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Genetic Transformation of *Eucalyptus*—Challenges and Future Prospects

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ABSTRACT

Improving *Eucalyptus* tree species through conventional breeding is constrained by long generation times and high genetic load. As an additional tool for introducing valuable traits, gene transfer technology can be applied directly to elite, clonally produced genotypes without the need for introgressive breeding and backcrossing. It is also a powerful scientific means for analyzing plant physiology and gene function. However, researchers require regeneration systems that are efficient, and preferably are workable in several species, genotypes and hybrids. During the past decade, genetic transformation has been performed successfully with the main commercially grown *Eucalyptus* species, *E. grandis* x *E. urophylla*, *E. urophylla*, *E. camaldulensis*, *E. tereticornis*, *E. globulus* and in the early flowering species *E. occidentalis*. Field trials have now demonstrated that transformation in the laboratory can give rise to healthy, productive trees with stable expression of new traits, including male sterility, cold resistance and modified lignin content. However, regeneration of stable transgenic plants is generally slow, occurs at very low rates, requires genotype-specific customization, and for many genotypes has been nearly impossible to achieve. Furthermore, no open, public sector-accessible method is yet available to a broad array of scientists. Here, we review the progress made in eucalypt transformation, emphasizing the factors that appear to have the greatest roles in causing recalcitrance and promoting successful regeneration and transformation.

Keywords: *Agrobacterium tumefaciens*, eucalypts, genetic transformation, *in vitro* regeneration, somatic embryogenesis, transformation rate

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Introduction

Eucalyptus trees are the most widely planted tropical and subtropical hardwoods in the world, with commercial plantations found throughout much of the southern hemisphere. This genus comprises of 894 taxa (Slee et al. 2006), including subspecies and natural hybrids (Blakely 1955, Penford and Willis 1961, Johnston and Marryatt 1965, Brooker and Kleinig 1996). Eucalypts are native to the Australian continent and islands to the north (Hall et al. 1963, Williams and Potts 1996). Globally, their cultivated area is increasing rapidly, now covering approximately 20 Mha (GIT Forestry 2009). Trees of this genus are most extensively grown in Brazil (4.2 Mha), India (3.9Mha) and China (2.6 Mha), where they are utilized for high-quality pulp production, timber, fuelwood, and essential oils.

Eucalyptus has abundant genetic diversity and many interspecies hybrids show remarkable yields. Several taxa can also be vegetatively propagated, enabling the most productive genotypes to be cloned (Grattapaglia and Kirst 2008). The development of high-throughput DNA genotyping resources (Petroli et al. 2012, Sansaloni et al. 2010) and implementation of genomic selection approaches can markedly improve the rate of genetic gain in breeding (Resende et al. 2012). However, traits not readily available in the gene pool can also be very valuable, as demonstrated by the rapid expansion of GMO crops in many countries. Thus, it is highly desirable to add genetic transformation to the array of tools available for eucalypt tree improvement. However, transformation and regeneration of healthy transgenic plants is extremely challenging in most eucalypt species. Here, we review the progress already made toward those goals and present options for future advancements.

Development of *in vitro* Regeneration Methods for *Eucalyptus*

In vitro plant regeneration is the most important component for successful implementation of various biotechnological techniques. It offers opportunities to produce superior genotypes through mutagenesis, somaclonal variation, and genetic engineering (Hajari et al. 2006). Tissue-culture of *Eucalyptus* was first reported by Jacquiot (1964a,b), who developed callus cultures from seedling explants and cambial tissue of *E. cladocalyx*, *E. gomphocephala*, *E. gunnii*, *E. tereticornis*, and *E. camaldulensis* (a summary of reports on *in vitro* regeneration is presented in Appendix 1). Sussex (1965) used hypocotyls to produce callus and cell suspension cultures of *E. camaldulensis*. Since then, many advances have been reported, including callus cultures originating from seedling-derived explants of most *Eucalyptus* species (Lakshmi Sita and Vaidyanathan 1979, Qin and Kirby 1990); lignotubers from *E. bancroftii* (de Fossard 1974, de Fossard et al. 1974), *E. citriodora* (Aneja and Atal 1969), *E. robusta* (Goncalves 1975), and *E. obliqna* and *E. viminalis* (Winton 1974); as well as anthers (Goncalves 1975, Bennett and McComb 1982, McComb and Bennett 1982, Boulay 1987). All showed that calli formation could be induced at 25°C in the dark or at a low light intensity. These earlier efforts have been reviewed in detail by Roux and van Staden (1991) and Jones and van Staden (1997). Typical stages of organogenic regeneration are shown in Fig. 1 (Chauhan and Myburg 2011, unpublished).

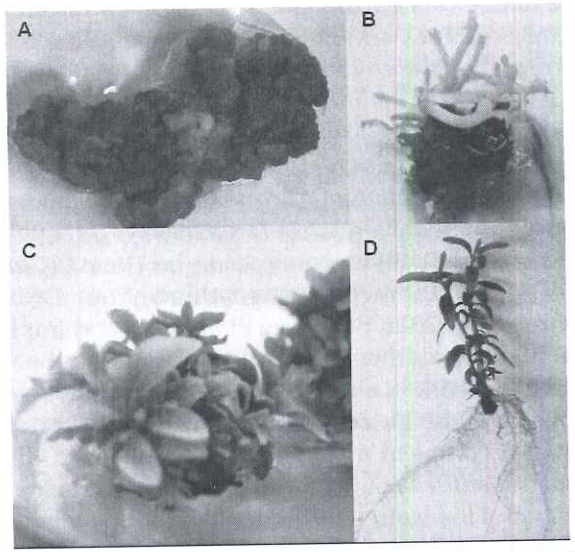

Figure 1. Indirect organogenesis from leaf explants of *Eucalyptus grandis*. Steps shown include (A) callus induction from leaf explants, (B) shoot regeneration from callus, (C) shoot multiplication and (D) rooting of plantlets. Images from Myburg laboratory (Chauhan and Myburg 2011, unpublished).

Tissues from mature *Eucalyptus* trees are especially recalcitrant to micropropagation (Roux and van Staden 1991). Successful disinfection of mature explants has been a critical step in obtaining cultures free of contamination (Warrag et al. 1991). Vitrification, manifested by translucent, thick leaves and poor tissue differentiation in established shoot cultures (George and Sherrington 1984) is often observed. This can be reduced by modifying the concentrations of growth regulators, such as cytokinin (Durand-Cresswell et al. 1982), or by selecting alternate gelling agents or carbon sources. For example, Boulay (1983) transferred vitrified shoots to a multiplication medium containing 15 g L^{-1} activated charcoal to promote the development of normal shoot buds. Alternatively, individual vitrified shoots, if not common, can simply be discarded during sub-cultures.

Generally, an MS basal medium (Murashige and Skoog 1962) is used for indirect organogenesis of *Eucalyptus* species. However, B5 media (Gamborg et al. 1968) or modified MS and WPM (Lloyd and McCown 1981) media have also been utilized. For calli development and subsequent shoot induction, various combinations of growth regulators can be used, most commonly NAA (Napthelene acetic acid) plus BA (Benzyladenine), 4CPPU (N-(2-Chloro-4-pyridyl)-N-phenylurea), or TDZ (Thidiazuron). Herve et al. (2001) combined 0.04 µM picloram with 1 µM BA for callus induction and shoot regeneration from *E. gunnii* explants. Huang et al. (2010) reported frequencies of 10 to 57% shoot induction from hypocotyl-derived calli of *E. urophylla* after 40 d of culture, and shoot regeneration has also been described from other *Eucalyptus* callus cultures (Mullins et al. 1997, Ho et al. 1998, Tournier et al. 2003, Aggarwal et al. 2010, Dibax et al. 2010). However, the lack of an efficient protocol for shoot organogenesis very often presents a bottleneck when undertaking the genetic manipulation of commercially important clones.

