

Effects of an inducible *aiiA* gene on disease resistance in *Eucalyptus urophylla* × *Eucalyptus grandis*

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Abstract *N*-acyl-homoserine lactones (AHLs) are metabolites of mostly gram-negative bacteria and are critical signaling molecules in bacterial quorum-sensing systems. At threshold concentrations, AHLs can activate the expression of pathogenic genes and induce diseases. Therefore, reducing AHL concentrations is a key point of disease control in plants. AHL-lactonase, which is expressed by *aiiA*, is widespread in *Bacillus sp* and can hydrolyze AHLs. In the present study, we cloned *aiiA* from *Bacillus subtilis* by PCR. A plant expression vector of *aiiA* was constructed and name Pcam-PPP3-*aiiA*, in which expression of *aiiA* was controlled by the pathogen-inducible plant promoter PPP3. The recombinant plasmid was transferred into *Eucalyptus* × *urophylla* × *E. grandis* by an *Agrobacterium*-mediated transformation. PCR and Southern blotting showed that *aiiA* was successfully integrated into the *E. urophylla* × *E. grandis* genome and its expression was induced by *Ralstonia solanacearum* 12 h after inoculation, as shown by reverse transcription-PCR. The transcription efficacy of *aiiA* increased 43.88-, 30.65-, and 18.95-fold after inoculation with *R. solanacearum*, *Erwinia carotovora ssp. zea* (*Sabet*) and *Cylindrocladium quinqueseptatum*, respectively as shown by RT-real-time PCR. Transgenic *E.urophylla* × *E.grandis* expressing the AIIA

protein exhibited significantly enhanced disease resistance compared to non-transgenic plants by delaying the onset of wilting and reducing the disease index.

Keywords *Eucalyptus urophylla* × *E. grandis* · *aiiA* · Genetic transformation · Inducible expression · Broad-spectrum disease resistance

Abbreviations

BA	6-Benzyladenine
NAA	Naphthalene acetic acid
IAA	Indole-3-acetic acid
MS	Murashige and Skoog
PBU	<i>N</i> -phenyl- <i>N'</i> -[6-(2-chlorobenzothiazol)-yl] urea

Introduction

Eucalyptus, which belongs to the Myrtaceae family, is the most widely planted hardwood crop in tropical and subtropical regions. The tremendous potential of *Eucalyptus* species to produce timber and fiber for pulp and paper industry has led to the rising commercial dominance of this genus, which comprises more than 1000 species (Doughty 2000; Potts and Dungey 2004; Prakash and Gurumurthi 2010; Torre et al. 2014). *Eucalyptus* is one of the most important forest trees in China, covering >3 million hectares of commercial plantations (Ouyang 2012a). *Eucalyptus*

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urophylla × *Eucalyptus grandis* can reach a height of 50 m. Besides the production of pulpwood, *Eucalyptus urophylla* × *Eucalyptus grandis* is also used for timber, veneer, firewood, shelter, ornamentals and essential oil production (Lu et al. 2010). However, diseases such as bacterial wilt, fungal infection, and gray mold have seriously endangered the *Eucalyptus* crop in China, especially that of the hybrid *E. urophylla* × *E. grandis* (Wu et al. 2007).

Traditional methods for the prevention and treatment of these diseases involve the use of chemical pesticides, which often cause environmental pollution and damage. Producing disease resistant trees through genetic engineering can potentially be faster, better controlled, predictable, and less expensive than traditional breeding (Giri et al. 2004). Powell et al. (2005) provided a thorough review of the techniques used to enhance fungal and bacterial resistance in transgenic trees.

Quorum sensing is a signaling mechanism utilized by bacteria to coordinate gene expression in response to changes in cell density. Quorum sensing is central to a number of physiological responses, including stationary phase induction (Lithgow et al. 2000), pathogenesis (Williams et al. 2000; Wu et al. 2001), biofilm formation (De Kievit et al. 2001), antibiotic production (Bainton et al. 1992), exoenzyme production (Chernin et al. 1998), nodulation (Rosemeyer et al. 1998), plasmid transfer (Piper and Farrand 2000), light production, and conjugation. *N*-acyl-homoserine lactones (AHLs) are metabolites of most gram-negative bacteria and are critical signaling molecules in bacterial quorum-sensing systems. At threshold concentrations, AHLs can activate the expression of pathogenic genes and induce diseases. Therefore, reducing AHL concentrations is a key point of the disease control in plants (Wopperer et al. 2006; Chen et al. 2009).

