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Somatic Embryogenesis and Genetic Engineering of *Acacia* Species

Xiangming Xie* • **Mingjia Yang** • **Xiaoqing He**

College of Biological Sciences and Biotechnology, Beijing Forestry University, P. O. Box. 162, 100083, Beijing, P.R. China

Corresponding author: * **xiexiangm@bjfu.edu.cn**

ABSTRACT

Acacia species are widely dispersed in tropical and sub-tropical regions of the world and many of these species are important for fuelwood, timber, shelterbelts, soil improvement and landscaping and garden ornamentals. *A. mangium*, *A. crassicarpa*, *A. auriculiformis*, and *A. hybrid* (*A. mangium* × *A. hybrid*) have become a preferred fibre source for the pulp and paper industry because of their rapid growth, high pulp yield, high fibre quality and their ability to thrive in degraded soils. These tree species have been expandingly planted for reforestation, reclamation of wasteland, and industrial material production in Southeast Asia, especially in Indonesia, and in China. Thus the increasingly expanding plantation of these species requires clonal propagation of elite clones and efficient techniques for *in vitro* regeneration. However, the recalcitrance of regeneration, long generation time of trees, and the prolonged period needed for evaluation of mature traits are strong limitations for classical breeding programs in *Acacia*. The development of methods for *in vitro* regeneration including micropropagation, organogenesis and embryogenesis and genetic engineering has provided a new alternative for producing *Acacia* elite trees or modified genotypes. This review focuses on somatic embryogenesis in *Acacia*, and briefly presents research advances in genetic engineering in *Acacia.*

Keywords: *Agrobacterium*-mediated transformation, soil restoration, somatic embryo, transgenic

Abbreviations: **ABA**, abscisic acid; **Ads**, adenine sulfate; **Asn**, L-asparagine monohydrate; **BA**, 6-benzylaminopurine; **B5**, Gamborg's medium (Gamborg *et al*. 1968); **CH**, casein enzymatic hydrolysate; **GA_3**, gibberellic acid; **Gln**, L-glutamine; **GUS**, β-Glucuronidase; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **Kn**, kinetin; **MS**, Murashige and Skoog (1962); **NAA**, α-naphthalene acetic acid; **NPT II**, neomycin phosphotransferase; **Pro**, L-proline 7; **TDZ**, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron); **Vc**, vitamin C (L-ascorbic acid); **WPM**, Woody Plant Medium (Lloyd and McCown 1981); **2,4-D**, 2,4-dichlorophenoxy acetic acid

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INTRODUCTION

The genus *Acacia*, a leguminous genus belonging to the Mimosaceae family, comprises approximately 1200 species that are widely dispersed in tropical and sub-tropical regions of Australia, South America, Asia and Africa (Simmons 1987; Jones *et al*. 1990). For the purpose of soil conservation, suitable nitrogen-fixing species that are well adapted to

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Table 1 Somatic embryogenesis of *Acacia* species.

Acacia species[Ref.]	Explant type	Embryogenic callus or somatic embryo production				Embryo maturation and germination			
		Medium	PGR	Suspension culture	Additive	Medium	PGR	Result	Additive
A.catechu[1]	immature cotyledons	WPM	KT, NAA	yes	Pro, Vc	½MS	free	plantlets	
A.mangium[2]	immature embryo axes	MS	TDZ, IAA	no	amino acids; AC	½MS	GA_3	plantlets	
A. arabica[3]	immature zygotic embryos	MS	BA, 2,4-D	semi-solid		½MS	BA, ABA	plantlets	
A. nilotica[4]	immature endosperm	MS	BA, 2,4-D	no	CH; AC	modified MS	free	plantlets	Gln; CH; CW
A. auriculiformis[5]	mature hypocotyls	B5	BA, 2,4-D	yes		B5	free	only roots, no shoots	Gln, CH
A. sinuata[6]	leaf	MS	BA, 2,4-D	yes	CM	MS	free	plantlets	Gln
A. farnesiana[7]	immature zygotic embryos	MS	2,4-D, KT	semi-solid	CH	MS	free	plantlets	Ads
A. schaffneri[7]	immature zygotic embryos	MS	2,4-D, KT	semi-solid		MS	free	plantlets	Ads