Shoot organogenesis that involves a callus phase is likely to result in somaclonal variation. Therefore, attempts have been made to regenerate shoots directly from explants (Tibok et al. 1995). Nugent et al. (2001) reported a high frequency (84%) of adventitious bud formation from seedling-derived explants of *E. globulus*. Similarly, Mamaghani et al. (2009) noted that 88% of green twigs taken from one year old greenhouse-grown seedling can directly produce shoot buds in *E. microtheca* F. Muell. The maximum number of direct shoots were observed from explants cultured on modified MS medium with half-concentrations of KNO_3 and NH_4NO_3 containing 1.0 mg L^{-1} NAA, 1.0 mg L^{-1} kinetin, and 0.01 mg L^{-1} TDZ. The cultures had been incubated at 27°C under light for 16 hrs and at 19°C in darkness for 8 hrs. Aggarwal et al. (2010) described shoot bud organogenesis (45%) from leaf explants of *E. tereticornis*, while other research groups used nodule cultures to regenerate and, subsequently, transform several *Eucalyptus* species (Warrag et al. 1991, Kawazu et al. 1996). Mamaghani et al. 2008 compared plant regeneration

from leaf and hypocotyl explants through indirect and direct regeneration pathways in *E. microtheca* F. Muell and found direct organogenesis better than the indirect method.

Somatic embryogenesis (Fig. 2; Chauhan and Myburg 2011, unpublished; Appendix 2) is an alternative regenerative procedure for producing complete plantlets. For example, somatic embryo-like structures have been formed from cotyledons of *E. citriodora* (Muralidharan and Mascarenhas 1987, Muralidharan et al. 1989), seedling leaves of *E. dunnii* (Boulay 1987), hypocotyl and seedling leaves of *E. gunnii* (Franclet and Boulay 1989), hypocotyl, cotyledon and seedling leaves of *E. dunnii* (Qin and Kirby 1990), shoots of *E. grandis* (Lakshmi Sita et al. 1986), hypocotyl of *E. camaldulensis* (Prakash and Gurumurthi 2010), mature zygotic embryos of *E. globulus* (Pinto et al. 2008) and *E. tereticornis* (Prakash and Gurumurthi 2005b) and other species (Ouyang et al. 1980, Watt et al. 1999). However, the small percentage of embryogenesis that occurs, the difficulty in germinating embryos, and the low frequency of normally developed plantlets are major drawbacks to this approach. Induction of embryogenic calli and somatic embryos depends upon the type of explant used, media composition, and lighting conditions during incubation (Ouyang et al. 1981, Muralidharan and Mascarenhas 1987, Termignoni et al. 1996, Prakash and Gurumurthi 2005b, Prakash and Gurumurthi 2010). The highest callus induction could be obtained from young explants (cotyledons from 10 day old seedling), but maximum somatic embryogenesis was achieved from mature zygotic embryos using MS basal medium supplemented with 0.5 mg L^{-1} benzyladenine (BA) and 0.1 mg L^{-1} NAA. Maturation of somatic embryos was increased by the addition of abscisic acid upto 1 mg L^{-1} and by lowering the sucrose concentration to 1% (Prakash and Gurumurthi 2010). Somatic embryogenesis was obtained in this well-defined medium, but only under low light conditions.

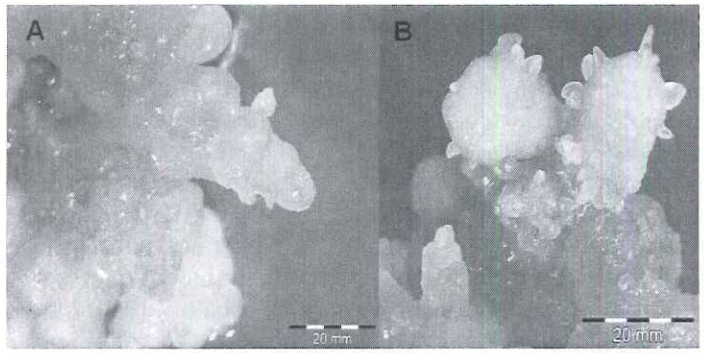

Figure 2. Somatic embryogenesis in *Eucalyptus grandis*. Panel A) shows initiation of primary somatic embryos from callus and B) shows secondary embryogenesis. Images from Myburg laboratory (Chauhan and Myburg 2011, unpublished).

Progress with Direct DNA Transformation

Direct gene transfer relies heavily on the ability to produce protoplasts. The efficiency of protoplast isolation varies from species to species and according to the physiology and genotype of the starting material. Although electroporation of protoplasts has been somewhat successful (Kawazu et al. 1990, Teulieres et al. 1990, 1991, Manders et al. 1992), it has been difficult to regenerate complete transgenic plants by this method (Appendix 3). Kawazu et al. (1990) used protoplasts of *E. saligna* for electroporation, while Teulieres et al. (1991) demonstrated that *E. gunnii* protoplasts could be transformed via electropulsation and treatment with polyethylene glycol. These groups have concluded that the physiological condition of the cells and the type of promoter associated with the reporter genes are important components of successful transformation. Teulieres et al. (1991) found that a gene-specific promoter from a protein synthesis elongation factor gene was more efficient than the 35S cauliflower mosaic virus promoter for polyethylene/glycol or electropulsation induced transformation in *Eucalyptus gunnii* protoplasts.

Particle bombardment had been tested with several *Eucalyptus* species, but only transformed calli were obtained that showed stable expression of the reporter gene (Sartoretto et al. 2002). Such stable transgene expression was also observed in transformed calli from zygotic embryos of *E. globulus* when a biolistic gun was used (Rochange et al. 1995, Serrano et al. 1996). There, cotyledons and axes of zygotic embryos served as target material for bombardment and transformed calli were obtained on a selective medium containing 100 mg L^{-1} kanamycin (kan). However, the shoots regenerated from those transformed calli were not kan-resistant and did not show GUS expression. Similarly, *gus* and *nptII* were introduced into calli obtained from cotyledon and hypocotyl explants of *E. grandis* x *E. urophylla* (Sartoretto et al. 2002). Although GUS was expressed in callus tissues after 105 days under selection pressure (200 mg L^{-1} kan), no transgenic plants were regenerated. Overall, direct gene transfer methods have had very limited success for *Eucalyptus* transformation.

Progress Using *Agrobacterium*-Mediated Genetic Transformation

For tree species, *Agrobacterium tumefaciens*-mediated gene transfer is often preferred over biolistic procedures because it seems less prone to the production of multicopy and fragmented gene insertions. Preliminary studies showed variable degrees of susceptibility of *E. gunnii*, *E. grandis*, *E. dunnii*, *E. nitens*, *E. globulus*, and *E. grandis* x *E. urophylla* to different strains of *Agrobacterium rhizogenes* and *A. tumefaciens* (Adam 1987, MacRae and van Staden 1993, Azmi et al. 1997, Machado et al. 1997). Shooty tumors were obtained when *E. grandis* x *E. urophylla* (Andrade and Brasileiro

1997) and *E. globulus* (Azmi et al. 1997) were transformed with wild-type *A. tumefaciens* strains. *Agrobacterium*-mediated genetic transformation has also been used to improve the rootability of *Eucalyptus* species (MacRae 1991), and disarmed strains of *A. tumefaciens* harboring foreign gene(s) have been exploited. *Agrobacterium*-mediated genetic transformation of *Eucalyptus* is now used in attempts to enhance wood quality (Chen et al. 2001, Tournier et al. 2003, Valerio et al. 2003) and resistance to abiotic (Dibax et al. 2010) and biotic stresses (Harcourt et al. 2000, Wang et al. 2007). Several patents have been issued that claim efficient *Eucalyptus* transformation procedures, including those filed by Kawazu (2000b; 6,563,024 B1), Chang et al. (2006; W02006/052554A2), and Teasdale et al. (1999; W01999/048355). Most published papers have described transformation using seedling-derived explants. The exceptions had been reports by Chen et al. (1996, 2001), Mullins et al. (1997), and Spokevicius et al. (2005), as well as United States patents by Kawazu (2000b) and Edwards et al. (1996; W096/025504). The Teasdale patent covered a method for transforming *E. grandis* and *E. camaldulensis* via seedling-derived explants, but its efficiency was low (2.2%). The Edwards patent demonstrated the transformation of *E. globulus* and *E. nitens*. A European Patent by Kawazu et al. (2000a; 1050209 A2) described a procedure, based on a vertical rotary culture system, for inducing the formation of transgenic primordia. The process presented in the patent from Kawazu et al. (2000b) also used rotary-culturing to produce transgenic *Eucalyptus* plants.

