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Short Communication

Regeneration and genetic engineering of a tropical tree, *Azadirachta excelsa*

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Abstract *Azadirachta excelsa* belongs to the family Meliaceae and is one of the most important silviculture trees in the tropics. In this study, we established a somatic embryogenesis system and succeeded in construction of transgenic lines expressing genes for *Bar*, *GUS* and *GFP* employing the *Agrobacterium*-mediated transformation technique. Somatic embryos were obtained after approximately two months of culture on Murashige-Skoog (MS) medium containing 5 μM 6-benzyladenine. Plantlets were then regenerated by transferring somatic embryos to modified 1/2 MS medium without phytohormones. We initially attempted genetic transformation using two different *A. tumefaciens* strains, and found only the strain LBA4404 to be active in infection to explants. The strain EHA101 was totally inactive, suggesting that a specific interaction between *Agrobacterium* sp. and host plant might be critical for gene transfer. The possibility is discussed of applying the methodology developed in this work to the practical propagation of tropical trees.

Key words: *Azadirachta excelsa*, Bar, GFP, GUS, somatic embryogenesis.

Global deforestation due to human activity has become increasingly evident over the past decades, resulting in serious environmental deterioration, including greenhouse effects, air pollution and water deficits (Bright 2000). One efficient countermeasure to cope with these problems is afforestation of waste lands, particularly in tropical areas, with the aim of increasing carbon absorption and maintaining the ecosystem. However, large scale planting of tropical tree species of high quality has often encountered difficulties because of the lack of propagation and culture technology. Seeds of tropical trees usually have a short-life, losing the capacity for germination within a few days. The cutting method is now widely adopted for, for example, *Dipterocarpaceae* species, but this also restricts efficient propagation due to limited plagiotropic shoots from mature trees (Nakamura 2006). Introduction of modern technology including tissue culture and gene transfer appears to be a promising alternative approach, but despite great advance in these techniques for herbaceous plants, applications for tropical tree species have been left far behind. Indeed, successful cases of genetic transformation have been reported for only a few species, like *Eucalyptus* (MacRae and Staden 1993), coffee (Hatanaka et al. 1999) and henna (Bakkali et al. 1997).

Our long-range aim is to establish common and efficient technology for improvement of tropical tree species. To this end, we here selected a model tree, *Azadirachta excelsa* Jacobs (sentang), and attempted to develop methods for somatic embryogenesis and gene transfer. *A. excelsa* Jacobs (sentang) belongs to the family Meliaceae (Lemmens et al. 1995), and is one of the most important silviculture trees in the tropics, naturally growing in Malaysia, Philippines, Borneo and Thailand. It is planted along roadsides and farm boundaries, and grows rapidly, reaching 15 to 20 m in height with a diameter of 30 cm within 8 years. The timber is utilized for furniture, carving, paneling and veneers. In addition, young shoots, leaves, flowers and seeds are dietetically used, for example as salads. Like neem *(Azadirachta indica*), the leaves and fruits contain a limonoid, named azadirachtin (Cui et al. 1998; Kanokmedhakul et al. 2005), which exhibits strong insecticidal effects. In Thailand, azadirachtin is extracted from seed kernels and used to protect cabbage and other related *Brassica* vegetables from insects, especially the diamondback moth. Because of its ease of propagation by seeding and cutting and fast growth, together with above-mentioned applications, *A. excelsa* is an optimal material for establishing simple protocols for tropical trees for somatic embryogenesis and *Agrobacterium*-mediated genetic transformation, by which additional

Abbreviations: Bar, bialaphos resistance; GFP, green fluorescence protein; GUS, β-glucuronidase.
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useful traits such as herbicide and/or pest tolerance could be conferred.

