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ORIGINAL PAPER

Agrobacterium tumefaciens-mediated genetic transformation of *Acacia crassicarpa* via organogenesis

Mingjia Yang · Xiangming Xie · Caixia Zheng · Fangqiu Zhang · Xiaoqing He · Zhiru Li

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Abstract A protocol was developed for *Agroacterium*-mediated genetic transformation of *Acacia crassicarpa* via organogenesis by using in vitro phyllode (leaf) as the explant. Phyllode (leaf) explants were co-cultured with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary vector pBI101 (harboring antisense *Pt4CL1* with respect to the Pt4CL1P promoter). The selection for transgenic shoots was performed through two consecutive steps on Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth regulators and antibiotics in the following order: 0.5 mg/l thidiazuron (TDZ), 0.5 mg/l α-naphthaleneacetic acid (NAA), 300 mg/l carbenicillin (Car) and 20 mg/l kanamycin (Km) for 10 days; 0.1 mg/l TDZ, 200 mg/l Car and 20 mg/l Km for 60 days; 0.5 mg/l indole-3-butyric acid (IBA), 100 mg/l Car and 20 mg/l Km 50 days. 21.7% of nodules produced multiple adventitious shoot buds, of which 27.7% survived in initial selection. The shoot buds were subjected to repeated selection on MS medium supplemented with 0.1 mg/l TDZ, 200 mg/l Car and 20 mg/l Km for 60 days. Transgenic plants were obtained after rooting on half-strength MS medium supplemented with 0.5 mg/l IBA, 100 mg/l Car 20 mg/l Km 50 days. Genomic PCR analysis confirmed the incorporation of the antisense *Pt4CL1* with respect to the Pt4CL1P promoter fragment into the host genome.

Keywords Lignin · 4CL1 · Phyllode

Abbreviations

BA	6-Benzylaminopurine
Car	Carbenicillin
CW	Coconut water
GUS	β-Glucuronidase
IBA	Indole-3-butyric acid
Km	Kanamycin sulphate
NAA	α-Naphthaleneacetic acid
NPT II	Neomycin phosphotransferase
TDZ	1-Phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron)

Introduction

Acacia crassicarpa Cunn. ex Benth is a tropical leguminous tree species which has become a preferred fibre source for the paper and pulp industry because of its rapid growth, high pulp yield, high fibre quality and its ability to thrive in degraded soils (Pan and You 1994). It has been

M. Yang · X. Xie (✉) · C. Zheng · X. He · Z. Li
College of Biological Science and Biotechnology, Beijing Forestry University, P.O. Box 162, Beijing 100083, Peoples' Republic of China
e-mail: xiangmingx@sina.com; xie-xiangming@163.com

F. Zhang
Guangdong Academy of Forestry, Guangzhou 510520, Peoples' Republic of China

expandingly planted in Indonesia and in South China for reforestation, reclamation of wastelands, and industrial material production (Pan and You 1994; Yang et al. 1995; Stephen 2000). The genetic modification of *A. crassicarpa* to confer desirable traits such as modified lignin content will be of commercial importance, which is hard to achieve through classical breeding owing to the recalcitrance of regeneration, long generation time of trees, and the prolonged period needed for evaluation of mature traits.

A key component of most functional genomics approaches is to establish a high-throughput transformation system useful for developing various gene identification strategies. Stable genetic transformation methods have been developed for legume trees such as those reported for *Acacia mangium* (Xie and Hong 2002), *Acacia sinuata* (Vengadesan et al. 2006) and *Robinia pseudoacacia* (Han et al. 1993; Igasaki et al. 2000; Kanwar et al. 2003; Zaragoza et al. 2004). However, there is no report of genetic transformation of *A. crassicarpa* thus far. Stable regeneration from phyllode (leaf) through organogenesis was previously reported by us in *A. crassicarpa*, which is superior to regeneration from immature cotyledons or embryo axes from seeds with considerable genetic diversity. In this paper, we describe a protocol for *Agrobacterium*-mediated genetic transformation of this important tropical leguminous tree species. Since lignin quantity is one of the two major barriers to wood-pulp production, the antisense 4CL gene encoding 4-coumarate–CoA ligase, which has been verified to induce lignin reduction in aspen (*Populus tremuloides*) (Hu et al. 1999), has been transferred into *A. crassicarpa.* Transgene integration has been verified by PCR on *A. crassicarpa* genomic DNA.