Transgenic modification of cellulose and lignin content

Lignin, a complex phenolic polymer in the secondary cell walls of higher plants, must be separated from cellulose during pulp and paper production. However, this process is costly, energy-consuming, and polluting (Sierra-Alvarez and Lettinga 1991, Luisa et al. 1996, Zhang et al. 2002, Pokhrel and Viraraghavan 2004). Research has been conducted to investigate the constituents of fiber secondary cell walls, as well as to elucidate the pathways for cellulose, hemicellulose and lignin biosynthesis (Mansfield 2009, Vanholme et al. 2012). The goal has been to clone the genes that encode key enzymes involved in these pathways so that commercially important *Eucalyptus* species can be genetically modified for enhanced cellulose biosynthesis or reduced lignin formation (Chen et al. 2001, Kawaoka et al. 2003, 2006, Shani et al. 2003, Tournier et al. 2003, Valerio et al. 2003).

Modification of proteins containing cellulose-binding domains (CBD, e.g., in expansins) was found to affect the *in vitro* elongation of plant cells by affecting the interaction between xyloglucan and cellulose (Shpigel et al. 1998). Down-regulation of late-expressed genes involved in monolignol biosynthesis, e.g., cinnamyl alcohol dehydrogenase (CAD), has been

studied extensively as a means for modifying lignin composition and content (Boudet 2000, Tournier et al. 2003, Valerio et al. 2003). Genetic transformation of *E. camaldulensis* with CAD-antisense constructs led to significant down-regulation of CAD activity (Valerio et al. 2003). In three transgenic lines obtained using cDNA from *E. gunnii*, transcript levels had reduced CAD expression, with activity being diminished in one-third of the transformed shoots. However, 10-month-old transgenic plants showed no significant changes in lignin profiles (quantity, composition, or pulp yield), indicating that the CAD-antisense construct was not stably expressed or that lignification is regulated at earlier steps of the phenylpropanoid pathway. Similarly, Tournier et al. (2003) transferred CAD antisense cDNA from *E. gunnii*, under the control of the 35S CaMV promoter with a double enhancer sequence into *E. grandis* x *E. urophylla*. Approximately 10% of the starting explants produced kan-resistant, PCR-positive shoots and 58% of the 120 transgenic shoots produced had significantly inhibited CAD activity; nine of these shoots showed what the authors considered to be substantial down-regulation. However, only two transgenic lines, with 26% and 22% residual CAD activity, could be successfully transferred to the greenhouse and tested further.

Cinnamate-4-hydoxylase (C4H) is the second enzyme involved in the monolignol biosynthetic pathway. Its down-regulation is followed by a reduction in lignin content. Chen et al. (2001) transferred the C4H gene from *Populus tremuloides*, in both sense and antisense orientations, into *E. camaldulensis* through *A. tumefaciens* transformation. Transgenic plantlets were obtained and acclimatized to greenhouse conditions.

Other components of the lignin biosynthetic pathway can be manipulated by modifying secondary cell wall related transcription factors such as MYB and LIM proteins. Kawaoka et al. (2003) introduced antisense *Ntlim1* (tobacco transcription factor) into *E. camaldulensis* and obtained plantlets that had a 20% reduction in lignin in their stem xylem tissues. The same research group also succeeded in isolating the homologous gene of *Ntlim1* from *E. camaldulensis* named *Eclim1*. Kawaoka et al. (2006) showed that *Eclim1* is one of the key transcription factors involved in lignin biosynthesis. The antisense *Ntliml* construct was introduced into *E. camaldulensis*, and transgenic *Eucalyptus* plants were studied under greenhouse conditions. The plants showed decreased expression of several lignin biosynthesis genes, including *C4H*, phenylalanine ammonialyase (*PAL*), and 4-hydroxycinnamate CoA ligase (*4CL*). Line LG12, in which *Ecliml* transcripts were most suppressed, exhibited a change in leaf shape, rapid abscission of the upper leaves, and a 29% reduction in lignin content. The authors speculated that due to vessel malfunction this transgenic line lacked the capacity to transport water normally.

Resistance to insects and pathogens

Several insects and pathogens have been reported to cause defoliation in *Eucalyptus* plantations (Whyte et al. 2011). Severe defoliation of trees can cause tree mortality, reduce growth and wood production, and increase rotation time (Harcourt et al. 2000). The transformation of *E. camaldulensis* with the insecticidal *cry 3A* gene and herbicide resistance *bar* gene resulted in generation of two transgenic events expressing 0.0025% to 0.01% Cry 3A protein (% total soluble protein). Based on greenhouse tests, the lines were resistant to early instar *Chrysomelid variicolis, C. bimaculata* and *C. agricola*, major pests of Australian *eucalyptus* plantations (Harcourt et al. 2000). A sweet pepper gene, *hrap*, was used to transform *Eucalyptus urophylla* plants for pest resistance (Wang et al. 2007). To enhance disease resistance, Shao et al. (2002) transferred the cecropin D gene into *E. urophylla* to obtain transgenic plants that showed marked resistance to *Pseudomonas solanacearum*.

Transgenic drought tolerance

The *Arabidopsis SHINE/WAX INDUCER* (*SHN/WIN*) genes were shown to be involved in drought tolerance as well as wax/cutin biosynthesis (Aharoni et al. 2004, Kannangara et al. 2007). An attempt to overexpress one of the *AtSHN* genes in rice resulted in an increase in cellulose content by 34%, reduced lignin content by 45% and increase in wood digestibly, but not an increase in drought tolerance. Two orthologs of the *AtSHN1* gene were identified in *Eucalyptus*, namely *EgSHN1a* and *EgSHN1b* (Marques et al. 2011), which could be used in future to generate drought and pathogen resistant plants in addition to modifying cell wall properties.

Tsuchihira et al. (2010) transformed callus cells of *E. grandis* x *E. urophylla* with two *Raphanus sativus*-plasma membrane aquaporin (PIP) genes, *RsPIP1;1* and *RsPIP2;1*. They obtained transformants that were either PIP2-overexpressing or PIP1-suppressing. Although most members of the PIP2 subgroup exhibit water channel activity, drought tolerance was not enhanced in transgenic lines containing either gene. Only one line, expressing *RsPIP2;1*, had high photosynthetic activity and an improved growth rate under normal conditions. These results suggested that, whereas down-regulation of PIP2 causes serious damage, up-regulation of PIP2 can enhance photosynthesis activity and growth in *Eucalyptus* trees.

Transgenic cold/freeze tolerance

An increase in proline biosynthesis can lead to increased cold tolerance in *E. saligna*. Dibax et al. (2010) transformed cotyledons and leaf explants with the Δ^1-pyrroline-5-carboxylate synthetase gene (*P5C5*). As a result, the mean proline content in leaf tissues from transgenic plants was 13.39 µmol g^{-1} fresh mass compared with 3.42 µmol g^{-1} fresh mass from control plants. However, the protocol applied provided a transformation efficiency of only 0.5%.

Navarro et al. (2011) developed transgenic lines of *E. urophylla* x *E. grandis* that constitutively over-expressed two C-repeat binding factor genes (*Egu*CBF1a/b) isolated from *E. gunnii*. Although the lines showed improved cold tolerance, one of them exhibited alterations in several phenotypic characters such as reduced leaf area, higher oil gland density and a wax deposition on the cuticle. Although the knowledge gained from all of these experiments have advanced the transformation of *Eucalyptus*, the rates of formation of transgenic calli and regeneration of transgenic plants have been inadequate.

Zhang et al. (2007) demonstrated that CBF genes driven by novel dehydrin promoters isolated from *Eucalyptus dunnii* and *Eucalyptus macarthurii* enhance cold tolerance. Later, US patent number 20120266326 A1 by Zhang et al. (2011) presented a protocol for successful production of freeze tolerant *Eucalyptus* transgenic lines expressing the C-Repeat Binding Factor (CBF2) gene. *E. grandis* x *E. urophylla* field trials conducted with freeze tolerant *E. grandis* x *E. urophylla* (AGEH427) carrying *CBF2* in eight different locations across the Southeast US [including USDA Hardiness Zones 8a (potential kill zone), 8b (target freeze-stress zone)], and 9a (freeze stress-free zone) revealed significant freeze hardiness as compared to non transformed control plants (Hinchee et al. 2011).