Define all abbreviations here:
Ref. = References: 1, Rout *et al*. 1995; 2, Xie and Hong 2001; 3, Rashmi *et al*. 2003; 4, Garg *et al*. 1996; 5, Zhang *et al*. 1997; 6, Vengadesan *et al*. 2002; 7, Ortiz *et al*. 2000. **ABA**, abscisic acid; **Ads**, adenine sulfate; **BA**, 6-benzylaminopurine; **B5**, Gamborg's medium (Gamborg *et al*. 1968); **CH**, casein enzymatic hydrolysate; **GA_3**, gibberellic acid; **Gln**, L-glutamine; **IAA**, indole-3-acetic acid; **IBA,** Indole-3-butyric acid; **Kn**, kinetin; **MS**, Murashige and Skoog (1962); **NAA**, α-naphthalene acetic acid; **Pro**, L-proline 7; **TDZ**, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron); **Vc**, vitamin C (L-ascorbic acid); **WPM**, Woody Plant Medium (Lloyd and McCown 1981); **2,4-D**, 2,4-dichlorophenoxy acetic acid

environmental stresses and constraints (water stress and soil properties) are attractive. Under increasing land degradation, *Acacia* species are favored in programs of soil restoration and fertility enhancement (Lal 2004). Furthermore, many of these species are important for fuelwood, timber and shelterbelts (Palmberg 1981) and some are rich in sources of protein, tannin, paint, ink, flavouring agents and gum. Therefore, they have been described as a multipurpose tree species (Vengadesan *et al*. 2002b). They also have become a preferred fibre source for the pulp and paper industry because of its rapid growth, high pulp yield, high fibre quality and its ability to thrive in degraded soils (Pan and You 1994). The increasing demands for fibre products, along with the environmental importance of the tree, need to be matched by enhancing its production and optimizing its quality. Finally, and not of least importance is their extensive landscaping and ornamental use.

However, the recalcitrance of regeneration, long generation time of trees and the prolonged period required for evaluation of mature traits are strong limitations for classical breeding and selection. To circumvent these obstacles, the most promising alternative is genetic transformation of *Acacia*. This technology has two main advantages with respect to conventional breeding: a) genes encoding specific proteins can be 'cut' from virtually any living being, from viruses to higher plants to animals and 'pasted' into *Acacia*, thus broadening the range of genes available outside the current boundaries of the genus; b) individual genotypes can be modified for one or a small number of well defined traits while preserving the rest of the genome intact, hence, targeted modification of commercial cultivars could add value to them without disrupting their genome (Confalonieri 2003).

Somatic embryogenesis, the production of bipolar structures from somatic cells is of considerable theoretical and practical importance because it can be used to combine efficient cloning with genetic modification (Sharp *et al*. 1980). *In vitro* somatic embryos can develop either from callus or directly from the explant without any intermediate callus stage. Since plant regeneration from callus cultures such as organogenesis is often associated with genetic and cytological variations (Larkin and Scowcraft 1981) which are not always desirable, direct somatic embryogenesis has better applicability in the improvement of *Acacia*. While the greatest importance of somatic embryos lies in its practical application in large scale vegetative propagation, embryogenic cultures are also an attractive target for genetic modification (Arnold *et al*. 2002). As compared to organogenesis, which is frequently used as another forest gene-transfer recipient system, somatic embryogenesis is more efficient because of the following advantages: a) It is the developmental pathway by which somatic cells develop into structures that resemble zygotic embryos (Jimenez 2001), which is more effective for accepting foreign DNA; b) Its single cell origin (or a group of cells) results in less mosaic transgenic plants; c) Its bipolar structures will develop not only the shoots but also the roots, which could save time in practice.

In addition, the genus *Acacia* demonstrates self- and cross-pollination, and occasional interspecies pollination within *Acacia* species (Sedgley *et al*. 1992; Sornsathapornkul *et al*. 1999). These reproductive characteristics create considerable genetic diversity within the progeny, which is undesirable for commercial propagation through seeds. Furthermore, the regeneration rate of leguminous trees in natural habitats is quite low (Dewan *et al*. 1992). Thus the expanding plantation of *Acacia* requires clonal propagation of elite clones and efficient techniques for *in vitro* propagation. Therefore, the establishment of highly efficient somatic embryogenesis could solve these problems, meeting the practical demands for the production of forestry.

