmed by PCR amplification of the Rs-AFP2 gene (the amplicon size is 250 bp) with the gene-specific forward primer 5'-ATCCAACAATGGCTAAGTTTGCT-3' and the reverse primer 5'-GCTCTCATTAACA AGGGGAAATA-3'. Plasmid pPBR-2 DNA was used as the positive control and the DNA from the non-transformed plants was used as the negative control. PCR was performed with the following amplification programme in a PTC-100TM thermocycler: 94 °C for 5 min, followed by 32 cycles of 94 °C for 1 min, 52 °C for 45 s, 72 °C for 45 s, 72 °C for 8 min and 4 °C until insertion into the gel. The amplified fragments were subjected to electrophoresis in 0.8% agarose gel containing 0.5 µg of ethidium bromide and examied under UV radiation (Brody & Kern 2004).

RNA extraction

The leaves of transformed and non-transformed control plants grown in green house were inoculated with *P. capsici*. After 60 hours, total RNA was extracted from 300 mg of leaf tissue from the transformed and non-transformed control plants according to the protocol described by Donald et al. (1997).

Reverse transcription-PCR

Reverse transcription (RT)-PCR was performed according to the manufacturer's instructions provided in the manual of Takara RT-PCR kit (TaKaRa Biotechnology Company, Japan).

Disease resistance testing

To assess the disease resistance of the transgenic plants, 5-month-old plants were inoculated with a conidial suspension (1×10^6 spores mL^{-1}) of *P. capsici* (provided by the China Eucalyptus Research Centre, Zhanjiang, China) using an atomiser and the inoculum was applied to run-off. The leaves were digitally photographed, the diseased areas electronically traced and their pixel areas calculated using Adobe Photoshop version (7.0) software. Disease level was expressed as the percentage of leaf necrotic area. Enzyme activities of phenylalanine aminolyase (PAL), polyphenol oxidase (PPO) and peroxidase (POD) were measured according to the protocol described by Agarwal (2007). Each experiment was performed twice with at least three replicates per treatment and appropriate controls.

RESULTS

Antibiotic study

Optimisation of Kan concentration

An increase in Kan concentration was correlated with a decrease in the number of adventitious buds and roots (Figure 1). The differentiation of adventitious buds was inhibited at Kan concentration of 55 mg L^{-1}.

Hypocotyl growth rate decreased after infection and the growth potential of the new adventitious buds was low. Kan concentration of 45 mg L^{-1} was selected for adventitious bud induction. No rooting was observed in tissue culture seedlings when Kan was used at concentration of 75 mg L^{-1}. Therefore, this concentration was used for rooting selection.

Optimisation of Cef concentration

Adventitious bud induction was mildly affected at low Cef concentrations (Figure 2) but was clearly inhibited by Cef concentrations of ≥ 400 mg L^{-1}. *Agrobacterium tumefaciens* growth was inhibited at Cef concentrations of ≥ 200 mg L^{-1} (Figure 3). The optimal Cef concentration was determined as 200 mg L^{-1}.

Optimisation of genetic transformation parameters

Pre-culture time

Figure 4 shows an increasing tendency of the rate of resistant bud induction in *E. urophylla* hypocotyls with time. The number of resistant buds peaked when the pre-culture time reached six days.

pH of the infection medium

The relation between pH of the infection medium used for the transformation and induction of resistant buds of *E. urophylla* hypocotyls is shown in Figure 5. The number of resistant buds initially increased, peaked at pH 5.6 and then showed a downward trend. At pH 5.6, the hypocotyls were most conducive to *A. tumefaciens* infection.

Co-culture time

The number of resistant buds was highest when the co-culture time was six days (Figure 6).

Plant tissue culture

After co-cultivation for six days, 96% explants formed calluses (Figure 7a). After 20 days, the calluses were transferred to MS medium containing 0.25 µM NAA and 4.4 µM BA to induce the formation of adventitious buds. The percentage of adventitious bud formation ranged from 50 to 80% (Figures 9b–e). Shoot elongation was then stimulated. The mean shoot length ranged from 2.5 to 3.0 cm after 28 days of cultivation and the shoots showed rooting at the end of the elongation phase (Figure 9f). The