Transgenic herbicide resistance

Transgenic *Eucalyptus pellita* and *E. grandis* x *E. dunnii* carrying the *bar* gene which encodes a phosphinothricin acetyl transferase was found to be strongly resistant to 200 gL^{-1} phosphinothricin at the level of 6.0 Lha^{-1} and 4.0 Lha^{-1} under greenhouse conditions (González et al. 2011). The patent by Chang et al. (2006) presented a protocol, claimed to be genotype-independent, for transforming *E. urophylla* using the acetolactate synthase (*ALS*) herbicide tolerance gene as a marker. Using RT-PCR, the authors tested eight transgenic plantlets of *E. grandis* and *E. urophylla* and confirmed that *ALS* was being expressed. The use of herbicide resistant trees could increase the productivity and reduce the cost of weed management (Llewellyn et al. 1999). In all of these reports, however, only low transformation efficiencies were achieved and only specific genotypes were successfully transformed. It would be highly valuable if the factors that limit transformation were understood, and thus more generalized methods developed.

Factors Influencing *Eucalyptus* Transformation Rates

Agrobacterium-mediated transformation is an effective and widely used approach to introduce foreign DNA into plants. Although several protocols are available for *in vitro* regeneration of complete plantlets of *Eucalyptus* species, they are all highly genotype-dependent. Various components must be optimized for each new genotype, and often for each new laboratory (Table 1, Appendix 4). These include the choice of *Agrobacterium* strains; explant type and physiology; acetosyringone concentration; methods for pre-conditioning; and the duration of co-cultivation. All of these factors interact with each other in determining the rate of gene transfer and ultimately the extent of integration and transgene expression.

Explant oxidation

The first step in development of a transformation system is usually to establish an efficient *in vitro* regeneration system (Figs. 1 and 2). This can be challenging in most eucalypt species and genotypes. Transformed shoots are produced primarily through adventitious organogenesis from transformed calli (Mullins et al. 1997, Ho et al. 1998, Harcourt et al. 2000, Chen et al. 2001, 2007b, Dibax et al. 2005, Quisen 2007). Explant establishment is often difficult as a result of the release of phenolic compounds into the medium, especially when incubated under high light intensity (e.g., Figs. 3 and 4). This can lead to necrosis and the death of explants (Laine and David 1994). Therefore, shoot regeneration is preferably induced under diffuse fluorescent light or in the dark to prevent oxidation at the wounding site (Kithahara and Caldas 1975, Lakshmi Sita and Vaidyanathan 1979, Laine and David 1994, Tournier et al. 2003). High cytokinin concentrations in the culture media can also contribute to explant oxidation (Barrueto-Cid et al. 1999). Antioxidants such as polyvinylpyrrolidone (PVP) (Lakshmi Sita and Rani 1985, Rao and Venkateswara 1985) and citric or ascorbic acids (Gerwal et al. 1980) may be added to the media to reduce the effect of phenolics (Dan 2008).

Agrobacterium strains

When *Agrobacterium* is used as part of a strategy for gene transfer, success depends upon the interaction between the bacterium and plant species or genotype. Adam (1989) was the first to study the induction of shooty tumors in *Eucalyptus* by different *A. tumefaciens* strains. Later, Machado et al. (1997) evaluated the susceptibility of *E. grandis* x *E. urophylla* hybrids to five *A. rhizogenes* and *A. tumefaciens* wild-type strains. These investigations revealed that the degree of virulence differed among strains, suggesting that eucalypts could be transformed if a genotype was carefully matched with

Table 1. Summary of transformation parameters in studies that reported successful transformation of *Eucalyptus species*.

Species	Explant source	Gene transferred	Marker gene	Strain of *A. tumefaciens*	Promoter	Pre-conditioning duration	Co-cultivation duration/ conditions	Bacterial cell density	Infection duration	Selection antibiotic	Disinfectant antibiotic	Reference
E. camaldulensis	Leaf	*uid A*	*npt II*	*Agrobacterium tumefaciens* A6, LBA 4404, GV31 11, AGL1, GV3850	CaMV 35S	-	2 d	109 cells per ml	-	9 mg L^{-1} kan	200 mg L^{-1} cefotaxime	Mullins et al. 1997
E. camaldulensis	Hypocotyl	*uid A*	*npt II*	CIB542 (derivative of EHA 101)	CaMV 35S	3 d	2 d	-	5 min	40 mg L^{-1}kan	500 mg L^{-1} cefotaxime+ 500 mg L^{-1} carbenicillin	Ho et al. 1998
E. globulus	Cotyledon and hypocotyl	*uid A*	*npt II*	LBA 4404, C58C1 and EHA 105	CaMV 35S	-	6 d	OD_{660} = 0.6	Overnight, 28°C, darkness, 100rpm	75 mg L^{-1} kan	500 mg L^{-1} cefotaxime	Moralejo et al. 1998
E. camaldulensis	Hypocotyl and cotyledon	*cry 3A*	*bar*	AGL1	CaMV 35S	-	2-3 d	-	-	10 mg L^{-1} PPT	200 mg L^{-1} cefotaxime	Harcourt et al. 2000
E. camaldulensis	Leaf from *in vitro* shoot culture	*Populus tremuloides C4H + gus*	*nptII*	CIB542 (derivative of EHA 101) containing pSC4H and pASC4H	CaMV 35S	-	2 d	-	5 min	40 mg L^{-1}kan	500 mg L^{-1} cefotaxime+ 500 mg L^{-1} carbenicillin	Chen et al. 2001
E. grandis x *E. urophylla*	Seedling	*uid A* *Lhcb1*2*	*npt II* *nptII*	LBA 4404	CaMV 35S	-	48 hr, 28°C	-	24 hr, 28°C, 100 rpm	10 mg L^{-1} genticin G-418	100 mg L^{-1} cefotaxime	Gonzalez et al. 2002
E. grandis x *E. urophylla*	Leaf from seedling-derived shoot culture	CAD antisense cDNA	*npt II*	AGL1	CaMV 35S	2 d	5 d	OD_{600} = 0.6	15s sonication + 5 min vacuum infiltration	50 mg L^{-1} kan	300 mg L^{-1} augmentin	Tournier et al. 2003

Table 1. contd....

Table 1. contd.

Species	Explant source	Gene transferred	Marker gene	Strain of *A. tumefaciens*	Promoter	Pre-conditioning duration	Co-cultivation duration/ conditions	Bacterial cell density	Infection duration	Selection antibiotic	Disinfectant antibiotic	Reference
E. camaldulensis	Young leaf of regenerated plantlet from individual seedling	CAD	*npt II*	EHA 105	CaMV 35S with double enhancer	-	4 d, diffuselight, 25°C	-	-	50 mg L^{-1}kan	250 mg L^{-1} claforan	Valerio et al. 2003
E. camaldulensis	Hypocotyl, cotyledon, mature leaf	*uid A*	*npt II*	CIB542 (derivative of EHA 101)	CaMV 35S	-	2 d, darkness, 25°C	OD_{600} = 1.0–1.5	10 min	40 mg L^{-1} kan	500 mg L^{-1}cefotaxime +500 mg L^{-1} carbenicillin	Chen et al. 2007b
E. occidentalis	Hypocotyl and cotyledon	*uid A*	*npt II*	AGL1	CaMV 35S	-	-	-	1 hr	10 mg L^{-1} genticin	100 mg L^{-1} timentin	Southerton 2007
E. tereticornis	Cotyledon and hypocotyl	*uid A*	*npt II*	LBA 4404	CaMV 35S	2 d	2 d	Bacterial cells in late log phase were used for infection	10 min	40 mg L^{-1} kan	300 mg L^{-1} cefotaxime	Prakash and Gurumurthi 2009
E. saligna	Leaf	*P5CSF129A + gus*	*npt II*	EHA105	CaMV 35S	-	5 d	OD_{600} = 0.5	30 min	50 mg L^{-1} kan	500 mg L^{-1} cefotaxime	Dibax et al. 2010
E. tereticornis	Leaf from shoot culture	*uidA*	*nptII*	EHA105 and LBA 4404 Maximum *gus* expression obtained with EHA105	CaMV 35S	2 d	2 d	OD_{600} = 0.8	0–30 min; after 10 min,	50 mg L^{-1} kan	500 mg L^{-1} cefotaxime	Aggarwal et al. 2011
E. grandis x *E. urophylla*	Leaf from micro-cutting	*EguCBF1a/b*	*npt II* and *sfp*	AGL1	CaMV 35S	2 d	5 d	OD_{600} = 0.6	15s sonication + 5 min vacuum infiltration	50 mg L^{-1} kan	300 mg L^{-1} augmentin	Navarro et al. 2011