As the biotechnology industry has entered the era of the 21st century, molecular cloning and genetic engineering is routine in agricultural crops, vegetables and ornamental plants, but the research in molecular breeding for forest trees, in particular *Acacia* has greatly lagged behind due to a number of constraints. This review focuses on somatic embryogenesis in *Acacia*, and briefly presents research advances in genetic engineering in *Acacia*.

SOMATIC EMBRYOGENESIS OF *ACACIA* SPECIES

Somatic embryogenesis has a single cell origin as opposed to other multicellular events such as organogenesis, micropropagation etc. (Becks-Husemann and Reinert 1970; Reinert *et al*. 1977). Somatic embryogenesis has been reported in a variety of woody trees (Lu S *et al*. 2003). Since asexual propagation of some tree species and hybrids has been found difficult to achieve using propagation by cuttings and/or root suckers, in this case somatic embryogenesis provides an alternative for a large scale commercial propagation. Currently there have been eight papers describing somatic embryogenesis in *Acacia* species (**Table 1**), but it is extremely difficult to define a general method reliable for all *Acacia* species and hybrids based on a limited number of research reports.

Explant type

Embryonal and immature or juvenile tissues were often used to induce embryogenic callus in many *Acacia* species. Immature zygotic embryos were used in *A. arabica* (Rashmi *et al*. 2003), *A. farnesiana* and *A. schaffneri* (Ortiz *et al*. 2000). It has also been reported that immature embryo axes and hypocotyls were used as explants for embryogenic callus in *A. mangium* (Xie and Hong 2001) and *A. auriculiformis* (Zhang *et al*. 1997) respectively. Other explants include immature cotyledons used in *A. catechu* (Rout *et al*. 1995)

and immature endosperm in *A. nilotica* (Garg *et al*. 1996).

As the immature-seed-derived somatic embryos might have a high level of genetic diversity, the mature leaves rather than cotyledons or hypocotyls were used as explants for *in vitro* shoot regeneration, particularly useful for the propagation of the superior tree clones with desirable traits and performances. Attempts were also made to induce embryogenic calli from higher differentiated tissues like the seedling leaf in *A. sinuate* (Vengadesan *et al*. 2002a). All the calli derived from different explants produced embryos successfully.

In addition, several authors have highlighted a role of embryo in the proliferation of mature endosperm (Bhojwani 1984). Interestingly, Garg *et al*. reported that immature endosperm generally does not require the association of an embryo to form callus (Garg *et al*. 1996), suggesting that in *Acacia* species, the presence of embryo was not only unnecessary but deleterious for the initial proliferation of endosperm callus (Garg *et al*. 1996).

Media

Media for embryogenic calli or somatic embryos production

Three types of media, namely, Woody Plant Medium (WPM, Lloyd and Mc Cown 1981), Gamborg's medium (B5, Gamborg *et al*. 1968) and Murashige and Skoog medium (MS, Murashige and Skoog 1962) have been employed in the induction of embryogenic callus or somatic embryos. Among the medium types used, B5 medium is much more efficient than MS medium in embryogenic callus induction in *A. auriculiformis*. And WPM is better than MS in the embryogenic callus induction in *A. catechu*. However, MS medium were still effective in most of *Acacia* species in the induction embryogenic calli or somatic embryos such as in *A. mangium* (Xie and Hong 2001), *A. arabica* (Rashmi *et al*. 2003), *A. nilotica* (Garg *et al*. 1996), *A. sinuate* (Vengadesan *et al*. 2002a), *A. farnesiana* and *A. schaffneri* (Ortiz *et al*. 2000).

Media for embryos maturation and germination

Maturation and germination of somatic embryos was achieved using MS, half strength MS medium and B5 medium, respectively. In *A. nilotica* (Garg *et al*. 1996), embryos germination was achieved on MS after 15 d pretreatment on modified MS medium in which major salts were replaced by those of B5 medium. Half-strength MS medium was also employed successfully in somatic embryos in *A. catechu* (Rout *et al*. 1995), *A. mangium* (Xie and Hong 2001) and *A. arabica* (Rashmi *et al*. 2003). B5 medium could only improve development of roots but no shoots in *A. auriculiformis* (Zhang *et al*. 1997).