Materials and methods

Plant material and culture condition

Mature seeds were collected from a natural grove of *A. crassicarpa* tree of 20–30 m in height at the seed orchard, Guangzhou, China. Seeds were sterilized following the protocol described by Yang et al. (2006). Then they were germinated on half-strength, sucrose free Murashige and Skoog (MS) medium (Murashige and Skoog 1962). Seeds germinated within 10–12 days showing cotyledons. Phyllodes (without any pinnate on the top of them) were excised from 1-year-old in vitro seedlings and cut into 0.3–0.5 cm pieces, which were used as explants. All media were autoclaved at 121°C for 15 min. NAA was added into the media before autoclaving. TDZ and indole-3-butyric acid (IBA) were filter sterilized with a 0.2 μm membrane and then added into media after autoclaving. After that all media were readjusted to pH 5.8 with 1 N NaOH (sterile).

All above media (except the medium for germination) were supplemented with 5% (v/v) coconut water (CW) and 30 g/l sucrose and solidified with 0.4% (w/v) agar. Unless otherwise specified, all the cultures were maintained at 28°C under cool-white fluorescent light at an irradiance of 26 μmol s^{-1} m^{-2} (PAR) at a 16-h day photoperiod.

Regeneration system

A previously developed stable regeneration system through organogenesis based on the production of shoots and roots from in vitro phyllode (leaf) (rather than cotyledons and hypocotyls) explants was used (Yang et al. 2006). These explants were cultured on MS media supplemented with 0.5 mg/l TDZ and 0.5 mg/l NAA for regeneration. They were transferred to fresh regeneration medium at 10-day intervals. Elongation of the clusters of adventitious shoots was performed on elongation medium (MS medium containing 0.1 mg/l TDZ). Shoots were rooted on rooting medium (half-strength MS medium with 0.5 mg/l IBA).

All cultures were maintained in the growth room under conditions mentioned above.

Km as selection agent for transgenic plants

Sensitivity of the phyllode explants to Kanamycin sulphate (Km) was determined by culturing the explants on the regeneration medium with 0, 0.5, 1 mg/1 Km for 2 months. The explants were transferred to fresh regeneration medium at 10-day intervals. The experiment was repeated three times with 50 phyllodes segments per treatment.

Sensitivity of the clusters of adventitious shoots to Km was determined by culturing the shoots on the

regeneration medium with 0, 5, 10, 15 and 20 mg/l Km for 10 days. The experiment was repeated two times with 30 explants per treatment.

Sensitivity of rooting of shoots to Km was determined by culturing the elongated adventitious shoots (2–3 cm) on the rooting medium with 0, 5, 10, 20 and 40 mg/1 Km for 2 months. The shoots were subcultured after 10 days, and subsequently were cultured in the same Erlenmeyer flask for 20 days. After that the shoots were not transferred again. Ten milliliters fresh half-strength MS medium liquid was added into the flask at an interval of 20 days. Before adding the fresh medium the prior liquid should be poured out. The experiment was repeated two times with 50 shoots per treatment. Subsequently, a concentration of Km suitable for selection was determined and used in transformation.

Agrobacterium tumefaciens strain, plasmid, and culture

Genetic transformation was performed using the *A. tumefaciens* LBA4404 strain (Ooms et al. 1981) harboring the binary vector (Li et al. 2003) derived from pBI101 by replaced β-glucuronidase and 35S promoter fragment with an antisense *Pt4CL1* with respect to the Pt4CL1P promoter. This binary vector carries a neomycin phosphotransferase (*npt*II) gene under the control of *nos* promoter, and the anti-sense 4CL1 gene under the control of Pt4CL1P promoter.

A. tumefaciens was grown overnight in the darkness in YEP medium (Chilton et al. 1974) containing 100 mg/l streptomycin and 50 mg/l Km at 28°C on a gyratory shaker set at 250 rpm. Ten milliliters of bacterial suspension was pelleted and resuspended in 50 ml liquid YEP medium containing 100 mg/l streptomycin and 50 mg/l Km and cultured at 28°C at 250 rpm in the dark until the OD600 reached 0.8. For transformation, the culture was centrifuged at 2,292*g* for 10 min, and the cells resuspended in MS liquid medium (pH 5.6) with 100 μM acetosyringone (Sigma). The bacteria suspension was cultured in the darkness at 120 rpm at 28°C for an hour before used for infection of plant cells.