Figure 3. Effect of light intensity on shoot regeneration rates from leaves of *E. grandis* x *E. urophylla* without *Agrobacterium* infection. A) Shoot formation after 40, 52, and 90 d in culture; B) Shoot regeneration under lowest light intensity (9–13 µmol photons m^{-2} s^{-1}).
Data and images from Strauss laboratory (Ma and Strauss 2011b, unpublished).

Figure 4. Effect of light intensity on shoot regeneration rates from leaves of *E. grandis* x *E. urophylla* co-cultivated with *Agrobacterium* AGL1 containing a p409S::GUS construct: A) Shoot regeneration after 40, 52, and 90 d of co-cultivation; lower light intensities (14–20 µmol photons m^{-2} s^{-1}) gave highest shoot regeneration; B) Shoot formation after 90 d of co-cultivation.
Data and images from Strauss laboratory (Adapted from Ma et al. 2011a).

the appropriate strain. The nopaline strains of *A. tumefaciens* produced large and vigorous tumors, indicating their potential as a vector for transforming *E. grandis* x *E. urophylla* hybrids with engineered T-DNA. For *E. camaldulensis*, five wild-type or disarmed strains of *A. tumefaciens* were tested: A6, LBA 4404, GV 3111, AGL1, and GV 3850 (Mullins et al. 1997). Rooted, transformed plants were obtained within six months when AGL1 and GV 3850 were used. Similarly, three *A. tumefaciens* strains—EHA 101, pEHA 101 (agropine); LBA 4404, pAL 4404 (octopine); and C58C1, pMP90 (nopaline)—were monitored for their capacity to infect *E. globulus* tissues (Moralejo et al. 1998). The supervirulent agropine strain yielded four-fold greater transient expression (2914.8 ± 712.8 pmol MU min^{-1} mg^{-1} proteins) than the octopine (631.2 ± 110.4) or nopaline (1690.8 ± 291.6) strains. High levels of virG expression lead to hypervirulence (Jin et al. 1987), and tree tissues generally responded

better to the nopaline strains than to the octopine (Riemenschneider 1990, de Bondt et al. 1994). Aggarwal et al. (2011) found EHA105 to be more efficient to transform *E. tereticornis* than LBA 4404.

Duration of pre-conditioning

Pre-conditioning duration has a marked effect on transformation frequency. During the pre-culture period, cells are prepared for division so that when the T-DNA is transferred during co-cultivation, the cells have already entered the regeneration phase (McHughen et al. 1989, Ainsley et al. 2001). A pre-conditioning duration of 0 to 5 d has commonly been used for *Eucalyptus* transformation (Machado et al. 1997, Ho et al. 1998, Moralejo et al. 1998, Tournier et al. 2003). In the case of *E. globulus*, GUS transient expression was higher after 4 to 6 d of pre-culture than after 8 d as compared to the control (no pre-culture) (Moralejo et al. 1998). However, 6 day preculture combined with wounding of explants and inoculation with *Agrobacterium tumefaciens* strain EHA105 lead to production of GUS expressing shoots with 1.2% transformation efficiency. For *E. grandis* hybrids, Machado et al. (1997) and Tournier et al. (2003) recommended 4 d and 2 d, respectively.

Duration of co-cultivation

The duration of co-cultivation has been shown to influence *Agrobacterium*-mediated genetic transformation in several plant species. Abundant evidence has shown that only a minority of cells that re-enter the division phase at wounding sites are competent for both stable transformation and shoot regeneration. For *Eucalyptus*, the period of co-cultivation used has commonly been 2 to 6 d (Mullins et al. 1997, Ho et al. 1998, Moralejo et al. 1998, Harcourt et al. 2000, Tournier et al. 2003, Valerio et al. 2003, Prakash and Gurumurthi 2009, Dibax et al. 2010, Navarro et al. 2011). Prolonged co-cultivation is generally recommended for tree species because it provides an opportunity for a larger number of induced bacteria to attach to the plant cells, making them more competent for *Agrobacterium* infection. However, this longer period can also lead to excessive bacterial growth, resulting in tissue necrosis and decreased transformation rates (Kutty et al. 2010). Therefore, one must balance these risks of prolonged co-cultivation against the potential for more successful transformation.

Using *E. camaldulensis*, Ho et al. (1998) infected hypocotyl explants with an *Agrobacterium* suspension for 5 min, followed by 2 d of co-cultivation, thereby achieving a putative transformation rate of 10%. Similarly, when leaf explants of *E. camaldulensis* were co-cultivated for 2 d to allow for T-DNA transfer and integration, efficiencies of 2.8 to 13.5% were obtained based on the number of confirmed transformed plants (Mullins et al. 1997, Chen et al. 2001). Cotyledon and hypocotyl explants excised from 50-day-old seedlings of the same species were co-cultivated with *Agrobacterium* for 2 to 3 d, resulting in two phosphinothricin (PPT)-resistant transgenic lines (Harcourt et al. 2000). To ensure stable transformation, other researchers have recommended co-cultivating for 4 d (Esmeraldo et al. 1997, Valerio et al. 2003) or 5 d in the cases of *E. grandis* and *E. urophylla* (Machado et al. 1997, Tournier et al. 2003) or *E. saligna* (Dibax et al. 2010). Finally, when seedling explants of *E. globulus* were co-cultured with *Agrobacterium* overnight in a liquid reactivation medium followed by 6 d on a solid medium, approximately 2.6% of the resultant plantlets exhibited kanamycin resistance (Moralejo et al. 1998).

Co-cultivation temperature

The temperature used for co-cultivation plays a critical role in T-DNA transfer (Dillen et al. 1997, Uranbey et al. 2005). The virulence (*vir*) region of *A. tumefaciens* is 30 kb long and contains six main operons necessary for T-DNA transfer to plant cells: *virA*, *virB*, *virC*, *virD*, *virG*, and *virE*. Alt-Morbe et al. (1989) demonstrated that high temperatures inactivate the *virD* operon, and that *virG* expression occurred only below 28°C. *Vir* genes were not expressed at temperatures greater than 32°C due to a conformational change in the folding of the virA protein (Jin et al. 1993). The optimal temperature at which plant cells are most receptive to bacterial infection is possibly related to cell division (Kudirka et al. 1986). Tumor development after *A. tumefaciens* infection had been most successful at approximately 22°C, and was not observed at temperatures above 29°C (Fullner and Nester 1996). When transforming *Eucalyptus*, co-cultivation temperatures should be maintained between 23°C and 28°C (Moralejo et al. 1998, Gonzalez et al. 2002, Valerio et al. 2003, Chen et al. 2007b). For successful integration of transgenes, *Agrobacterium*-infected explants from *E. globulus*, *E. grandis*, *E. urophylla*, and *E. camaldulensis* were co-cultivated either under darkness at 28°C (Moralejo et al. 1998) or 23°C (Tournier et al. 2003), or in diffuse light at 25°C (Valerio et al. 2003) or 26°C (Harcourt et al. 2000). Besides these experiments, we are not aware of any comprehensive studies of the relationship of co-cultivation temperature to transformation rate in *Eucalyptus*.