Media state

The embryogenic calli can be kept on semi-solid medium in such *Acacia* species as in *A. arabica* (Rashmi *et al*. 2003), *A. sinuate* (Vengadesan *et al*. 2002a), *A. farnesiana* and *A. schaffneri* (Ortiz *et al*. 2000). For large scale propagation, it is usually optimal to establish suspension cultures, where the proliferation rate is higher and the cultures become more synchronized. In suspension cultures, single cells and cell aggregates develop as separate structures. Thus the cells can easily be separated by sieving or centrifugation, and thereafter, subcultured and manipulated as required (Arnold *et al*. 2002). Up to now, it has been reported that the suspension cultures were employed in embryogenic calli or somatic embryos production of *A. catechu* (Rout *et al*. 1995), *A. auriculiformi*s (Zhang *et al*. 1997) and *A. sinuate* (Vengadesan *et al*. 2002a).

Growth regulators

The various developmental stages of explants in embryogenesis in *Acacia* are largely regulated by auxins, cytokines or other PGRs for the initiation of somatic embryogenesis.

Growth regulators in embryogenic calli or somatic embryos production

It has been shown in many research reports that a range of auxins and cytokinins played a vital role in embryogenic calli or somatic embryos production in many *Acacia* species. Usually embryogenic callus is formed in medium containing auxin (Arnold *et al*. 2002). Synthetic auxins, like 2,4-dichlorophenoxy acetic acid (2,4-D), which is particularly effective for promoting establishment and proliferation of embryogenic cultures, are usually less metabolized by the cells than other auxins. 2,4-D combined with kinetin (Kn), 6-benzylaminopurine (BA) was widely employed in embryogenic calli or somatic embryos production in many species of *Acacia* such as *A. nilotia* (Garg *et al*. 1996), *A. auriculiformis* (Zhang *et al*.), *A. arabica* (Rashmi *et al*. 2003), *A. sinuata* (Vengadesan *et al*. 2002a), *A. farnesiana and A. schaffneri* (Ortiz *et al*. 2000). The combination of 2,4-D and BA promoted embryogenic callus formation and proliferation in *A. nilotia* (Garg *et al*. 1996).

Other kinds of auxins played an important role in embryogenic calli or somatic embryo production. α-Naphthalene acetic acid (NAA) and indole-3-acetic acid (IAA) have been successfully employed in *A. catechu* (Rout *et al*. 1995) and *A. mangium* (Xie and Hong 2001) for somatic embryo induction. Nodular callus induced on the media supplemented with combinations of NAA with BA or Kn could not become embryogenic *A. arabia*. Rout *et al*. (1995) reported that the combination of 2.7 μM NAA and 13.9 μM Kn promoted *A. catechu* (Rout *et al*. 1995) somatic embryogenesis from immature cotyledon. However, a similar combination of NAA and Kn did not induce embryogenic callus from *A. mangium* (Xie and Hong 2001) immature embryonic axes. Also, the other tested combinations of NAA and BA or NAA and Kn induced adventitious roots or loose white-yellowish callus instead of embryogenic callus in *A. mangium*.

Three kinds of cytokinins, namely Kn, 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) and BA were involved in embryogenic calli or somatic embryos production in *Acacia*. BA was predominantly used for growth regulator for induction of embryogenic calli or somatic embryos production in *A. nilotia* (Garg *et al*. 1996), *A. sinuata* (Vengadesan *et al*. 2002a), *A. auriculiformis* (Zhang *et al*. 1997) and *A. arabica* (Rashmi *et al*. 2003). Kn was also reported to induce somatic embryos in *A. catechu* (Rout *et al*. 1995), *A. farnesiana and A. schaffneri* (Ortiz *et al*. 2000), respectively. Xie and Hong (2001) have produced embryogenic calli in the presence of TDZ (5.0 μM) and IAA (1.1 μM). Torpedo and cotyledonary stage embryos were attained in the presence of gibberellic acid (GA_3).