Transformation and plant regeneration

Phyllodes (without any pinnate on the top of them) excised from 1-year-old seedlings were cut into 0.3–0.5 cm pieces and pre-cultured for 3 days on the regeneration medium supplemented with no antibiotic. Then the explant pieces were immersed in the activated *Agrobacterium* suspension (OD600 = 0.8) for 15 min at 28°C on a gyratory shaker set at 50 rpm, and then washed once with sterile distilled water, blotted dry on sterile filter paper. After that, they were co-cultured for 3 days on the regeneration medium without Km at 28°C in the dark. One hundred and fifty explants were used for one experiment, and this experiment was repeated twice.

After 3 days of cocultivation the explants were washed three times in sterile distilled water supplemented with 300 mg/l Carbenicillin (Car), blotted dry, and transferred to the regeneration medium with 300 mg/l Car. The explants were subcultured at 10-day intervals to fresh medium of the same composition. After 60 days of regeneration culture explants with adventitious shoots were initially selected on regeneration medium supplemented with 300 mg/l Car and 20 mg/l Km as the selection agent for 10 days, then on elongation medium supplemented with 20 mg/l Km and 200 mg/l Car for 50 days. Transgenic shoots were rooted on rooting medium supplemented with 20 mg/l Km and 100 mg/l Car. The putative transgenic shoots were subcultured by adding liquid MS medium (supplemented with 40 mg/l Km) as mentioned before.

DNA extraction PCR analysis

DNA was extracted from the fresh phyllodes of individual plants using the method described by Xie and Hong (2002).

PCR analysis of putative transgenic shoots was performed to prove integration of the transgene into the plant genome. PCR was used to screen 30 putative plants. Primer sets used were: Pt4CL1P promoter-specific sense primer (5′-CAGGAATGCTCTGCACT CTG-3′) coupled with *Pt4CL1* 5′-end sense primer (5′-ATGAATCCACAAGAATTCAT-3′). The predicted sizes of the amplified DNA fragments were 1.6 kb. PCR was performed on an Applied biosystems geneamp PCR system 2700 with 30 cycles at 94°C for 30 s, 60°C for 1 min, and 72°C for 3 min. All reactions were preceded by a primary denaturation step at 94°C for 10 min.

Non-transgenic plant as the negative control and plasmid DNA of modified pBI101 harbouring

antisense *Pt4CL1* with respect to the Pt4CL1P promoter fragment as the positive control were used.

Results and discussion

Regeneration system

Successful regeneration was previously obtained by our protocol (Yang et al. 2006). Green compact nodules and adventitious shoots were induced in 10 and 40 days, respectively, on the regeneration medium. Fifty-six percent (56/100) of the nodules developed adventitious shoots. Shoot elongation was achieved by transferring the clusters of adventitious shoots to elongation medium for 2 months. The elongated adventitious shoots were rooted at a rate of 96.5% (55/57) on rooting medium for 1 month. Stable regeneration from phyllode (leaf) through organogenesis in *A. crassicarpa* is superior to regeneration from immature cotyledons or embryo axes from seeds with considerable genetic diversity which is undesirable for commercial propagation. Stem segments from rejuvenated adventitious shoots were used as explants in genetic transformation of *A. mangium* (Xie and Hong 2002). This similarly avoided genetic diversity in contrast to the use of hypocotyls as explants in *A. sinuata* (Vengadesan et al. 2006).

Km as selection agent for transgenic plants

Our experiments showed complete inhibition of regeneration of adventitious shoots at 1 mg/l Km (Fig. 1). Km above 1 mg/l didn't lead to progressive browning of the explants and subsequent shoot death as described in *A. sinuate* (Vengadesan et al. 2006), but prolonged the green nodule induction to 15d, and the explants remained in the green-nodule stage for 2 months.

Clusters of adventitious shoots showed more tolerance to Km. They turned brown when cultured on the regeneration medium with 20 mg/l Km for 10 days. With a lower concentration of Km (0, 5, 10, 15) the clusters of shoots grew well and no significant difference was found.

Well developed shoots showed much higher tolerance to Km. When Km reached 20 mg/l, the percentage of root induction was reduced to 47% (47/100; Fig. 2). Km above 40 mg/l didn't lead to shoot death, but caused inhibition of lateral and secondary root induction (Figs. 3 and 4f–i).