Co-cultivation media

Prior to transformation, *Agrobacterium* is typically grown overnight in an LB medium, then centrifuged and re-suspended in a liquid regeneration

medium. Wounded explants are infected with these bacterial suspensions for 5, 10, or 30 min (Ho et al. 1998, Chen et al. 2001, 2007b, Prakash and Gurumurthi 2009, Dibax et al. 2010, Aggarwal et al. 2011) after which the explants are blotted to remove excessive *Agrobacterium* followed by transfer to co-cultivation media. In early experiments, Moralejo et al. (1998) treated the explants under darkness overnight in a liquid medium containing *Agrobacterium* (OD_{660nm} = 0.6) with gentle shaking (100 rpm), followed by co-cultivation on a solid medium for 6 d. Co-cultivation in an auxin-rich medium was reported to increase the proportion of actively dividing cells in the S-phase significantly, the stage at which cells are more prone to the integration of foreign DNA (Sangwan et al. 1992). Conditioning with a plant growth regulator namely, BAP, 2,4-Dichlorophenoxyacetic acid (2,4 D), indole-3-acetic acid (IAA), 2-isopentenyl adenine (2 iP) has also improved transformation efficiencies in *Arabidopsis* (Sangwan et al. 1992) and pear (Negri et al. 2002). Proline, an osmotically active compound, can also be added to the bacterial suspension before explants are inoculated in order to promote transformation. For example, Tournier et al. (2003) reported success when 1 µM of proline was mixed into an *Agrobacterium* suspension prior to infecting leaf explants from *E. grandis* x *E. urophylla*. However, Rochange (1996) noted that supplementing the culture media with proline did not affect transformation frequency for *E. globulus*. Addition of surfactants to the co-cultivation media has been effective in enhancing transformation efficiency in several plant species (Wu et al. 2003). It is also effective in *Eucalyptus*, but must be carefully optimized as it also causes tissue necrosis (Ma and Strauss 2011b, unpublished data).

Phenolic compounds/wound response

By promoting release of phenolics and providing routes for bacterial ingress, wounding of explants is generally done prior to co-cultivation. Phenolic compounds, such as acetosyringone and alpha hydroxyl acetosyringone, are released at the wounding site and essential for the expression of *vir* genes (Miguel and Oliveira 1999). During the induction process, such genes are first activated in the presence of these phenolics (Winans et al. 1989). The autophosphorylation of *virA* leads to the phosphorylation of *virG*, which then induces the expression of other *vir* genes. To transform *E. grandis* x *E. urophylla*, Tournier et al. (2003) recommended that 50 µM acetosyringone be added to the solid pre-conditioning and co-cultivation media. Southerton (2007) transformed *E. occidentalis* by incorporating 50 µM acetosyringone into the bacterial suspension 2 hr prior to explant inoculation in order to activate the *vir* genes. For *E. grandis* and *E. grandis* x *E. urophylla* tissues, 100 µM acetosyringone was recommended (Gonzalez et al. 2002).

Supplementing the pre-conditioning medium with 100 µM acetosyringone significantly increased transient GUS expression (by 62.2%) in leaf explants of *E. tereticornis* (Aggarwal et al. 2011).

As a wounding procedure, bombardment with naked microprojectiles was evaluated by Moralejo et al. (1998) for *E. globulus*. Although transient expression was observed as a result of the biolistic treatment with a GUS expressing construct, no improvement of stable transformation was observed.

Plants treated with ultrasound manifest acoustic cavitations that generate microscopic wounds, thereby exposing their internal cell membranes to *Agrobacterium* better (Rivera et al. 2012). Generally, a bath sonicator (40 kHz) has been used. Gonzalez et al. (2002) used a sonication-assisted *Agrobacterium* transformation (SAAT) system to transform intact seeds from *E. grandis* x *E. urophylla*. Of the 500 seeds that were inoculated, only two callus pieces were recovered and 27 plantlets were obtained from the calli. Based on genomic DNA hybridization analysis, only four were found to be independent events. Another method used to facilitate T-DNA transfer is vacuum infiltration, which eliminates the air present in intercellular spaces and helps the bacteria penetrate into tissues. During inoculation, Tournier et al. (2003) subjected explants to 15 s of sonication and 5 min of infiltration under vacuum. They obtained an impressive integrative and regenerative transformation rate of approximately 10%.

Cell density of bacterial suspensions

Though not essential for transformation (Quoirin and Quisen 2006), *Agrobacterium* liquid cultures are generally centrifuged and the pellets re-suspended in a liquid regeneration medium prior to co-cultivation. To infect leaf explants from *E. camaldulensis*, Mullins et al. (1997) suspended *Agrobacterium* in a callusing medium, at 109 cells per milliliter. Moralejo et al. (1998) co-cultivated the wounded seedling organs of *E. globulus* overnight in a liquid reactivation medium with an *A. tumefaciens* suspension at OD_{660} = 0.6, while Chen et al. (2007b) used a suspension at OD_{600} =1.0 to 1.5 for only 10 min. Leaf explants of *E. camaldulensis* were immersed in a suspension at OD_{600} = 0.5 and exposed to 15 s sonication and 5 min infiltration under vacuum resulted in 10% transformation efficiency (Tournier et al. 2003); when those of *E. saligna* were immersed for 30 min at the same density, only a 0.5% transformation efficiency was achieved (Dibax et al. 2010). Because explants may become necrotic when treated with more highly concentrated bacteria (Bhaskar et al. 2009), a diluted suspension—generally in the range of 0.2 to 0.5 OD_{600} is usually employed.

Choice of genotype

The difficulty in transforming *Eucalyptus* genotypes prevents many elite clones from being employed in research, and requires extensive genotype screening as part of the development of transformation systems. The regenerability of a particular genotype and its susceptibility to *Agrobacterium* infection are important determinants of success. The best transformation performance has been achieved with *E. camaldulensis*, followed by *E. grandis* x *E. urophylla*. For example, Tibok et al. (1995) reported that gene transfer and integration in an *E. grandis* x *E. urophylla* hybrid greatly depended upon the genotype that was chosen. Of the 23 clones of *E. camaldulensis* tested for transformation, only 13 were validated as transformable and regenerable by Mullins et al. (1997). Five of them exhibited regeneration from more than 60% of the explants. Under selection pressure, however, only 3.6% produced putative transformants, and rooted plants were obtained from only one clone, CML43. In *E. globulus*, 66 initial seedlings produced only 15 kan-resistant shoots, with only seven (1.2%) having GUS activity (Moralejo et al. 1998). An average putative transformation rate of 10%, based on callus formation from co-cultivated explants, was achieved for *E. camaldulensis* (Ho et al. 1998). There, only two lines of putative transformed calli produced shoots, and shoot regeneration was accomplished after four to six months. Southern blot analysis confirmed that the two resultant lines, from separate callus clumps, were positive transformants.

Type of explant

Rather than using explants from mature plants (Mullins et al. 1997, Chen et al. 2001, 2007b, Dibax et al. 2010), most studies of *Eucalyptus* transformation have used seedling-derived explants (Azmi et al. 1997, Machado et al. 1997, Ho et al. 1998, Moralejo et al. 1998, Bandyopadhyay et al. 1999, Harcourt et al. 2000, Gonzalez et al. 2002, Tournier et al. 2003, Valerio et al. 2003, Chen et al. 2007b, Southerton 2007, Prakash and Gurumurthi 2009, Quisen et al. 2009). This is because juvenile tissue is more amenable to tissue culture manipulation than mature tissues. However, it may not be possible to obtain juvenile explants from established elite clonal genotypes, which are often only available as mature trees, or as rooted cuttings. When seedlings are used as the explant source, large numbers of transgenic genotypes are needed so that both conventional selection for growth properties, and the desirable traits that are conferred via transgene expression, can be obtained.