AHL-mediated quorum-sensing systems have recently been viewed as new targets for anti-infective therapies (Chen et al. 2009). In contrast to traditional drug designs that are either bactericidal or bacteriostatic, disruption of the AHL-mediated quorum sensing mechanisms, known as quorum quenching, aims to shut down the expression of virulence rather than kill the organisms. Therefore, quorum quenching has the potential to overcome drug-related toxicities, complicated superinfections, and antibiotic resistance in antibiotic therapy. Dong et al. (2000) isolated a novel lactonase gene, *aiiA*, from a *Bacillus* sp. This gene

encodes an enzyme that renders AHL biologically inactive. Since many pathogenic bacteria use quorum sensing to regulate virulence, it is thought to interfere with the bacterial communication system by disrupting quorum sensing to treat or prevent infection. As one of the anti-quorum-sensing strategies, AHL-signaling molecule degradation could have potential application in attenuating plant disease. This group created *aiiA* gene-transformed tobacco and potato plants, both of which acquired soft rot resistance (Dong et al. 2001).

Some *Bacillus thuringiensis* strains generate an endocellular acyl homoserine lactonase (*aiiA*), which has an inhibitory effect on pathogens by disrupting the signal molecules (AHL) of their quorum sensing system. *Agrobacterium tumefaciens*-mediated transformation can be used to introduce genes from other species into the target plant genome. These features make *A. tumefaciens* a widely used agent for the 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; Lepikson-Neto et al. 2014; Sykes et al. 2015). There are few publications about the genetic transformation of *E. urophylla* × *E. grandis* but none reported the introduction of *aiiA* into *E. urophylla* × *E. grandis* tissues. By introducing *aiiA*, we aimed to enhance the resistance of *E. urophylla* × *E. grandis* to *Phytophthora capsici* and *R. solanacearum*, which causes devastating diseases in many crop species, including eucalyptus (Liang et al. 2004).

Materials and methods

Plant materials

Stem segments from aseptic clonal seedlings of *E. urophylla* × *E. grandis* 32–29 were kindly provided by the China Eucalyptus Research Center (Guangdong, China). For callus induction, stem segments (4–8 mm) were excised from aseptic seedlings and inoculated on MS medium supplemented with 100 mg/L of vitamin C, 30 g/L of sucrose, 7 g/L of agar, and 13.2 μM *N*-phenyl-*N'*-[6-(2-chlorobenzothiazol)-yl] urea (PBU) and 0.285 μM indole-3-acetic acid (IAA) for pre-culturing. 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 quorum-quenching *aiiA* was cloned from *Bacillus subtilis* by PCR in our laboratory (Ouyang et al. 2012b). The sequence analysis indicated that the gene consisted of 751 nucleotides (nt) coding 250 amino acids. The nt sequence shared 87–96 % identity with other *aiiA* (Ouyang et al. 2012b). *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA1301-PP3-*aiiA* was used for the genetic transformation of *E. urophylla* × *E. grandis*. This binary vector contains *aiiA* under the control of the pathogen-inducible plant promoter PPP3 that can be induced by a pathogen (Peng et al. 2004). At the same time, the pCAMBIA1301-CaMV35S-*aiiA* vector with the hygromycin (Hyg) selection marker gene was constructed.

Plant tissue culture and genetic transformation system

To facilitate optimization of *A. tumefaciens*-mediated transformation of *E. urophylla* × *E. grandis*, five factors were studied using an $L_{16}(4^5)$ orthogonal design (Chen 2005). A total of 16 treatments was selected. Each with five replicates of 30 explants. The extreme deviation (R) was calculated to evaluate the effects of each factor, and analysis of variance (ANOVA) was performed to show the significant differences among the factors and identify the optimal conditions for transfer DNA (T-DNA) delivery.

Hypocotyl segments were pre-cultured on MS medium and then immersed in a bacterial solution. 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 for co-culturing. After the co-cultivation, the *A. tumefaciens* was removed by swirling of the explants in sterile water. The explants were then 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 6-benzyladenine [BA] and 4.4 μM NAA) supplemented with 200 mg/L cefotaxime (Cef) for decontaminating the residual *A. tumefaciens*, and 9 mg/L hygromycin (Hyg) sulfate was used to select the transformed resistant buds. After 30 days, the rate of adventitious

bud induction in each case was calculated, and the impact of different pre-culture times, the pH of the infection medium, concentration of *Agrobacterium*, infection time, and co-culture times on the rate of resistant bud induction in *E. urophylla* × *E. grandis* hypocotyls was tested.