Aseptic seedlings of *A. excelsa* were provided by Tsukuba Research Institute, Sumitomo Forestry Co., Ltd., and maintained at 25±1°C under a 16-h/8-h light/dark cycle with fluorescent illumination at 30–35 μmol m^{-2} s^{-1}. Explants were aseptically excised from leaves and petioles, and used for induction of somatic embryogenesis and transformation. For direct somatic embryogenesis, leaf explants were transferred to Murashige and Skoog (MS) medium (Murashige and Skoog. 1962) containing 3% sucrose, and combinations of 6-benzyladenine (BA), α-naphthylacetic acid (NAA) and 0.3% gellan gum. The medium was adjusted to pH 5.7 before autoclaving at 121°C for 15 min. The cultures were then maintained under continuous dark conditions at 25±1°C for 4 weeks. Twenty explants were used for each treatment and all experiments were repeated three times. When apparently normal somatic embryos appeared, they were transferred to half-strength inorganic salts MS (modified 1/2MS) medium containing vitamins, 3% sucrose and 0.3% gellan gum, pH 5.7. Cultures were maintained at 28±1°C with a 16-h/8-h light/dark cycle under the fluorescent illumination, and after 8–12 weeks, regenerated plantlets were transferred to culture boxes containing Florialite (Nisshinbo Industries, Inc., Japan) or to plastic pots containing Vermiculite (Nittai Inc., Japan) and Metro-Mix® 350 (Sun Gro Horticulture Distribution Inc., USA) (1 : 1 v/v). For genetic transformation, *Bar*, *GFP* and *GUS* and *bar* genes were used. The *Bar* gene originates from *Streptomyces hygroscopicus*, and encodes an enzyme phosphinothricin acetyltransferase (PAT), which inactivates phosphinothricin-related herbicide (Thompson et al. 1987). To date, it has widely been utilized to transform various plants, including tobacco, tomato and potato (De Block et al. 1987) to confer resistance against herbicides, such as bialaphos. *Agrobacterium tumefaciens* strain LBA4404 (Hiei et al. 1994) or EHA101 (Hood et al. 1986) harboring modified pSMAB (Toki 1997), pBI121 or *sGFP* (Ito et al. 2003) were cultured in LB medium containing 50 mg l^{-1} spectinomycin or kanamycin at 28°C until the absorbancy at 600 nm had reached 0.5–0.8. Bacterial suspensions were centrifuged, and the pellets were suspended in liquid MS medium. Leaf explants (approximately 5–8 mm^2) and petioles were excised from aseptic seedlings, and co-cultured with bacteria for 10 min at room temperature. After aspiration, infected explants were dried on a sterilized paper and transferred to co-culture medium (MS medium containing 5 μM BA). After culture continuously in the dark at 28±1°C for 2 days, explants were transferred to MS medium (5 μM BA, 300 mg l^{-1} cefotaxim and 5 mg l^{-1} bialaphos or kanamycin), which was then exchanged at 2-week intervals. Explants were cultured on MS medium containing 5 μM BA and 5 mg l^{-1} bialaphos or kanamycin and subcultured at 2-week intervals onto fresh medium of the same composition. Explants and somatic embryos were finally selected on MS medium containing 5 μM BA and 25 mg l^{-1} bialaphos or kanamycin. Total RNAs were extracted from somatic embryos or plantlet leaves (Chang et al. 1993) and RT-PCR was performed with an RNA PCR Kit (AMV) Ver.3.0 (Takara, Japan) and the following primers: for the *Bar* gene, 5′-GGTCTGCACCATCGTCAA-3′ and 5′-TCCAGCTGCCAGAAACC-3′; for the *GFP* gene, 5′-GGAGAAGAACTTTTCACTGG-3′ and 5′-GTGGTC-TCTCTTTTCGTTGG-3′; for the *GUS* gene, 5′-CGGGGAAACTCAGCAAG-3′ and 5′-ACATATCCA-GCCATGCAC-3′; and for the rRNA gene (accession number AY695594), 5′-GAGGAAGGAGAAGTCGT-AAC-3′ and 5′-CTCGAGAGGCTTGTTCTCAC-3′. PCR was carried out under conditions of up to 40-cycles of denaturation at 96°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s. The PCR products were analyzed on 1% agarose gel. Expression of *GFP* in somatic embryos was visualized under a fluorescence stereo microscope (MZ FL III; Leica Microsystems, Heerbrugg, Switzerland) with a GFP filter. Fluorescence images were photographed using a digital camera system (Hamamatsu Photonics Co. Ltd., Japan). GUS activity in somatic embryos was histochemically determined after 16h incubation at 37°C (Jefferson et al. 1987).

The optimal culture condition for somatic embryogenesis from leaf disks was first determined by varying concentrations of BA and NAA in MS medium (Figure 1A). Among the various combinations tested, 5 μM BA was found to be the most efficient to induce milky-white and normal somatic embryos, which were directly formed on the cut edges within two to four months (Figure 1B). After two to four months of culture, 431 somatic embryos from 50 leaf disks were obtained (10.3±2.8/disk on average). Since a total of 60 disks were subjected to embryo induction, the efficiency of somatic embryo formation was about 83% (50 out of 60 leaf disks). When NAA was present in addition to BA in the medium, most leaf sections formed calli but fewer somatic embryos than those cultured with BA alone (Figure 1A). Another auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) showed similar effects as NAA (data not shown). These results indicated BA to be sufficient to efficiently induce somatic embryos. When the initial embryos were cultured further, secondary somatic embryos were directly formed without callus formation (Figure 1C). These somatic embryos were transferred onto modified 1/2MS medium without phytohormones, and cultured further. After 4 months, they successfully regenerated at a frequency of about 25%, forming roots and leaves (Figure 1D). Plantlets were transplanted to