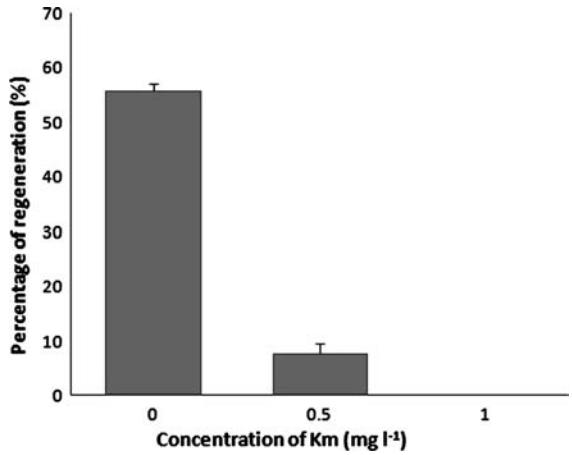

Fig. 1 Tolerance of *A. crassicarpa* explants to Km as indicated by percentage of regeneration at the end of a 60-day incubation on the selection medium. Average values with standard deviations of three independent experiments are given (each with 50 explants)

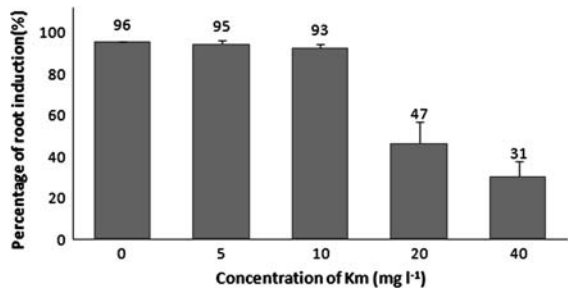

Fig. 2 Tolerance of *A. crassicarpa* explants rooting to Km as indicated by percentage of root induction at the end of a 60-day on the selection medium. Average values with standard deviations of two independent experiments are given (each with 50 explants)

Our experiment showed that both regeneration and rooting of *A. crassicarpa* were sensitive to Km, whereas *A. mangium* explants had a resistance to Km that extended to 300 mg/l in the medium (Xie and Hong 2002).

Transformation and plant regeneration

Successive selection steps were applied using different concentrations of Km to provide optimum selection pressure. The production of transgenic plants involves the stable integration of foreign

DNA into the host genome and the subsequent regeneration of whole plants from the transformed cells. Although these two independent processes are critical to the successful generation of transgenic plants, competence for regeneration is the first limitation (Pena et al. 2004). Because of the high sensitivity of regeneration of explants to Km (Fig. 1), explants were cultured on regeneration medium supplemented with only 300 mg/l Car after 3 days of cocultivation, which allowed a higher percentage of regeneration and provided a chance for

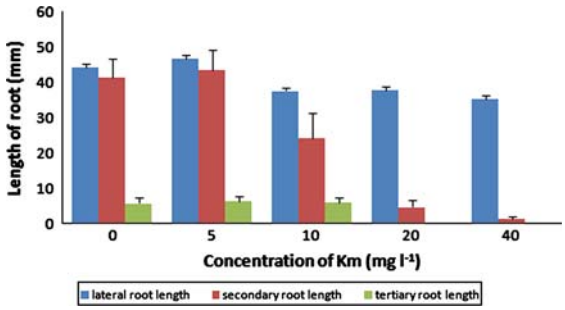

Fig. 3 Tolerance of *A. crassicarpa* explants rooting to Km as indicated by length of lateral root, secondary root, and tertiary root at the end of a 60-day on the selection medium. Average values with standard deviations of two independent experiments are given (each with 50 explants)

Agrobacterium infection. Green compact nodules and adventitious shoots developed after 15 and 60 days (Fig. 4a, b), respectively. When we used this scheme, 21.7% (65/300) of the explants formed adventitious shoots. Every explant developed more than 20 shoots. Although there was no selection pressure until now, the regeneration frequency was much lower than in non-transgenic explants (56%), which might be caused by the infection of *Agrobacterium* and the toxicity of Car, suggesting that the regeneration of *A. crassicarpa* from phyllode explants via organogenesis was very sensitive.

After 10 days of initial selection on the regeneration medium supplemented with 300 mg/l Car and 20 mg/l Km the growth of non-transgenic tissues were effectively inhibited (Fig. 4c), 27.7% (360/1300) of adventitious shoots survived.

Efficient shoot elongation (Fig. 4d) was achieved by transferring the clusters of adventitious shoots to elongation medium supplemented with 300 mg/l Car and 20 mg/l Km with the response percentage of 42% (151/360) in 60 days.