Besides cytokinin and auxin, abscisic acid was also utilized in somatic embryo induction. Ortiz *et al*. (2000) achieved 345 and 198 somatic embryos per gm of callus of *A. farnesiana* and *A. schaffneri*, respectively, in media without growth regulators but inclusion of abscisic acid (ABA).

Growth regulators in embryos maturation and germination

A major problem associated with somatic embryogenesis is the poor conversion into plants. Therefore, in order to stimulate further growth of the somatic embryos, it is necessary to transfer the embryogenic cultures to the medium lacking auxin (Arnold *et al*. 2002). With the depletion of auxin, the block on the expression of those genes required for the transition to the heart stage is removed (Zimmerman 1993). The successful embryos maturation and germination

were achieved in *A. nilotia* (Garg *et al*. 1996), *A. sinuata* (Vengadesan *et al*. 2002a), *A. catechu* (Rout *et al*. 1995), *A. farnesiana and A. schaffneri* (Ortiz *et al*. 2000) on plant growth regulator free medium.

Garg *et al*. (1996) only obtained two triploid *A. nilotica* plantlets as the somatic embryos formed by the endosperm callus of *A. nilotica*, which formed roots more readily than shoots. The similar result was achieved in *A. auriculiformis* (Zhang *et al*. 1997).

Rashmi *et al*. (2003) obtained the maximum percentage (67.8%) of germination with 0.94 μM ABA and 0.04 μM BA in *A. catechu*. The medium with BA alone did not promote germination. The somatic embryos produced green cotyledons and a plumule and radicular zone without showing secondary callus when ABA was applied. It is indicated that ABA promoted maturation and germination of somatic embryos of *Acacia arabica*. The similar phenomenon was also observed in *A. farnesiana and A. schaffneri* (Ortiz *et al*. 2000). On half-strength MS basal medium containing 5.0 mg/l GA_3 (AM-424) (**Table 1**), 11% of the cotyledonary-stage somatic embryos germinated into seedlings with roots, elongated hypocotyl and the first pinnate leaf *in A. mangium* as reported by Xie and Hong (Xie and Hong 2001).

Culture conditions

Various explants in most of *Acacia* species were cultured at 25-28°C in a 12-16 h photoperiod at a light intensity of 23-55 μmol m^{-2} s^{-1} for somatic embryos production and germination such as in *A. farnesiana* (Ortiz *et al*. 2000), *A. catechu* (Rout *et al*. 1995) and *A. mangium* (Xie and Hong 2001). There was still exception that the calli incubated in the dark exhibited somatic embryogenesis in *A. nilotia* (Garg *et al*. 1996). In subsequent subcultures of the embryogenic calli in the dark, the proliferation of somatic embryos increased, and the embryogenic cultures could be maintained at the interval of 8-week subcultures.

Additives

Addition of organic supplements is a common phenomenon in tissue culture literature.

Additives in embryogenic calli or somatic embryos production

Casein hydrolysate (CH) was widely used in *A. nilotica* (200 mg/l) (Garg *et al*. 1996), *A. mangium* (100 mg/l) (Xie and Hong 2001) and *A. auriculiformis* (500 mg/l) (Zhang *et al*. 1997) callus culture. The addition of 0.9-3.5 μM L-proline (Pro) to the medium was observed to influence development of somatic embryos and also promote secondary somatic embryogenesis in *A. catechu* (Rout *et al*. 1995). And Pro was found to stimulate proliferation of embryogenic callus while L-glutamine (Gln) not to stimulate embryogenic callus proliferation *in A. catechu* (Rout *et al*. 1995). Embryogenic callus was induced on MS medium containing a mixture of amino acids 100 mg/l Vc, 150 mg/l L-asparagine monohydrate (Asn), 100 mg/l CH, 150 mg/l Gln, 150 mg/l Pro as reported by Xie and Hong (2001). Ascorbic acid (Vc) and activated charcoal (AC) were the commonly used anti-oxidants and adsorbents for preventing browning in *A. nilotica* (Garg *et al*. 1996), *A. mangium* (Xie and Hong 2001) and A. *catechu* (Rout *et al*. 1995). In addition, 10% coconut water (CW) was supplemented in the somatic embryos production medium in *A. sinuate* (Vengadesan *et al*. 2002a).