After four subcultures, the Cef was omitted from the selection medium to continue the screening for putative transformants in the presence of Hyg. The Hyg-resistant cultures were subcultured every 2 weeks until the 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. Approximate 1.5-cm lengths of the shoots that regenerated from the Hyg-resistant callus clumps were excised and cultivated in modified 1/2MS medium containing 2.46 μM indole-3-butyric acid for rooting. Subsequently, the rooted plantlets grown in conical flasks covered with ventilate pellicle were transferred to a greenhouse (25 \pm 2 °C and 80 % relative humidity) for 7 days, then the plantlets were transferred to a 2:1 mixture of soil and fine sand (by volume) in the greenhouse and allowed to fully develop.

Plant genomic DNA extraction and PCR analysis

Total genomic DNA was isolated from the transformed and non-transformed control plants grown in the greenhouse and processed according to the protocol described by Doyle (1990). The presence of transgenes was confirmed by PCR amplification of PPP3-*aiiA* (amplicon size, 1100 bp) and 35S-*aiiA* (amplicon size, 750 bp) with gene-specific primers (Ouyang et al. 2012b). Plasmids pCAMBIA1301-PPP3-*aiiA* and pCAMBIA1301-35S-*aiiA* was used as positive controls while DNA from non-transformed plants were used as negative control. PCR was performed with the following amplification program in a PTC-100TM thermocycler (MJ Research, Waltham, MA, USA): 94 °C for 5 min, followed by 32 cycles at 94 °C for 1 min, 52 °C for 45 s, 72 °C for 45 s, 72 °C for 8 min, and 4 °C in storage. The amplified fragments were subjected to electrophoresis in a 0.8 % agarose gel containing 0.5 μg of ethidium bromide and visualized under UV radiation (Brody and Kern 2004).

Southern blot analysis

For the Southern blot analysis, 20 µg of genomic DNA of the transgenic event and control were digested with *Hind*III at 37 °C for 16 h. The DNA samples were then subjected to electrophoresis in agarose gel and stained with 1 % (v/v) ethidium bromide for 30 min. After electrophoresis, the DNA was transferred onto a Hybond-N⁺® membrane (GE Healthcare, UK) by capillarity and fixed by incubation at 80 °C for 2 h (Sambrook et al. 1989). Digoxigenin labeled probes using a random priming technique. After hybridization, the membranes were washed twice at 42 °C with saline-sodium citrate (SSC; 2 %) and sodium dodecyl sulfate (SDS; 0.1 %) solutions and twice with SSC (0.1 %) and SDS (0.1 %) solutions at 42 °C. Hybridization patterns were detected by exposure on a plate reader for 1 h and the images were captured by a FLA-3000 system (Fuji film).

RNA extraction and reverse transcription-PCR (RT-PCR)

Leaves of the transformed and non-transformed control plants grown in a greenhouse were inoculated with *Ralstonia solanacearum*. After 60 h, total RNA was extracted from 300 mg of leaf tissue from the transformed and non-transformed control plant leaf tissue according to the protocol described by Donald et al. (1997).

RT-PCR was performed according to the instructions provided with a Takara RT-PCR kit (TaKaRa Biotechnology Company, Japan) using primers (Primer 1': GCGAATTTCGAAAAGGGTCCAGTCCT; and Primer 2' AGAGTACTATTTATAGTGATATA-TATGCT.) synthesized by Shangai Biotechnology Company (China).

RNA blot analysis

The RNA was electrophoresed on a 1.2 % formaldehyde/MOPS (3-[*N*-morpholino] propane sulfonic acid) gel and transferred onto a nylon N⁺ membrane (Amersham) according to the manufacturer's instructions. The membrane was then hybridized with digoxigenin labeled probes fragment of the *aiiA* gene. All solutions and glassware were treated with 0.1 % diethyl pyrocarbonate.

Analysis for real-time PCR amplification efficiency of *aiiA* expression

Quantitative PCR was performed in a 96-well format using an Agilent Technologies/Stratagene Mx3000P. The qPCR primers and probes were designed using Primer5. Primer oligonucleotides were synthesized by Invitrogen, and probes were synthesized by Biosearch Technologies. The primers of *aiiA*: 5'gtgcactcagc-cggggaaa 3', 5'aggtcgtccggctcatacct 3'; The primers of reference gene: 5' acattgtgctcagtgtgga 3', 5' caagatagactccgatcc 3' Each 25 µL reaction contained 3 pmol of the appropriate probe oligonucleotide and 5 pmol of each corresponding primer oligonucleotide, plus 12.5 µL of Maxima Probe/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific #K0233) and cDNA equivalent to 1–5 ng of total RNA. The amplification efficiency of real-time PCR was analyzed according to the protocols of Schmittgen et al. (2004).