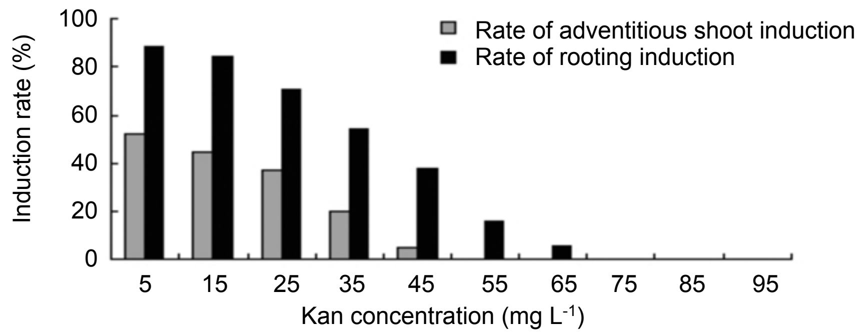

Figure 1 Relation between Kan concentrations and adventitious shoot induction and rooting of *Eucalyptus urophylla* explants; Kan = kanamycin

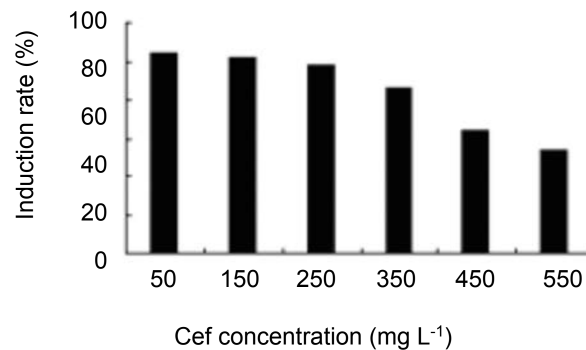

Figure 2 Effect of Cef concentration on adventitious shoot induction from hypocotyls; Cef = cefotaxime

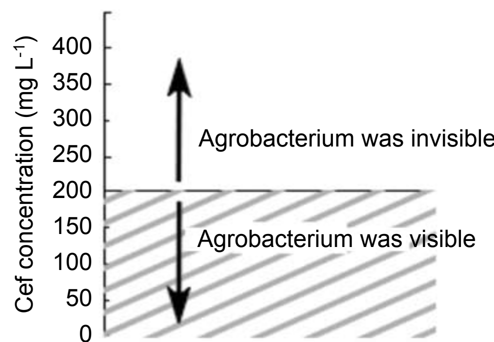

Figure 3 Determination of the optimal Cef concentration; Cef = cefotaxime

percentage of rooted microcuttings was 76%, the average number of roots per plant was 3 and the mean length of the main root was 4 cm (Figure 9g–h). After the acclimatisation period, the plant survival rate was 80% and the mean shoot size was 5 cm. No obvious phenotypic changes were observed (Figure 9i).

PCR analysis of plant genomic DNA

The PCR results are shown in Figure 8. The samples loaded in lanes 7, 13 and 30 displayed bands corresponding to approximately 250 bp, which were consistent with the size of the positive control. This indicated that the Rs-AFP2 gene was successfully integrated into the genome of *E. urophylla*.

RNA extraction and RT-PCR

Total RNA was extracted from the leaves (Figure 9a), which suggested that total RNA degradation was slight.