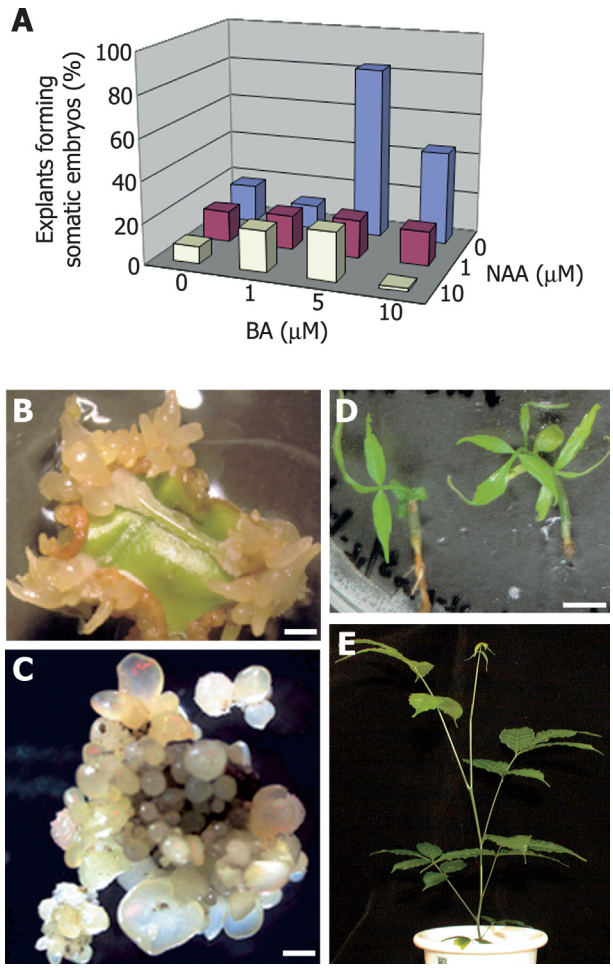

Figure 1. Establishment of somatic embryogenesis in *Azadirachta excelsa*. (A) Effects of concentrations of BA and NAA on somatic embryogenesis. Leaf disks were cultivated on MS medium containing the indicated hormones and frequency of somatic embryo formation was estimated 1 month later. (B) Somatic embryos directly forming at the edges of leaf explants 40 days later. (C) Secondary somatic embryos derived from the initial embryos as shown in B after 3 months. (D) Germination of somatic embryos showing roots and leaves after 2 months. (E) Mature plants grown in a greenhouse after 4 months. Scale bars indicate 1 mm in (B) and (C), and 1 cm in (D).

culture boxes containing Florialite with liquid modified 1/2MS medium, and incubated until leaves reached the lid. They were then transferred to plastic pots and grown to the maturity in a greenhouse (Figure 1E). These procedures are easier, simpler and more efficient than those previously reported (Liew et al. 1998, 1999, Techato et al. 2000).

In order to establish an efficient transformation method for *A. excelsa*, three genes were selected: *Bar*, *GFP* and *GUS*. The last two were used for confirmation, and the first as a model for practical transgenics. Plasmids containing each gene were introduced into *A. tumefaciens* strain EHA101 and used for transformation of leaf explants or petiols. Approximately 1–2 months after infection, all samples turned brown, and no initiation of somatic embryogenesis was observed.

Table 1. Different efficiency of *Agrobacterium*-mediated transformation between leaf disks and petioles

Transgenic line	Number of leaf disks forming somatic embryos/total leaf disks (%)	Number of petioles forming somatic embryos/total petioles (%)
Bar	2/22 (9.1)	1/2 (50.0)
GFP	1/23 (4.3)	1/3 (33.3)
GUS	3/20 (15.0)	3/8 (37.5)

Subsequently, we changed the host bacteria to *A. tumefaciens* strain LBA4404, and continued transformation experiments. One month after infection, leaf and petiole sections turned brown as previously observed. However, in contrast to the EHA101 case, somatic embryos did appear on leaf and petiole edges. Explants were further cultivated on selection medium and after 2 months, somatic embryogenesis was observed with 9% of leaf and 38% of petiole sections in total (Table 1). After selection of somatic embryos in the presence of bialaphos for the *Bar* gene, and kanamycin for the *GUS* and *GFP* genes, their expression was confirmed by RT-PCR, only transformed tissues accumulating transcripts of the relevant genes (Figure 2, left panel). Expression of *Bar* was evaluated with reference to resistance of transgenic plantlets to bialaphos (Figure 2A, right panel). Expression of the *GFP* gene was detected in transformed somatic embryos showing green fluorescence, while non-transformed control tissue was yellow (Figure 2B, right panel). Similarly *GUS* expression was confirmed by X-Gluc reactions, showing deep-blue staining (Figure 2C, right panel). The results of these experiments indicated that the introduced genes were stably expressed in somatic embryos and also in regenerated plantlets.