Disease resistance test

To assess the disease resistance levels of the transgenic plants, 30 whole 2-month-old T₀ plants were inoculated with a conidial suspension of *R. solanacearum*, *Phytophthora capsici* and *Erwinia carotovora* spp. *zeae* respectively (China Eucalyptus Research Center, Zhanjiang, China), using an atomizer, and the inoculum was applied to run-off. Ten non-transformed plants were used in disease resistance evaluation assays. The leaves were digitally photographed, the diseased areas were electronically traced, and their pixel areas were calculated using Adobe Photoshop version 7.0 software.

A plant virulence assay was performed as described by Toth et al. (1999). Blackleg symptoms were scored according to the following numerical index: 0 = no reaction; 1 = slight browning around the inoculation site; 2 = slight blackening around the inoculation site; 3 = small black rot spreading from the inoculation site; 4 = medium black rot spreading from the inoculation site; and 5 = large black rot (Toth et al. 1999). To compare the resistance of the transgenic plants expressing PPP3-*aiiA* and 35S-*aiiA*, the ability of *aiiA* to suppress disease and its effect on *aiiA*-producing transgenic plants were evaluated using disease indexes described by He et al. (1983).

In addition, the enzyme activities of phenylalanine aminolyase (PAL), polyphenol oxidase (PPO), peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), hydrogen peroxide (H₂O₂); and malondialdehyde (MDA) contents were measured according to the protocol described by Agarwal (2007). Each experiment was performed twice with at least three replicates per treatment and the appropriate controls.

Results

Establishment of a highly efficient genetic transformation system

We tested the impact of different variables on the rate of resistant bud induction and rate of histochemical staining of β -glucuronidase (GUS) in *E.urophylla* \times *E.grandis* hypocotyls using an orthogonal design. The percentage of hyg-resistant shoot induction and positive histochemical staining rate of GUS were significantly different in all 16 treatments. All the five factors had significantly different effects on T-DNA delivery ($p < 0.01$).

We conclude that the most favorable conditions to achieve *Agrobacterium*-mediated transformation using stem segments from aseptic clonal seedlings of *E.urophylla* \times *E.grandis* were: explants pre-cultured for 6 days and then immersed in the bacterial suspension of OD₆₀₀ = 0.3 with full-strength MS medium at pH 5.8 in the dark at 23–25 °C for 2 h. Then co-culturing under desiccation conditions in the dark at 25 \pm 2 °C for 2 days yielded the best results. The highest frequency of induction of hyg-resistant shoots was 64.36 %, while the highest positive histochemical staining frequency of GUS was 59.8 %.

Plant tissue culture

After co-cultivation for 6 days, 96 % of the explants formed a callus (Fig. 1a–c). After 20-d cultivation, calli were transferred to MS medium containing 0.25 μ mol/L naphthalene acetic acid (NAA) and 4.4 μ mol/L BA to induce adventitious bud formation. The percentage of adventitious bud formation was 30–60 % (Fig. 1d–e). Shoot length was 2.5–3.0 cm after 28 days of cultivation, and the shoots showed rooting at the end of the elongation phase (Fig. 1f). The percentage of rooted microcuttings was 76 %, the

average number of roots per plant was three, and the mean length of the main root was 8 cm (Fig. 1g–h). After the acclimatization period, the plant survival rate was 80 % and the mean shoot size was 5 cm; and no obvious phenotypic changes were observed (Fig. 1i).

PCR analysis of plant genomic DNA

Genomic DNA was extracted from ten regenerated Hyg-resistant plantlets. The presence of transgenes was confirmed by PCR amplification of a 750-bp region of *aiiA* using gene-specific forward and reverse primers, confirming a successful integration into the *E.urophylla* \times *E.grandis* genome.

Further, we determined whether T-DNA integration had occurred and determined how many copies of T-DNA were present in the plant genome by Southern blot analysis. Single major hybridization bands were found in five transgenic lines while no hybridization band was found in untransformed plants. These results confirmed that PPP3-*aiiA* and 35S-*aiiA* were stably integrated into the genome of the transgenic plants.

RNA extraction and RT-PCR

We compared *aiiA* expression before and after inoculation with *R. solanacearum* in southern blot hybridization-positive plants by RT-PCR and Northern blot hybridization using total RNA extracted from leaves. The results showed that *aiiA* transcripts were not detected in transformed and non-transformed plants before inoculation with *R. solanacearum*. However, after inoculation a 750-bp fragment was amplified in transgenic plants expressing PPP3-*aiiA* while expression was constitutive in transgenic plants expressing 35S-*aiiA*, confirming that the PPP3 promoter is induced by *R. solanacearum* infection. These results were confirmed by Northern blot hybridization experiments.