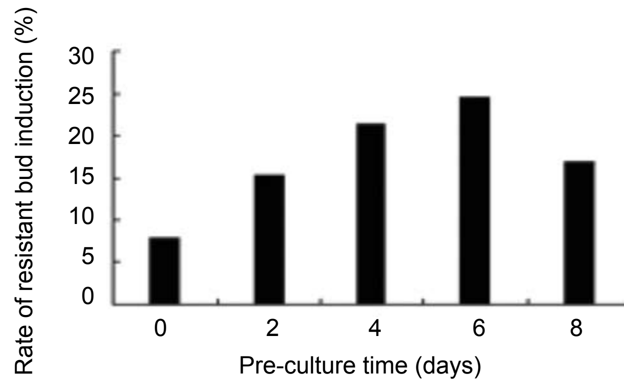

Figure 4 Effect of pre-culture time on the rate of resistant bud induction

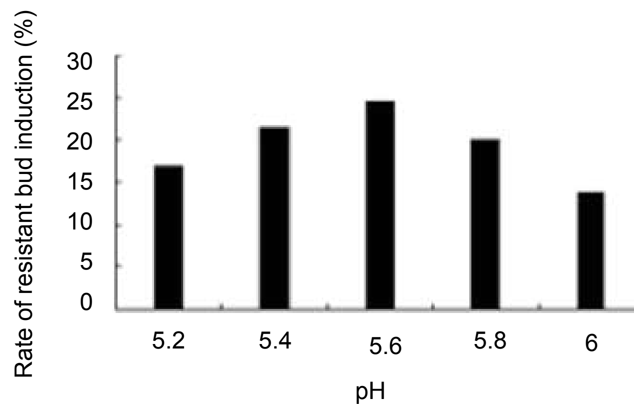

Figure 5 Effect of pH on the rate of resistant bud induction

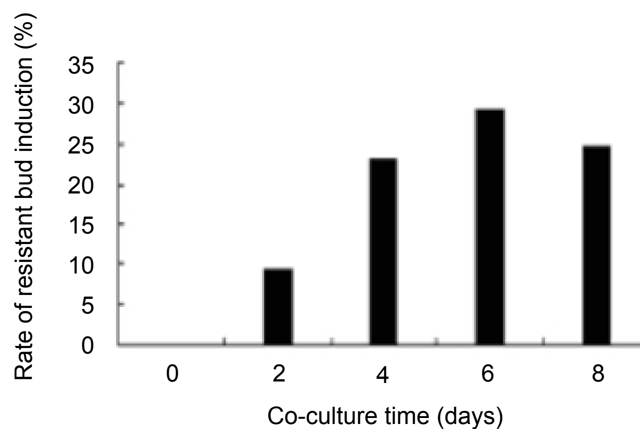

Figure 6 Effect of co-culture time on the rate of resistant bud induction

The results showed that Rs-AFP2 transcripts were not detected in transformed or non-transformed plants, indicating that the Rs-AFP2 gene was not expressed.

After inoculation with *P. capsici*, changes were observed in Rs-AFP2 expression (Figure 9c). RT-PCR performed in plants that yielded positive PCR effects resulted in the amplification of an 250-bp fragment, which was consistent with the size of the positive control. This result confirmed that the *prp1-1* promoter of the Rs-AFP2 gene was induced by infection with *P. capsici*.

Disease resistance

After *P. capsici* inoculation, non-transformed controls showed the highest level of infection (Figure 10). The leaves of non-transgenic plants