Selection genes and agents

The neomycin phosphotransferase (*npt II*) and bialaphos (*bar*) selection systems are commonly used for *Eucalyptus* transformation. The former has been particularly successful for generation of transgenic events having desired genes (Chen et al. 1996, 2001, 2007b, Mullins et al. 1997, Ho et al. 1998, Maralejo et al. 1998, Gonzalez et al. 2002, Tournier et al. 2003, Valerio et al. 2003, Prakash and Gurumurthi 2005a, 2009, Quisen 2007, Southerton 2007, Quisen et al. 2009, Dibax et al. 2010). Originating from *Escherichia coli*, *nptII* confers resistance to aminoglycoside antibiotics, such as kanamycin, neomycin, or geneticin. NPT-II catalyzes ATP-dependent phosphorylation of the 3′-hydroxyl group from the amino-hexose portion of those aminoglycosides that generate non-toxic molecules (Curtis et al. 1995, Seabra and Pais 1997, 1999, Balestrazzi et al. 2000). Theoretically, non-transformed cells would die in a medium containing these antibiotics, which strongly inhibit protein synthesis, and only transformed cells would survive (Curtis et al. 1995, Scorza et al. 1995, Mourgues et al. 1996, Bhatnagar and Khurana 2003).

Kanamycin has been used widely to select for *nptII* containing transformants of *Eucalyptus*. Concentrations of 9.0 to 50 mg L^{-1} have been tested with *E. camaldulensis* (Mullins et al. 1997, Ho et al. 1998, Chen et al. 2001, 2007b, Valerio et al. 2003). Although shoot regeneration in that species was inhibited when the concentration is above 9 mg L^{-1} (Mullins et al. 1997), as much as 75 mg L^{-1} was used to select *E. globulus* transformants (Moralejo et al. 1998). For *npt II*-transformed tissue of *E. grandis* x *E. urophylla*, the media have contained a range of concentrations and antibiotics, including 50 mg L^{-1} kan (Tournier et al. 2003), 200 mg L^{-1} kan (Sartoretto et al. 2002), or 10 mg L^{-1} geneticin G-418 (Gonzalez et al. 2002). Nevertheless, the low transformation efficiencies reported from those experiments might be attributed to the strong influence of these selection agents, which can severely inhibit shoot development (Gonzalez et al. 2002). In trials by Dibax et al. (2010), tissues of *E. saligna* were selected on a medium containing 50 mg L^{-1} kan, but only 0.5% of the infected explants regenerated putatively transformed shoot buds. Kanamycin can also be detoxified by developing calli, leading to large numbers of "escapes" (i.e., non-transgenic regenerants under antibiotic selection) compared to more potent selection agents (unpublished data). Geneticin G-418 has been shown to have a strong inhibitory effect on shoot regeneration (Gonzalez et al. 2002), thus usually producing fewer escapes than kanamycin does. Other selectable markers have been used less frequently, but successfully, in Eucalypts. In studies with *E. grandis* x *urophylla*, the optimum concentration of hygromycin (hyg) for callus formation and shoot induction was 5 mg L^{-1} whereas 2 mg L^{-1} hyg

was sufficient for root development (Figs. 5 and 6); this antibiotic was found to be much more effective than kanamycin at improving transformation frequency and reducing the incidence of non-transgenic escapes in *E. grandis* x *urophylla* hybrids (C. Ma and S. Strauss, unpubl. data).

The *bar* gene, derived from *Streptomyces hygroscopicus*, was used as a selectable marker for transforming *E. nitens*, *E. globulus* (Bandypopadhyay et al. 1999), and *E. camaldulensis* (Harcourt et al. 2000). This gene confers resistance to the broad-spectrum herbicide glufosinate ammonium or phosphinothricin (PPT). In tests by Harcourt et al. (2000), transformed cells were selected on a medium containing 10 mg L^{-1} PPT.

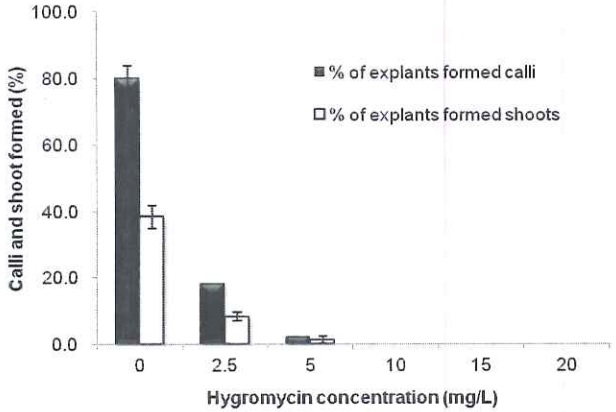

Figure 5. Hygromycin kill curve established in calli induction medium (CIM) and shoot induction medium (SIM) for *E. grandis* x *urophylla*: Calli formation (30 d in culture) and shoot regeneration (120 d) was from leaves. Data from Strauss laboratory (Adapted from Ma et al. 2011a).

Disinfectants

A disinfecting antibiotic is necessary for eliminating contaminating *Agrobacterium* overgrowth which in turn helps in achieving optimal transformation rates, but it must be chosen carefully to avoid inhibiting regeneration from the transformed tissues. The most commonly used antibiotics for killing *Agrobacterium* are cefotaxime and carbenicillin. Both are classified as β-lactam antibiotics, blocking biosynthesis of the peptidoglycan component of the bacterial cell wall through the action of penicillin-binding proteins (Asbel and Levison 2000). They are generally considered to have little or no plant toxicity because peptidoglycan is specific to prokaryotic cell walls. At cefotaxime concentrations of up to 200 mg L^{-1}, regeneration of transformed tissues from *E. camaldulensis* was unaffected (Mullins et al. 1997, Harcourt et al. 2000). Furthermore, transformed calli and shoots of that

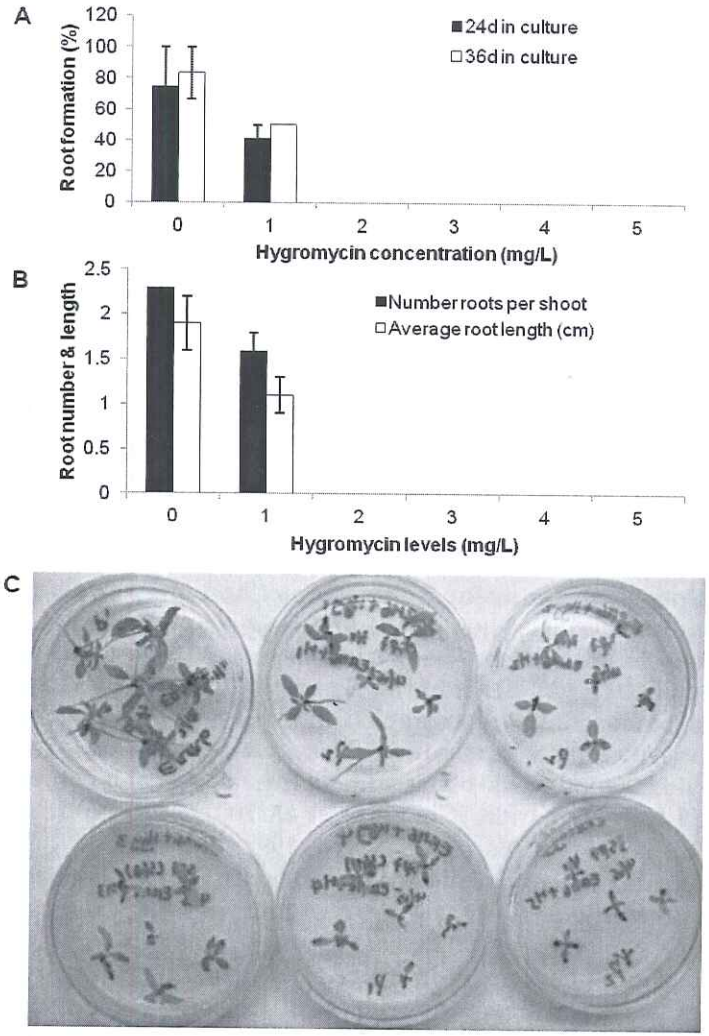

Figure 6. Hygromycin kill curve established in rooting for *E. grandis* x *urophylla* A) Percentage of shoots producing roots after 24 and 36 d in culture, B) Root numbers and lengths (cm) after 36 d in culture and C) Shoots rooted after 36 d in culture., Top (left to right): 0, 1, and 2 mg L^{-1} hygromycin; Bottom (left to right): 3, 4, and 5 mg L^{-1} hygromycin. A concentration of 2 mg L^{-1} hygromycin proved optimal for rooting. Data and images from Strauss laboratory (Ma and Strauss 2011b, unpublished).

species can be obtained on media containing 500 mg L^{-1} carbenicillin plus 500 mg L^{-1} cefotaxime (Ho et al. 1998, Chen et al. 2001, 2007b). Moralejo et al. (1998) used 500 mg L^{-1} cefotaxime to select for transformants of *E. globulus*. For *E. grandis* x *E. urophylla*, Gonzalez et al. (2002) treated explants with

a liquid medium containing 200 mg L^{-1} cefotaxime, and followed up with their transfer to a solid medium containing 100 mg L^{-1} cefotaxime. Finally, Southerton (2007) supplemented the shoot regeneration medium with 100 mg L^{-1} timentin to eliminate *Agrobacterium*.