After inoculation with *R. solanacearum*, changes were observed in *aiiA* expression (Fig. 2). RT-PCR performed in the plants that yielded positive PCR results resulted in the amplification of a 750-bp fragment, a finding that is consistent with the size of the positive control. However, regardless of whether inoculation with *R. solanacearum*, *aiiA* transcripts were detected in transgenic plants with the 35S-*aiiA* gene (Fig. 2). This result confirmed that the PPP3 promoter of *aiiA* is induced by *R. solanacearum* infection.



Fig. 1 Genetic transformation of *Eucalyptus urophylla* × *Eucalyptus grandis*. **a, b** Callus inoculated; **c, d** Differentiation of hygromycin-resistant adventitious buds; **e** Proliferation of hygromycin-resistant adventitious buds inoculated; **f** Elongation

of hygromycin-resistant adventitious buds; **g, h** Rooting of elongated shoot; **i** Plants in a plastic pot in the greenhouse. (Color figure online)

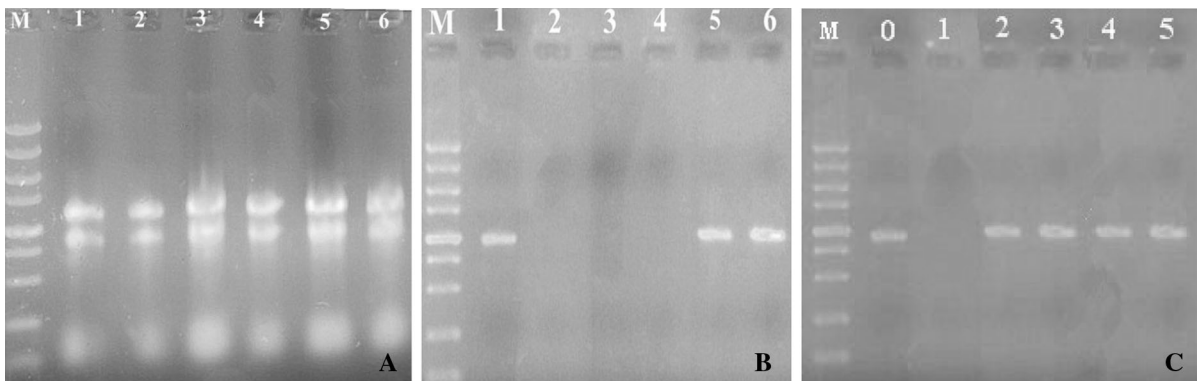


Fig. 2 RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR) analysis. **a** Total RNA samples from *Eucalyptus urophylla* × *Eucalyptus grandis* leaves; **b** RT-PCR expression analysis of transgenic plants with PPP3-*aiiA*. M: 5000-bp DNA marker; 1: positive control; 2: non-transgenic plants; 3–4: transgenic plants before inoculation with *Ralstonia*

solanacearum; 5–6: transgenic plants after inoculation with *R. solanacearum*; **c** RT-PCR expression analyses of transgenic plants with the 35S-*aiiA*. M: 5000-bp DNA marker; 0: positive control; 1: non-transgenic plant; 2–3: transgenic plants before inoculation with *R. solanacearum*; 4–5: transgenic plants after inoculation with *R. solanacearum*

The Northern blot hybridization result indicated that the *aiiA* transcripts were not detected in the transformed plants prior to inoculation with *R. solanacearum*, whereas after

inoculation with *R. solanacearum*, *aiiA* expression was observed. This study further shows that the PPP3 promoter of *aiiA* is induced by *R. solanacearum* infection.

RT-qPCR of *aiiA*

The transcription efficiency of *aiiA* was determined using real-time quantitative PCR. The transcription efficiency of *aiiA* under control of PPP3 promoters increased 43.88-, 30.65-, and 18.95-fold after inoculation with *R. solanacearum*, stalk rot disease, and *C. quinqueseptatum*, respectively. In contrast, the transcript efficiency of *aiiA* under the control of 35S promoters did not increase after inoculation with *R. solanacearum*, stalk rot disease, or *C. quinqueseptatum*. The real-time quantitative PCR results further showed that the PPP3 promoter of the *aiiA* gene is induced by infection with pathogenic bacteria.