Transgenic shoots with the length of 3–4 cm were rooted on rooting medium supplemented with 20 mg/l Km and 100 mg/l Car developed six lateral roots per shoot on average in 50 days. The putative

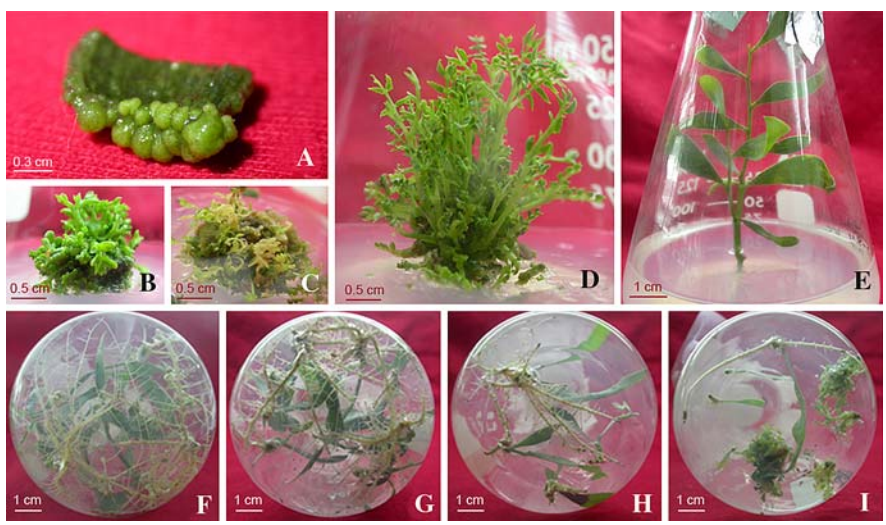

Fig. 4 Regeneration of transgentic *A. crassicarpa* plants (**a**–**e**) and shoots rooting tolerance to Km (**f**–**i**). (**a**) Nodule developed directly from phyllode explants on regeneration medium. (**b**) Putative transgenic clusters of adventitious shoots cultured on regeneration medium with 20 mg/l Km. (**c**) Clusters of shoots with no Km tolerance turned to brown in the 10 days initial selection. (**d**) Putative transgenic adventitious shoot elongation. (**e**) Whole transgenic plantlet with roots. (**f**) Shoots rooting on the medium supplemented with 0 and 5 mg/l Km. (**g**) Shoots rooting on the medium supplemented with 10 mg/l Km. (**h**) Shoots rooting on the medium supplemented with 20 mg/l Km. (**i**) Shoots rooting on the medium supplemented with 40 mg/l Km

transgenic shoots were subcultured by adding liquid MS medium (supplemented with 20 mg/l Km).

In this protocol the use of 20 mg/l Km after regeneration of clusters of adventitious shoots allowed host cells to survive long enough for *Agrobacterium* infection and cell division to take place. According to sensitivity of the clusters of adventitious shoots, 20 mg/l Km was used and efficiently inhibited the growth of non-transgenic tissues which turned brown after this selection step. Although the elongated shoots could tolerate higher Km concentrations, Km above 40 mg/l, although not leading to shoot death, caused the inhibition of lateral and secondary root induction. Therefore 20 mg/l Km was used in the subsequent selection process.

DNA extraction PCR analysis

The successful incorporation of the transgene was proven by the genomic PCR (Fig. 5). No positive result occurred with DNA isolated from control plants (non-transformed, CK−). Ten transformed plants were produced.

Although *A. tumefaciens* has been successfully used to transfer genes to a wide range of plant species, it has received little attention for transformation of legume trees (Xie et al. 2007). To our knowledge, this is the first report describing a detailed protocol for *Agrobacterium*-mediated transformation of *A. crassicarpa* via organogenesis, using phyllodes as explants for this ecologically and economically important tropical leguminous tree species. In summary, a protocol for *Agrobacterium*-mediated transformation of *A. crassicarpa* via organogenesis is described. It could be very useful to incorporate desirable traits through genetic modification into individuals of a known genetic background and proven performance as mentioned by Xie and Hong (2002). In addition, it could be an essential tool

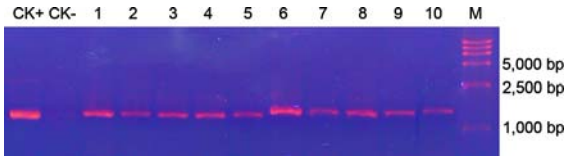

Fig. 5 Genomic PCR of transformed plants. Lanes 1–10, Transgenic plants; CK, non-transgenic plant as the negative control (CK−); plasmid DNA of modified pBI101 harbouring antisense *Pt4CL1* with respect to the Pt4CL1P promoter fragment as the positive control (CK+)

to investigate gene function of *Acacia*. The follow-up lignin content analysis of transgenic lines is under way, attempted to reduce lignin quantity which is important to improve wood-pulp production efficiency.

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