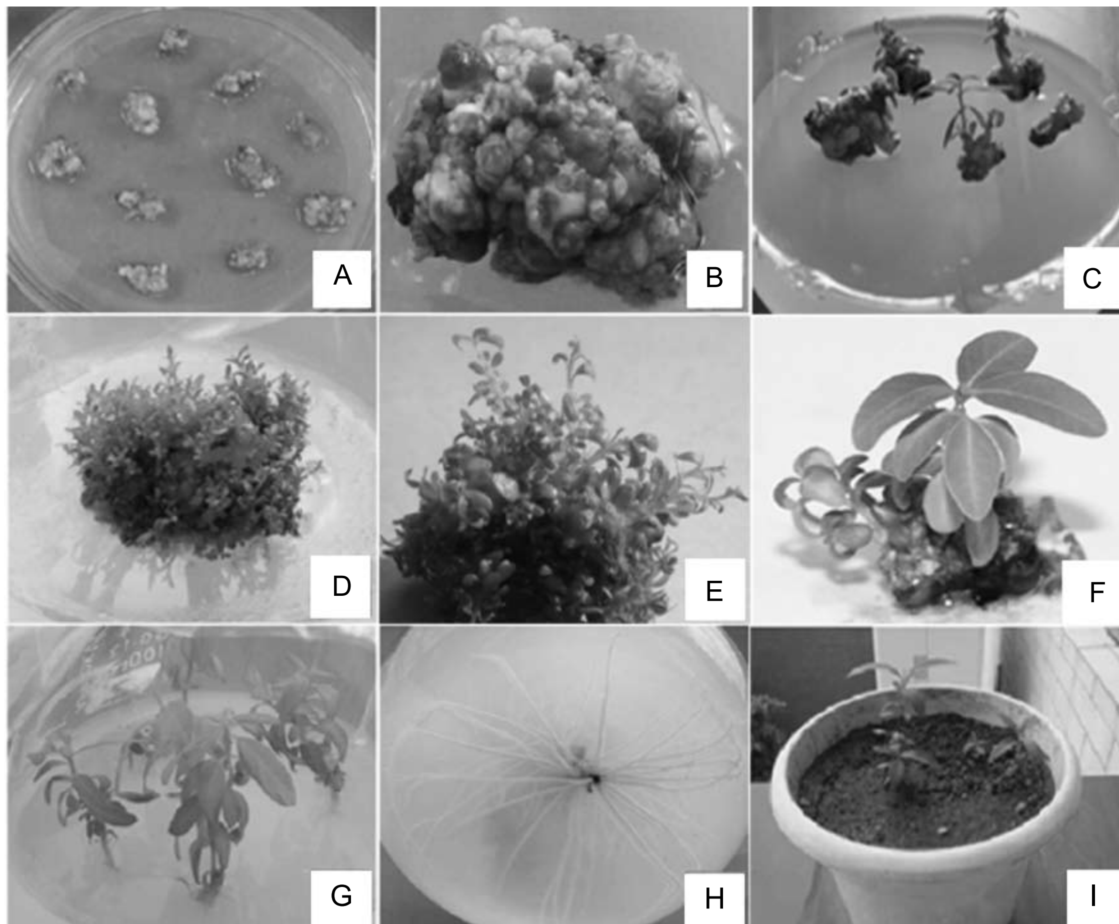

Figure 7 Regeneration of transgenic *Eucalyptus urophylla* from hypocotyls; A = callus inoculated on MS medium with 6.6 µM PBU and 0.285 µM IAA, B–E = differentiation of resistant adventitious buds after transfer to MS medium with 4.4 µM BA and 0.25 µM NAA and 45 mg L^{-1} Kan, F = elongation of resistant adventitious buds inoculated on MS/2 medium supplemented with 6.6 µM PBU and 0.285 µM NAA, G, H = rooting of elongated shoot cultivated in modified MS/2 medium supplemented with 2.46 µM IBA and 75 mg L^{-1} Kan, I = plants in a plastic pot in greenhouse

suffered severe blighting, necrosis and wilting six days after inoculation with *P. capsici* (Figure 10). Although none of the transformed lines showed complete disease resistance, transgenic plants only showed weak signs of necrosis in the inoculation area (near the leaf stalk). The degree of leaf necrosis ranged from 10 to 20% in transgenic plants.

The PAL, PPO and POD activities five days after inoculation with *P. capsici* were higher than those before inoculation (Figure 11). After inoculation with *P. capsici*, the activity of PAL and POD increased 25 and 45% in the transgenic leaves respectively. However, the increase in the PAL, POD and PPO activities obtained from transgenic leaves were less than those obtained from non-transgenic leaves.

DISCUSSION

Transgenic *Eucalyptus* plants have been generated by *A. tumefaciens* inoculation into explants such as hypocotyls and cotyledons (Prakash & Gurumurthi 2009, Dibax et al. 2010b). Successful *A. tumefaciens*-mediated transformation in most plants depends on the effectiveness of the tissue culture methods used. Therefore, the development of efficient, simple and rapid transformation systems that are easy to manipulate, and if possible, devoid of tissue culture steps is important (Li et al. 2000). The *A. tumefaciens*-mediated transformation system has some advantages such as simple operation, high transformation efficiency and easy DNA integration. In an earlier study, Huang et al.

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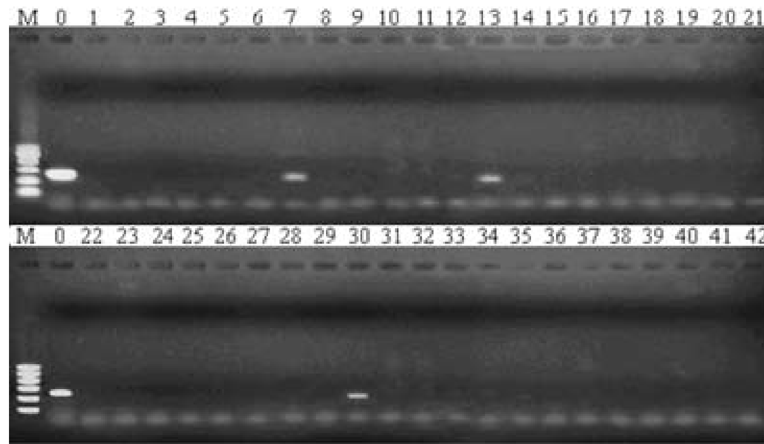

Figure 8 PCR analysis of putative transgenic plants of *Eucalyptus urophylla;* M = 100-bp marker, 0 = positive control, 1 = negative control, 2–42 = putative transgenic plants