Disease resistance

After *R. solanacearum* inoculation, the non-transformed controls showed the highest level of infection. None of the transformed lines showed complete disease resistance but they were tolerant to the initiation of infection (Fig. 4a, b). There seem to be a 4-day delay in infection between transgenic and control plants and the effect seems to be more pronounced for 35S-*aiiA* than for PPP3-*aiiA* with a 1-day difference between the two types of transgenic plants. According to the method of He et al. (1983), the relative disease index value was 2.5, indicating that the expression of *aiiA* in transgenic *E. urophylla* × *E. grandis* could enhance resistance to *R. solanacearum* (Fig. 3).

After *P. capsici* inoculation, the non-transformed controls showed the highest level of infection (60 %). The leaves of non-transgenic plants exhibited blighting necrosis, and wilting within 10 days after inoculation (Fig. 4c, d). Although none of the transformed lines showed complete disease resistance, the transgenic plants showed weak signs of necrosis in the inoculation area (near the leaf stalk). The degree of leaf necrosis was 10–20 % in the transgenic plants.

Activity-changes of defensive enzymes

Five days after inoculation with *R. solanacearum*, the SOD, PAL, PPO, and POD activities were higher than those before inoculation. The increase in these activities in the transgenic leaves after inoculation was remarkable compared to those obtained from the transgenic leaves before inoculation with increases of

55.7, 72.3, 84.2 and 42.5 %, respectively. After inoculation, the content of H₂O₂ increased 60.2 % in the transgenic leaves. However, CAT activity and MDA content decreased in the transgenic leaves after inoculation.

Difference in resistance levels between transgenic plants with PPP3-*aiiA* and 35S-*aiiA*

To test the difference in resistance between the plants transformed with PPP3-*aiiA* and those with 35S-*aiiA*, we compared the ability of the *aiiA* gene to cause disease. Even after prolonged incubation (6 days) with the pathogen, transgenic plants with PPP3-*aiiA* were clearly more tolerant. After prolonged incubation (15 days) with the pathogen, the disease index of the transgenic plants with PPP3-*aiiA* was less than that of the transgenic plants with 35S-*aiiA*. However, the average relative disease index value of the transgenic plants with PPP3-*aiiA* was 56.1 %, which was higher than that of the transgenic plants with 35S-*aiiA* (47.4 %). These results demonstrated that the inducible expression of *aiiA* in transgenic plants with PPP3-*aiiA* was higher than its constitutive expression, thus could delay the wilt symptom development and reduce the disease index (Table 1).

Discussion

Genetic engineering is a powerful tool for controlling plant diseases as well as a suitable alternative to the use of costly and environmentally undesirable chemical methods of disease control. Transgenic Eucalyptus plants have been generated by *A. tumefaciens* inoculation into explants such as hypocotyls and cotyledons (Prakash and Gurumurthi 2009; Dibax et al. 2010b; Ouyang et al. 2012c; Torre et al. 2014). 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, we reported an efficient regeneration system for *E. urophylla* × *E. grandis*

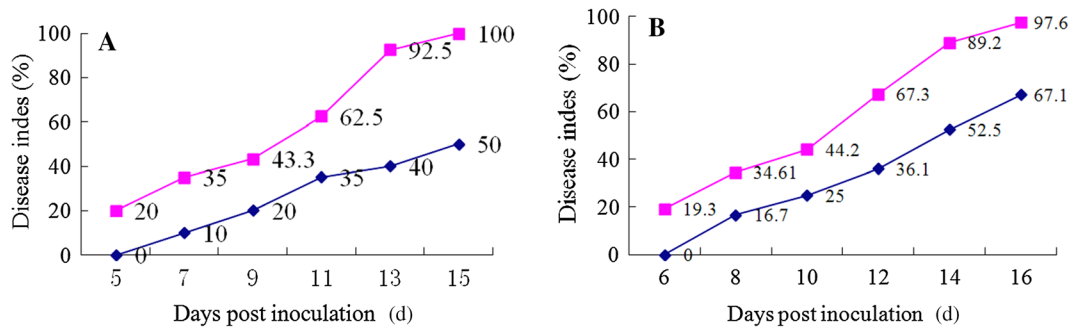


Fig. 3 Resistance evaluation of transgenic *Eucalyptus urophylla* × *Eucalyptus grandis* after inoculation with *Ralstonia solanacearum*. **a** Resistance evaluation of transgenic plants with the PPP3-*aiiA* gene after inoculation with *R. solanacearum*. NT non-transgenic plant, T transgenic plants with the PPP3-*aiiA* gene

b Resistance evaluation of transgenic plants with the 35S-*aiiA* gene after inoculation with *R. solanacearum*. NT non-transgenic plant, T transgenic plants with the 35S-*aiiA* gene