Additive in embryos maturation and germination

The embryos germinated on MS only after 15 d pre-treatment on modified MS medium in which major salts were replaced by those of major salts of B5 medium and supplemented with glutamine, CH and coconut water (10% CW) in *A. nilotica* (Garg *et al*. 1996). The addition of Gln, CH to the medium could promote embryos maturation in *A. auriculiformis* (Zhang *et al*. 1997), but the sole addition of glutamine to the medium without CH promoted embryos maturation in *A. sinuate* (Vengadesan *et al*. 2002a).

Carbon sources

Shoot and root growth was found to be affected by carbon sources in *Acacia* species.

Carbon sources in embryogenic calli or somatic embryos production

Irrespective of the types of media and explants, 30 g/l sucrose was universally used in *Acacia* species, such as in *A. arabia* (Rashmi *et al*. 2003). A noticeable variation of sucrose concentration in two-step maturation phase was observed in *A. mangium* (Xie and Hong 2001). Nearly 42% of embryogenic cultures with globular embryos produced torpedo- and cotyledonary-stage embryos by a two-step maturation phase. The first stage occurred on medium containing 30 g/l sucrose, followed by the second stage medium containing 50 g/l sucrose, which means somatic embryo maturation was completed in the presence of a relatively high sucrose concentration during the second maturation step.

Carbon sources in embryos maturation and germination

For embryos maturation and germination, lower concentrations of sucrose have been employed. 20 g/l sucrose has been used in *A. catechu* (Rout *et al*. 1995) and *A. arabia* (Rashmi *et al*. 2003). While 30 g/l sucrose was supplemented in germination of somatic embryos in *A. mangium* (Xie and Hong 2001), it was much lower than anterior stage usage of 50 g/l sucrose.

Proliferation of somatic emborys

Commercial mass propagation through embryogenic culture requires efficient protocols for repetitive or recurrent somatic embryogenesis. Embryogenic callus is maintained and proliferated in a medium similar to that used for initiation, which has only been reported for the following *Acacia* species.

Secondary somatic embryogenesis was achieved by repeatedly subculturing the embryogenic mass at 4-week intervals on fresh medium and the cultures were maintained for 18 months without any loss in the regenerative capacity in *A. catechu* as reported by Rout *et al*. (1995).

It was also reported that in *A. nilotica* (Garg *et al*. 1996), the proliferation of somatic embryos increased in subsequent subcultures of the embryogenic calli at 8-week intervals. And the embryos occurred in large clusters, and the adjacent embryos appeared interconnected.

Somatic embryos proliferated rapidly by secondary somatic embryogenesis after transfer to MS medium supplemented with BA and 2,4-D. The maximum number of somatic embryos per callus was 72.6 after 8 weeks of culture on medium containing 6.66 μM BA and 6.78 μM 2,4-D in *A. arabia* (Rashmi *et al*. 2003).

In *A. mangium* (Xie and Hong 2001) secondary embryogenesis induction was observed when embryogenic callus or heart-shaped embryos were subcultured on the solid induction medium containing 2.0 mg/l TDZ and 0.25 mg/l IAA. The capacity of repetitive somatic embryogenesis was maintained for over 6 months by subculturing once a month (data not shown).

For the successful establishment of embryogenesis protocol in *Acacia* species, the basic medium composition, plant growth regulators and their combination, the photoperiod and other culture conditions have to be taken into account. This technique has low probability of success, low