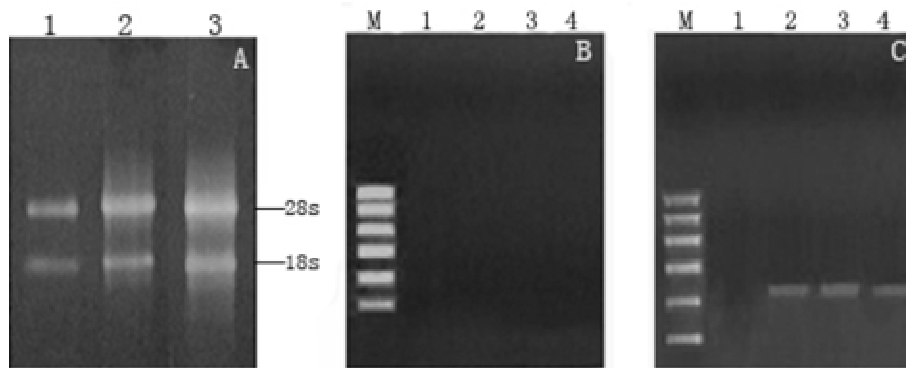

Figure 9 RNA extraction and RT-PCR analysis; A = total RNA samples from *Eucalyptus urophylla* leaves, B, C = RT-PCR analysis of transformants before and after inoculation with *Phytophthora capsici*; M = 100-bp DNA marker, 1 = non-transgenic *Eucalyptus urophylla,* 2–4 = transgenic *Eucalyptus urophylla*

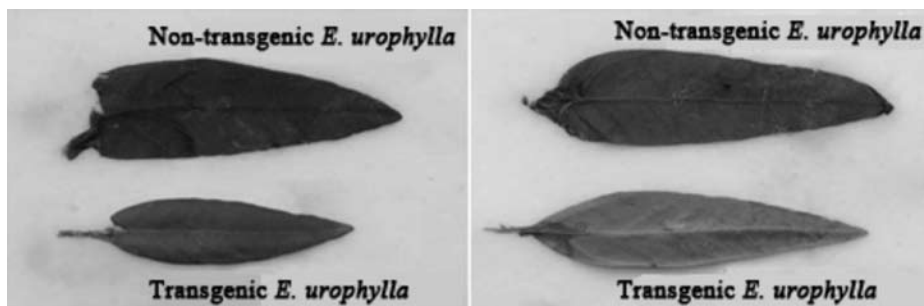

Figure 10 The incidence of *Eucalyptus urophylla* leaves, six days after inoculation with *Phytophthora capsici;* left is front, right is back

(2010) reported an efficient regeneration system for *E. urophylla* hypocotyls. In the present study, transgenic *E.urophylla* plantlets were generated after *A. tumefaciens*-mediated transformation using hypocotyl explants as the target material. Moralejo et al. (1998) obtained transgenic *E. globulus* plants using mature seeds as explant material. The transformation frequency increased after pre-culturing the explants. Plantlets were obtained through regeneration from the explants, which was similar to the results obtained in the present study. Pre-culturing before inoculation can effectively increase the transformation rate. The phenols secreted by plants upon wounding

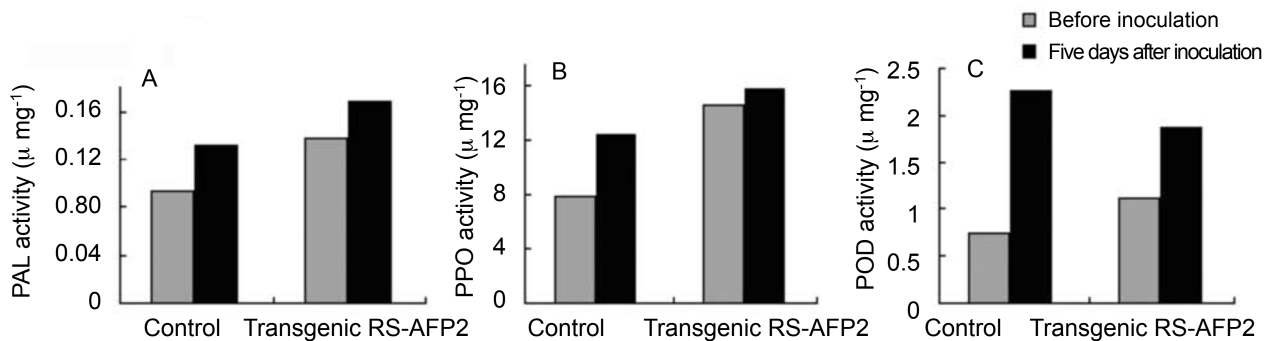

Figure 11 The enzyme activity analysis in transgenic and non-transgenic *Eucalyptus urophylla*; PAL = phenylalanine aminolyase, PPO = polyphenol oxidase, POD = peroxidase

can induce the *vir* genes in *A. tumefaciens* and promote the transfer of T-DNA. In the present study, explants were pre-cultured for six days before co-cultivation with *A. tumefaciens*, which in turn reduced the hypersensitive response and increased transformation efficiency. *Agrobacterium tumefaciens* needs time for T-DNA integration. Therefore, co-culture time is important to *A. tumefaciens*-mediated transformation system. Tumour induction and T-DNA transfer occurred only if *A. tumefaciens* was present at the wounded site for more than 16 hours (Sykes & Matthysse 1986, Gelvin 2000). In this study, the number of resistant buds was optimum when the co-culture time was six days.