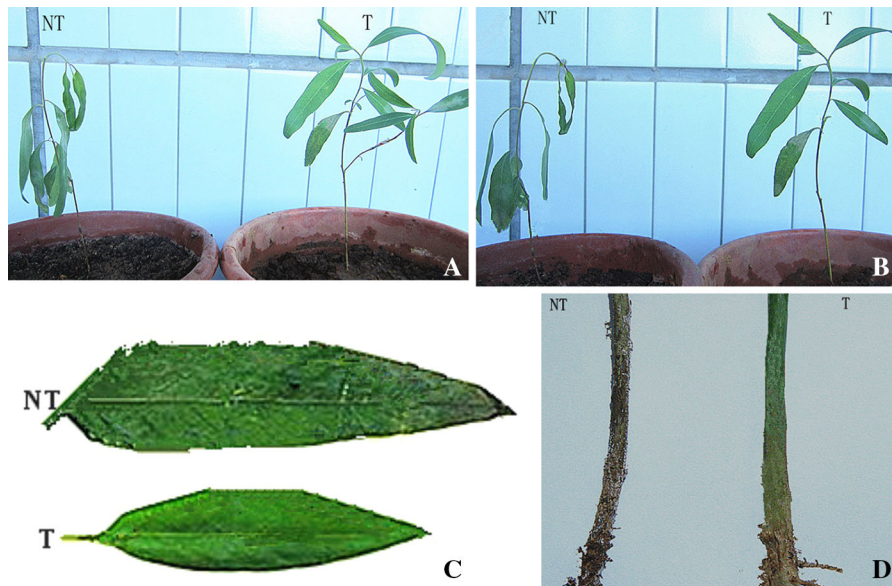


Fig. 4 Phenotypes of *Eucalyptus urophylla* × *Eucalyptus grandis* after inoculation. **a, b** Phenotypes on the 6th day after inoculation with *R. solanacearum*. **c, d** Phenotypes on the 10th

day after inoculation with *Phytophthora capsici*. NT non-transgenic plant, T transgenic plants with the PPP3-*aiiA* gene

Table 1 Resistance difference between the transgenic plants with the PPP3-*aiiA* gene and the transgenic plants with the 35S-*aiiA* gene

Different transgenic plants	Relative values of disease index (%)	Resistance classes	Resistance increase scale
PPP3 + <i>aiiA</i>	56.1**	Median level resistance	2.5**
35S + <i>aiiA</i>	47.4	Median level resistance	2.3

** Significant obvious difference ($p < 0.01$) with *t* test

hypocotyls (Ouyang et al. 2012a). In the present study, transgenic *E. urophylla* × *E. grandis* plantlets were generated after *A. tumefaciens*-mediated

transformation using hypocotyl explants. Moralejo et al. (1998) obtained transgenic *Eucalyptus globulus* plants by using stem segments. The transformation

frequency increased after pre-culturing of the explants and the plantlets were obtained through regeneration from the explants, similar to the results obtained in the present study.

In the present study, explants were pre-cultured for 6 days before co-cultivation with *A. tumefaciens*, which in turn reduced the hypersensitive response and increased the transformation efficiency. *A. tumefaciens* requires time for T-DNA integration; as such, co-culture time is important to the *A. tumefaciens*-mediated transformation system. Tumor induction and T-DNA transfer occurred only when *A. tumefaciens* was present at the wounded site for more than 16 h (Sykes and Matthysse 1986; Gelvin 2000). In this study, the number of resistant buds was optimum after a co-culture time of 2 day. We tested the impact of different facts on the rate of resistant bud induction and the positive rate of GUS histochemical staining in *E. urophylla* × *E. grandis* hypocotyls using an orthogonal design. The highest frequency of induction-resistant shoots was 64.36 %, while the highest frequency of positive GUS histochemical staining was 59.8 %, which was higher than that reported in other studies (Teulières et al. 1991; Deepika et al. 2011; Prakash and Gurumurthi 2009; Silva 2011).

The use of appropriate selection conditions is very important in plant transformation. Hyg is widely used as a selectable marker; therefore, its sensitivity should be determined at the initial stages of the development of a plant transformation system in which a Hyg-resistance gene is used (Fiola et al. 1990; James et al. 1990; Escandon and Hahne 1991). Ho et al. (1998) used 20 mg/L Hyg to select transgenic *Eucalyptus camaldulensis* plants after transforming the hypocotyl explants with *A. tumefaciens*. Prakash and Gurumurthi (2009) reported that 40 mg/L Hyg was enough to select *E. camaldulensis* transgenic plants. In the present study, the T-DNA of pCAMB1301 contains the *HPT* gene, which confers the Hyg resistance to the transformed plants. We used 9 mg/L Hyg to select the adventitious bud induction and 15 mg/L for rooting selection.