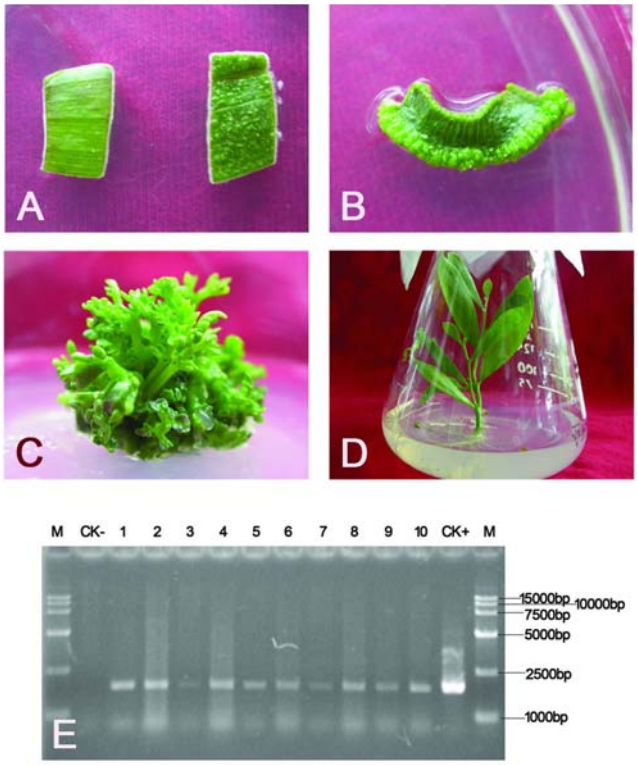

Fig. 1 *Agrobacterium*-mediated genetic transformation of *Acacia crassicarpa*. (**A**) Leaf plates cocultured with *Agrobacterium*. (**B**) Nodules developed directly from phyllode explants on selection media (MS + 5 mg/l kanamycin). (**C**) Adventitious shoot elongation. (**D**) Whole transgenic plantlet with roots. (**E**) PCR result verified the integration of transgene.

reproducibility and abnormal morphology in the induced embryos, which might derive from altered patterns of gene expression associated with somaclonal variation. Further studies are needed to optimize *in vitro* culture methods, eventually understanding the critical events in the somatic embryogenesis of *Acacia*.

ESTABLISHMENT OF TRANSFORMATION SYSTEM IN *ACACIA* SPECIES

In recent years, great effort has been made to develop transformation systems for *Acacia* species, heavily relying on the establishment of their efficient regeneration systems. Unfortunately, most *Acacia* species tested have proved to be recalcitrant to regeneration, and so far there have been a limited number of reports detailing the genetic engineering of *Acacia*. Two methods including *Agrobacterium*-mediated transformation and biolistics have been used to transfer foreign DNA into the *Acacia* genome (Xie X *et al*. 2006). The species involved *A*. *mangium*, *A*. *crassicarpa*, *A*. *sinuate* and *A*. *hybrid* (*A*. *mangium* × *A*. *auriculiformis*).

Acacia mangium

A protocol was developed for *Agrobacterium*-mediated genetic transformation of *A*. *mangium* (Xie and Hong 2002). Axillary buds and shoot apices of adult trees were rejuvenated by culturing them on MS medium, and stem segments of rejuvenated shoots were co-cultured with *Agrobacterium tumefaciens* strain LBA4404 harbouring binary vector pBI121. The selection for transgenic shoots was performed through five consecutive steps on MS medium supplemented with 1.0 mg/l TDZ, 0.25 mg/l IAA and different concentrations of geneticin (G418; 12-30 mg/l) and timentin (T; 50-300 mg/l). Thirty-four percent of the stem segments produced resistant multiple adventitious shoot buds, of which 30% expressed the β-glucuronidase gene. The shoot buds were subjected to repeated selection on MS medium supplemented with 2.0 mg/l BA, 2.5 mg/l GA_3 and 20 mg/l G418. Transgenic plants were obtained after rooting on half-strength MS medium supplemented with 2.0 mg/l, 0.1 mg/l Kn and 20 mg/l G418. Genomic Southern blot hybridization confirmed the incorporation of the *npt*II gene into the host genome.

Acacia crassicarpa

Based on our regeneration system in *A*. *crassicarpa* (Yang *et al*. 2006), a successful *Agrobacterium*-mediated genetic transformation of *A*. *crassicarpa* has been developed (**Fig. 1**). *A*. *crassicarpa* has become a preferred fibre source for the paper and pulp industry (Pan and You 1994). 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*) (Li *et al*. 2003), has been transferred into *A*. *crassicarpa* by using an *A*. *tumefaciens* mediated system. Transgene integration has been verified by PCR on *A*. *crassicarpa* genomic DNA. The following enzyme-activity analyses, lignin histochemical analysis, protein immunolocalization and lignin analysis should be carried out until the maturation of transgenetic plantlets.