The use of appropriate selection conditions is very important in plant transformation. Kan is widely used as a selectable marker. Therefore, Kan sensitivity should be determined at the initial stage of the development of a plant transformation system in which a Kan-resistance gene is used (Fiola et al. 1990, James et al. 1990, Escandon & Hahne 1991). Ho et al. (1998) used 40 mg L^{-1} Kan to select transgenic *E. camaldulensis* plants after transforming the hypocotyl explants with *A. tumefaciens*. Prakash and Gurumurthi (2009) reported that 40 mg L^{-1} Kan was enough to select *E. camaldulensis* transgenic plants. In the present study, the T-DNA of pPBR-2 contains the *nptII* gene, which confers Kan-resistance to transformed plants. We used 45 mg L^{-1} Kan to select the adventitious bud induction and 75 mg L^{-1} for rooting selection. After co-culturing with *A. tumefaciens*, the surface of the explants contained large numbers of bacteria. The control of bacterial growth is important because it can adversely affect explant growth and differentiation, leading to browning and death. However, the use of bacteriostatic antibiotics has adverse effects on plant cells. Ho et al.(1998) used 500 mg L^{-1} Cef for decontaminating residual *A. tumefaciens*. In the present study, we aimed at identifying a Cef concentration to effectively inhibit *A. tumefaciens* growth without affecting plant growth, and the results indicated that 200 mg L^{-1} was the optimal Cef concentration.

The RT-PCR results indicated that the *prp1-1* promoter of the Rs-AFP2 gene was induced by *P. capsici* 60 hours after inoculation, suggesting that disease resistance to *P. capsici* could be enhanced through the induction of Rs-AFP2 expression. These results indicated that the present protocol was successful at achieving efficient transformation, which achieved 7% efficiency in the genetic transformation (number showing positive results in the PCR analysis/number of PCR analysis of putative transgenic plants) and regeneration of *E. urophylla*. Kong et al. (2010) developed a *A. tumefaciens*-mediated transformation system for *E. urophylla* U6 and achieved only 0.59% transformation rate.

There has been an increasing interest in the development of regulated expression systems where transgenic expression occurs only in the presence of particular pathogens (Moreno et al. 2005). In the present study, we described a strategy for incorporating resistance to pathogenic bacteria. This approach relies on the use of an inducible rather than a constitutive promoter, resulting in expression of an antifungal gene at a sufficiently high level for protecting transgenic *E. urophylla* plants against *P. capsici*. The Rs-AFP2 gene is a member of the subfamily of plant defensins isolated from radish seeds (Terras et al. 1995). The protein is found predominantly in the outer cell layers lining different seed organs and

is released preferentially during seed germination after disruption of the seed coat. Rs-AFP2 gene expression was shown to confer enhanced resistance to the foliar pathogen *Alternaria longipes* in transgenic tobacco (Melchers & Stuiver 2000). Jha and Chattoo (2010) demonstrated that transgenic rice plants expressing the Rs-AFP2 gene showed enhanced resistance to *Magnaporthe oryzae* and *Rhizoctonia solani,* two of the most important pathogens of rice. In the present study, non-transformed controls showed the highest level of disease (60%) six days after inoculation. In contrast, the degree of leaf necrosis ranged from 10 to 20% in transgenic plants. In addition, the PAL, POD and PPO activities, which were associated with the levels of disease tolerance, were higher five days after inoculation with *P. capsici* in transgenic *E.urophylla* compared with the activity levels before inoculation. These results indicated that the levels of Rs-AFP2 gene expression in transgenic plants were sufficiently high enough to reduce fungal growth, which resulted in reduction in disease development. This suggested that this method could be applied to other crop plants to enhance resistance to fungal pathogens.

In conclusion, this is the first study of an efficient *A. tumefaciens*-mediated transformation system for *E. urophylla* with the Rs-AFP2 gene. The information presented here may constitute the basis for further optimisation of the protocol for the *Agrobacterium*-mediated transformation of *E. urophylla* and for future studies on the expression of this antifungal gene.