In the present study we established an efficient regeneration system with *A. excelsa*, a plant which is widely planted and utilized in Southeastern Asian countries due to highly useful traits as a source of timber, dietary and pharmaceutical agents. After examining a combination of phytohormones, we found that somatic embryogenesis was optimal with 5 μM BA, without any other hormones. Such a condition was also reported in coffee plants (Ogita et al. 2004). Using this condition, we could successfully transform *A. excelsa* with three different genes. Although our initial attempts using *A. tumefaciens* strain EHA101 totally failed, subsequent trials with strain LBA4404 turned out to be effective, yielding a rather high transformation efficiency of 14% on average. This suggested a specific interaction between *Agrobacterium* sp. and the host plant might be critical for gene transfer. It is generally considered that, upon infection, *Agrobacterium* sp. recognizes low molecular weight compounds, such as phenolic compounds and monosaccharides released from the host. Consequently, bacteria release a variety of chemical compounds, which

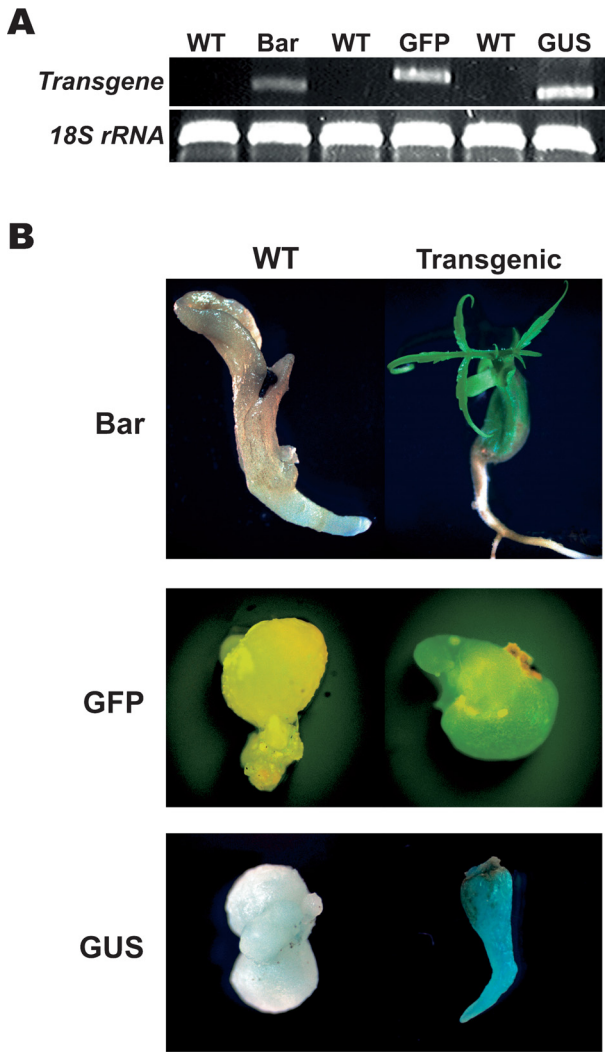

Figure 2. Expression of transgenes. (A) Identification of transcripts by RT-PCR. Total RNA was isolated from the wild type and the indicated transgenic lines expressing *Bar*, *GFP* or *GUS*, and accumulation of respective transcripts was detected using gene-specific primer sets as described in the text. RNA from non-transformed wild-type plants was used as the control. As a further internal control, transcripts for 18S rRNA were simultaneously detected for the indicated lines. (B) Activity of transgene products in somatic embryos and plantlets. One-month old plantlets of the wild type (left) and a transgenic line (right) were transferred onto MS medium containing 25 mg l^{-1} bialaphos, incubated at 28°C for 2 months, and photographed (top panel). Wild type (left) and transgenic (right) somatic embryos were observed for GFP under florescent light (middle panel). Wild type (left) and transgenic (right) somatic embryos were subjected to X-Gluc staining to estimated GUS activity (bottom panel).

might affect the host response (Winans 1992). From our and earlier findings, such exchange of chemical compounds might be critical, independent of whether *Agrobacterium* sp. can infect the host plants, and further detailed analysis should be performed to determine specific factors that play roles in *Agrobacterium*-plant interactions. Overall, our work suggests that determination of optimal regeneration condition and selection of *Agrobacterium* sp. are critical for successful transformation of tropical woody plants. Also *Bar*-expressing transgenic lines might be practically useful as herbicide tolerant stocks after appropriate safety assessment. The knowledge and techniques thus obtained will be of a great help for reforestation in tropical areas as an effective counterplan to cope with increasing environmental deterioration.

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