Other gene transfer techniques such as biolistics have also been tried, obtaining a great many putative transgenic *A*. *crassicarpa* plantlets (Xie X *et al*. 2006). This method is proved to be effective, but the selection process is tedious, involving a lot of identification work.

Acacia sinuata

Transgenic herbicide tolerant *A*. *sinuate* (Vengadesan *et al*. 2006) plants were produced by transformation with the *bar* gene conferring phosphinothricin resistance. Precultured hypocotyl explants were infected with *A*. *tumefaciens* strain EHA105 in the presence of 100 μM acetosyringone and shoots regenerated on MS medium with 13.3 μM BA, 2.6 μM IAA, 1 g/l AC, 1.5 mg/l phosphinothricin, and 300 mg/l cefotaxime. Phosphinothricin at 1.5 mg/l was used for selection. Shoots surviving selection on medium with phosphinothricin expressed GUS. Following Southern hybridization, eight independent shoots regenerated from 500 cocultivated explants were demonstrated to be transgenic, which represented a transformation frequency of 1.6%. The transgenic plantlets carried one to four copies of the transgene. Transgenic shoots were propagated as microcuttings in MS medium with 6.6 μM BA and 1.5 mg/l phosphinothricin. Shoots elongated and rooted in MS medium with GA_3 and IBA, respectively both supplemented with 1.5 mg/l phosphinothricin. Micropropagation of transgenic plants by microcuttings is proved to be a simple means to bulk up the material. Several transgenic plants were found to be resistant to leaf painting with the herbicide Basta.

Other *Acacia* species

Up to now, there are only a limited number of reports concerning the transgene into *Acacia* species, due to the unavailability of efficient and reliable regeneration systems. The genetic transformation is beset with the recalcitrance of regeneration of some *Acacia* species, especially those widely planted varieties. For example, a consortium jointed by some institutions and international pulp and paper companies launched a big project, conducting the regeneration and transformation of *A*. *hybrid* (*A*. *mangium* × *A*. *auriculiformis*), but the extremely low regeneration rate yielded nearly no transgenic plants (unpublished results). Both extensive and intensive research should be undertaken, covering efficient techniques for *in vitro* regeneration and genetic transformation of a wide range of important *Acacia* species.

CONCLUDING REMARKS

In order to efficiently regulate the formation of plants via somatic embryogenesis, it is important to understand how somatic embryos develop and how the development is influenced by different physical and chemical treatments. Such knowledge can be gained through the construction of fate maps representing an adequate number of morphological and molecular markers, specifying critical developmental stages (Arnold *et al*. 2002). Based on this fate map, it is possible to make a model of the process (Arnold *et al*. 2002). The mechanisms that control cell differentiation during somatic embryogenesis are far from clear. Secreted, soluble signal molecules, however, play an important role. It has long been observed that conditioned medium from embryogenic cultures can promote embryogenesis. Active components in the conditioned medium include endochitinases, arabinogalactan proteins and lipochitooligosaccharides (Arnold *et al*. 2002).

Acacia species and hybrids are intensively cultivated as sources of woody biomass for the forest products industry and for reforestation of lowlands in temperate regions of the world. However, the recalcitrance of regeneration, long generation time of trees and the prolonged period required for evaluation of mature traits are strong limitations for classical breeding and selection. The genetic transformation, avoiding the sexual process, offers opportunities for creating new *Acacia* varieties with important traits such as improved wood quality, improvement in growth, pest and disease resistance, etc. Although a limited progress was achieved during the past decade, many *Acacia* research programs are at a preliminary stage, with transgene focused on a few species. Hence, both *in vitro* regeneration and genetic transformation protocols need to be further optimized for some ecologically and economically important but recalcitrant *Acacia* tree species, such as *A*. *mangium*, *A*. *hybrid* (*A*. *mangium* × *A*. *auriculiformis*), which are currently planted over large areas in Southeast Asia and in China (Yang *et al*. 2006; Xie X *et al*. 2006). Furthermore, the combination of molecular techniques and classical breeding will help create forest trees with positive effects on the environment. It is noted, however, that risks associated with the biotechnological applications (concerning the impact on biodiversity, long-term adaptation, transgene inheritance and stability) should be carefully evaluated and field tests performed with transgenic *Acacia*.

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