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REFERENCES

Agarwal S. 2007. Increased antioxidant activity in *Cassia* seedlings under UV-B radiation. *Biologia Plantarum* 51: 157–160.

Brody JR & Kern SE. 2004. Sodium boric acid: a tris-free, cooler conductive medium for DNA electrophoresis. *Biotechniques* 36: 214–216.

Dibax R, Deschamps C, Bespalhok FJC, Vieira LGE, Molinari HBC, Campos De MKF & Quoirin M. 2010a. Organogenesis and *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus saligna* with P5CS gene. *Biologia Plantarum* 54: 6–12.

Dibax R, Eisfeld CL, Cuquel F, Koehler H & Quoirin M. 2005. Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis. Scientia Agricala* 62: 406–412.

Dibax R, Quisen RC, Bona C & Quoirin M. 2010b. Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis* Dehn and histological study of organogenesis in vitro. *Brazilian Archives of Biology and Technology* 53: 311–318.

Donald JM, Morven AM, Srima M & Margaret G. 1997. Improved RNA extraction from woody plants for the detection of viral pathogens by reverse transcription-polymerase chain reaction. *Plant Disease* 81: 222–226.

Doughty RW. 2000. *The* Eucalyptus*: A Natural and Commercial History of the Gum Tree.* John Hopkins University Press, Baltimore.

Doyle JL. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15.

Escandon AS & Hahne G. 1991. Genotype and composition of culture medium are factors important in the selection for transformed sunflower (*Helianthus annus*) callus. *Physiologia Plantarua* 81: 367–376.

FAO. 2001. *Global Forest Resources Assessment 2000.* FAO Forestry Paper 140. FAO, Rome.

Fiola JA, Hassan MA, Swartz HJ, Bors RH & McNicols R. 1990. Effect of thidiazuron, light fluence rates and kanamycin on *in vitro* shoot organogenesis from excised *Rubus* cotyledons and leaves. *Plant Cell Tissue and Organ Culture* 20: 223–228.

Gelvin SB. 2000. *Agrobactertum* and plant genes involved in T-DNA transfer and integration. *Plant Biology* 51: 223–256.

Giri CC, Shyamkumar B & Anjaneyulu C. 2004. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. *Trees* 18: 115–135.

Grattapaglia D & Kirst M. 2008. *Eucalyptus* applied genomics: from gene sequences to breeding tools. *New Phytologist* 179: 911–929.

Gustavo AR, Gonzalez-Cabrera J, Vazguez-Padron R & Ayra-Pardo C. 1998. *Agrobacterium tumefaciens*: a natural tool for plant transformation. *Electronic Journal of Biotechnology* 1: 118–133.

Ho CK, Chang SH, Tsay JY, Tsai CJ, Chiang VL & Chen ZZ. 1998. *Agrobacterium tumefaciens* mediated transformation of *Eucalyptus camaldulensis* and production of transgenic plants. *Plant Cell Reports* 17: 675–680.

Hood EE, Gelvin SB, Melchers LS & Hoekema A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* 2: 208–218.

Hong Z, Lakkineni K, Zhang Z & Verma DPS. 2000. Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. *Plant Physiology* 122: 1129–1136.

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Huang ZC, Zeng FH & Lu XY. 2010. Efficient regeneration of *Eucalyptus urophylla* from seedling-derived hypocotyls. *Biologia Plantarum* 54: 131–134.

James DJ, Passey AJ & Barnera DJ. 1990. Regeneration and transformation of apple and strawberry using disarmed T-DNA binary vectors. Pp 239–249 in Lycett GW & Griesone D (eds) *Genetic Engineering of Crop Plants*. Butter Worth, London.

Jha S & Chattoo BB. 2010. Expression of a plant defensin in rice confers resistance to fungal phytopathogens. *Transgenic Research* 19: 373–384.

Kong H, Guo AP, Guo YL, Liu EP & He LK. 2010. Lignin biosynthesis regulated by antisense 4CL gene in *Eucalyptus urophylla* U6. *Chinese Journal of Tropical Crops* 31: 1959–1963.

Lai JY, Shi HM, Liu K, Yin CY, Wei PX, Cen XF & Luo DP. 2007. Studies on the establish genetic transformation of *Eucalyptus urophylla* × *grandis*. *Journal of Sichuan University* (Natural Science edition) 47: 415–419.

Li W, Guo G & Zheng G. 2000. *Agrobacterium*-mediated transformation: state of the art and future prospect. *Chinese Science Bulletin* 45: 1537–1546.