The control of bacterial growth is important since 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 Cef to decontaminate residual *A. tumefaciens*. In the present study, we aimed to identify a Cef concentration that

would effectively inhibit *A. tumefaciens* growth without affecting plant growth, and the results indicated that 200 mg/L was the optimal Cef concentration.

The elongated shoots were transferred to the rooting medium containing Hyg, and we further confirmed the integration of the T-DNA containing the *aiiA* gene into the genome of *E. urophylla* × *E. grandis* using PCR and Southern blot analysis. The RT-PCR results indicated that the PPP3 promoter of the *aiiA* gene was induced by *R. solanacearum* 12 h after inoculation. However, regardless of *R. solanacearum* inoculation, *aiiA* transcripts were detected in transgenic plants transformed with 35S-*aiiA*. This result confirmed that the PPP3 promoter of *aiiA* is induced by *R. solanacearum* infection. The Northern blot hybridization results further showed that the PPP3 promoter of *aiiA* is induced by *R. solanacearum* infection. In this study, the transcript efficiency of *aiiA* under the control of PPP3 promoter increased 43.88-, 30.65-, and 18.95-fold after inoculation with *R. solanacearum*, *Erwinia carotovora* ssp. *zeae* (Sabet), and *C. quinqueseptatum*. These results demonstrated that the PPP3 promoter of *aiiA* is induced by many kinds of pathogenic bacteria.

The present protocol achieved efficient transformation (40 %; number showing positive results on PCR analysis/number of PCR analyses of putative transgenic plants) and the highest frequency induction of resistant shoots (64.36 %), findings that are higher than those reported in other studies. Kong et al. (2010) developed an *A. tumefaciens*-mediated transformation system for *E. urophylla* U6 and achieved only a 0.59 % transformation rate.

Interest has increased in the development of regulated expression systems in which transgene expression occurs only in the presence of particular pathogens (Moreno et al. 2005). Constitutive overexpression of defense components can lead to increased disease resistance, but the plants might have reduced size or altered morphology or show disease symptoms in the absence of pathogens. In the present report, we described a strategy for incorporating resistance to pathogenic bacteria. This approach relies on the use of an inducible rather than a constitutive promoter and results in the expression of an antifungal gene at a sufficiently high level for protecting transgenic *E. urophylla* × *E. grandis* plants against pathogenic bacteria.

As one of the anti-quorum-sensing strategies, degradation of AHL-signaling molecules could have potential application for attenuating plant disease. The AIIA protein, encoded by *aiiA*, can inactivate the AHL signals that regulate the quorum-sensing system within bacteria by hydrolyzing its lactone bond so that it can release the disease within the plant. Dong et al. (2000, 2001) obtained *aiiA* gene-transformed tobacco and potato plants, both of which had acquired resistance to soft rot. Zhang et al. (2005) demonstrated that transgenic plants expressing *aiiA* maintained strong antifungal activity against several kinds of plant pathogenic fungi but could also effectively control soft rot diseases in potato and Chinese cabbage plants.

In the present study, after *R. solanacearum* inoculation, the non-transformed controls showed the highest level of infection. Although none of the transformed lines showed complete disease resistance, the transgenic plants showed only weak signs. The resistance increase scale was 2.5, which indicated that the expression of *aiiA* in transgenic *E. urophylla* × *E. grandis* could enhance resistance to *R. solanacearum* disease and that the resistance classes can reach the median resistance level. In addition, the inducible expression resistance of *aiiA* in transgenic plants with PPP3-*aiiA* was higher than its constitutive expression and could delay wilt symptom development and reduce the disease index.

To the best of our knowledge, no study has been published on the introduction of the *aiiA* gene into forest tree tissues or about the resistance difference between the inducible and constitutive expression of *aiiA*. The SOD, PAL, POD, and PPO activities, which are associated with disease tolerance levels, were higher 2 days after inoculation with *R. solanacearum* in the transgenic *E. urophylla* × *E. grandis* than prior to the inoculation. These results indicate that the levels of *aiiA* gene expression in transgenic plants were high enough to reduce the pathogenic bacterial growth and resulted in reduced disease development, which suggests that this method can be applied to other crop plants to enhance resistance to pathogenic bacteria.

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