Li ZF & Luo FY. 2001. Synthesis and characterization of N-substituted phenyl-N'-[6-(2-chlorobenzothiazol)-yl] urea. *Chemical Research Application* 13: 80–82.

Liang XL, Feng NJ, Du JD, Ding XW, Song BQ, Zheng DF & Zuo YH. 2004. Status and prospect of *Phytophthora capsici*. *Rain Fed Crops* 26: 354–358.

Lu ZH, Xu JM, Li GY, Bai JY, Huang HJ & Hu Y. 2010. Study on multi-characters genetic analysis and selection index of 93 *Eucalyptus urophylla* clones. *Eucalypt Science and Technology* 27: 1–8.

Luo QJ, Zeng FH & Wu YR. 2004. Construction of fungi inducible plant expression vector pPB. *Journal of Zhan Jiang Normal College* 26: 37–40.

Martini N, Egen M, Runtz I & Strittmatter G. 1993. Promoter sequences of a potato pathogenesis-related gene mediate transcriptional activation selectively upon fungal infection. *Molecular General Genetics* 236: 170–186.

Melchers LS & Stuiver MH. 2000. Novel genes for disease-resistance breeding. *Current Opinion in Plant Biology* 3: 147–152.

Moreno AB, Peñas G, Rufat M, Bravo JM, Estopà M, Messeguer J & Segundo BS. 2005. Pathogen-induced production of the antifungal AFP protein from *Aspergillus giganteus* confers resistance to the blast fungus *Magnaporthe grisea* in transgenic rice. *Molecular Plant Microbe Interaction* 18: 960–972.

Moralejo M, Rochange F, Boudet AM & Teulieres C. 1998. Generation of transgenic *E. globulus* plantlets through *Agrobacterium tumefaciens* mediated transformation. Australian. *Journal of Plant Physiology* 252: 207–212.

Murashige T & Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.

Powell WA, Maynard CA, Boyle B & Seguin A. 2005. Fungal and bacterial resistance in transgenic trees. In Flaunding M & Ditrich E (eds) *Transgenic Trees*. Springer, Heidelberg.

Potts BM & Dungey HS. 2004. Interspecific hybridization of eucalypts: key issues for breeders and geneticists. *New Forests* 27: 115–138.

Prakash MG & Gurumurthi K. 2009. Genetic transformation and regeneration of transgenic plants from precultured cotyledon and hypocotyl explants of *Eucalyptus tereticornis* Sm. using *Agrobacterium tumefaciens. In vitro Cellular and Developmental Biology Plant* 45: 429–434.

Quoirin M & Quisen R. 2006. Advances in genetic transformation of *Eucalyptus* species. In Franche C (ed) *Molecular Biology of Tropical Plants*. Research Signpost, Kerala.

Samblanx De GW, Fernandez Del CA, Sijtsma L, Plasman HH, Schaaper WM, Posthuma GA, Fant F, Meloen RH, Broekaert WF & Van Amerongen A. 1996. Antifungal activity of synthetic 15-mer peptides based on the Rs-AFP2 sequence. *Journal of Reptide Research* 9: 262–268.

Shao ZF, Chen WY, Luo HL & Ye XF. 2002. Studies on the introduction of the Cecropin D gene into *Eucalyptus urophylla* to breed the resistant varieties to *Pseudomonns solanacearum. Scientia Silvae Sinicae* 38: 92–98.

Sykes LC & Matthysse AG. 1986. Time required for tumor induction by *Agrobacterium tumefaciens. Applied and Environmental Microbiology* 52: 597–598.

Terras FRG, Eggermont K, Kovaleva V, Raikhel NV, Osborn, RW, Kester A, Rees SB, Torrekens S, Leuven FV, Vanderleyden J, Cammue BPA & Broekaert WF. 1995. Small cysteine-rich antifungal proteins from radish: their role in host defence. *Plant Cell* 7: 573–588.

Terras FRG, Torrekens S & Van Leuven F. 1993. A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species. *Federation of European Biochemical Societies Letters* 316: 233–240.

Tournier V, Grat S, Marque C, Kayal EW, Penchel R, Andrade G, Boudet AM & Teulières C. 2003. An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* × *E. urophylla*). *Transgenic Research* 2: 403–411.

Wu ZH, Xie YJ, Luo LF & Zhang WY. 2007. Advances in research on bacterial wilt caused by *Ralstonia solanacearum* in *Eucalyptus* spp. in China. *Forest Research* 20: 569–575.

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