Promoters

Promoters that initiate transcription in all parts of a plant are most commonly used for *Agrobacterium*-mediated genetic transformation. The constitutive promoter CaMV35S has generally been used with *Eucalyptus*. Nevertheless, depending upon the objective of the transformation effort, a variety of other promoters can be used, such as those that are tissue-specific or -preferred, cell type-specific, inducible, or repressible. For example, when the goal is modification of wood properties, one could choose promoters that will drive specific spatial/temporal expression or activate genes of interest that have the desired expression pattern in wood-forming tissues.

Potential Means for Overcoming Challenges in Genetic Transformation of *Eucalyptus* Species

A major obstacle to the creation of transgenic eucalypt plants has been the low rate of shoot regeneration, which consequently reduces transformation efficiency. Attempts to increase this efficiency have included wounding plant tissue via microprojectile bombardment (Moralejo et al. 1998), sonication-assisted transformation (Gonzalez et al. 2002), and modifications to culturing parameters (Barrueto-Cid et al. 1999). Nevertheless, reproducible transformation events mediated by *Agrobacterium* for *Eucalyptus* species remain sporadic and efficiencies must still be significantly improved. Although recent work on *Eucalyptus* transformation using a range of important genes has been promising (Dibax et al. 2010, Navarro et al. 2011), technical aspects still require more attention. Some of the major hurdles are discussed below.

Poor shoot regeneration

Among the commercially important species and clonal genotypes of *Eucalyptus*, we must identify those most amenable to regeneration and transformation. Adjusting our protocols for culturing media and growing conditions will also be helpful. For example, as discussed above, incubating cultures under low levels of light (9–20 µmolphotons m^{-2} s^{-1}) can result in

enhanced shoot formation when compared with cultures that are maintained under a higher intensity (27–40 µmolphotons m^{-2} s^{-1}, Figs. 3 and 4). Oxidation and browning of explants can also be reduced by adding antioxidants to the media for callus induction and shoot regeneration. Herve et al. (2001) described a two-step regeneration procedure to reduce oxidation of explants where the explants were maintained on MS medium supplemented with 0.04 µM picloram and 1 µM BA for 1 week followed by an increase in BA concentration to 2.25 µM BA. This resulted in regeneration of shoot buds from 53.5% of the nodal explants within eight weeks of culture.

Addition of 6-benzylamino purine (BAP: Gloke et al. 2006) alone or in combination with auxins namely NAA (Dibax et al. 2005) improves shoot regeneration in *Eucalyptus* species. Other researchers were successful in generating organogenic callus using BAP in combination with IAA (Muralidharan and Mascarenhas 1987, Warrag et al. 1991, Laine and David 1994, Tibok et al. 1995, Azmi et al. 1997, Mullins et al. 1997, Bandyopadhyay et al. 1999, Barrueto Cid et al. 1999). Cotyledon explants treated in this way showed 54% shoot regeneration (Dibax et al. 2005). The highest shoot formation (98%) was observed on cotyledon node-derived calli as compared to hypocotyl and cotyledon explants on SP medium supplemented with Zeatin and NAA (Barrueto Cid et al. 1999). Earlier reports demonstrated successful shoot proliferation in *Eucalyptus* species on TDZ containing media (Tibok et al. 1995). These protocols could be tested for genetic transformation of *Eucalyptus* species to improve shoot regeneration from transformed tissue.

Transgene stability

Although there have been many reports of highly stable expression over years in eucalypts (e.g., male-sterility: Zhang et al. 2012), instability has also been observed in some cases. Once the transgenic plants are established in the greenhouse and then transferred to field conditions, transgene expression may change. During their life cycles, trees adapt to seasonal climatic changes as well as to biotic stresses. The most common promoter used for *Eucalyptus* transformation is CaMV35S, which has been found to be silenced in a variety of plant species (Kooter et al. 1999, Hull et al. 2000, Potenza et al. 2004). Eucalypt promoters, due to their homology to endogenous genes, might even be more prone to silencing. Because the rate of gene silencing varies widely among genes, promoters, genotypes, and environmental conditions without obvious predictors a case by case empirical evaluation will be necessary for the foreseeable future.

Conclusions and Suggestions for Future Research

Genetic engineering of *Eucalyptus* has been accomplished in a variety of laboratories, and a number of traits of value to science and management have been demonstrated. A number of field studies have demonstrated the production of healthy trees with stable trait expression. Despite these successes, transformation remains costly, difficult, or impossible for most genotypes, achievable by only the largest and most experienced research laboratories. Many of these problems are inherent to the biology of *Eucalyptus* and not easily solved with the empirical, trial and error methods employed to date. Advances in developmental biology have led to the discovery of many genes that directly affect shoot regeneration; however, to our knowledge none have been tested in eucalypts. A more developmental science approach may lead to larger and more general advances than have been possible to date, but will require a large, concerted effort as significant advancements are unlikely to come easily. A major reason for the slow progress in transformation may also be the very limited sharing of information among companies and privately funded scientists. Much more open sharing of detailed protocols and genotypes would stimulate transgenic research in eucalypts, which by attracting high caliber scientific activity should lead to further innovations in transformation approaches for these important tree species.

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APPENDIX 1

Summary of work on organogenesis in *Eucalyptus* species.

Species	Explant source	Callus induction medium	Shoot regeneration medium	Rooting medium	Outcome	Reference
E. grandis	Leaf from *in vitro* shoot culture	-	-	-	-	Laine and David 1994
E. urophylla	Hypocotyl	MS + 20.0 g L^{-1} sucrose, 2.0 g L^{-1} phytagel, BAP (0.1–1.0 mg L^{-1}), and NAA (0–0.5 mg L^{-1}) or NAA (0–2.0 mg L^{-1}) + TDZ (0.01–2.0 mg L^{-1})	MS + 20 g L^{-1} sucrose, 2.0 g L^{-1} phytagel, 0.2 mg L^{-1} NAA, and 0.2 mg L^{-1} BAP MS + 20.0 g L^{-1} sucrose, 2.0 g L^{-1} phytagel, and 1.0 mg L^{-1} zeatin	MS + 1.0 mg L^{-1} NAA and 0.01 mg L^{-1} BAP	Maximum 37% of explants regenerating shoots on MS + 0.2 mg L^{-1} NAA and 0.2 mg L^{-1} BAP However, 27% more shoots per explant were obtained from MS + 1.0 mg L^{-1} zeatin	Tibok et al. 1995
E. grandis x *E. urophylla*	Leaf from micropropagated plantlet	MS + 1.0 mg L^{-1} kinetin and 10.0 mg L^{-1} IBA	-	-	-	Chen et al. 1996
E. globulus	Hypocotyl	-	MS medium + NAA (0.01 or 1.0 mg L^{-1}) and 0.2 mg L^{-1} BA or 0.2 mg L^{-1} BA and 0.2 mg L^{-1} TDZ	Incubation of shoots in MS medium containing 3mg L^{-1} IBA in dark, followed by transfer to MS basal medium	About 70% of regenerating hypocotyls showed induction of 5 to 20 buds	Azmi et al. 1997
E. camaldulensis	Leaf	WPM + 1.0 g L^{-1} casein, 50.0 g L^{-1} sucrose, 3.0 mg L^{-1} NAA, 0.1 mg L^{-1} BA, and 0.5% Phytagar	Same as callus induction medium, with 0.3 mg L^{-1} BA but without NAA or casein	WPM	Rooted plants	Mullins et al. 1997
E. camaldulensis	Hypocotyl	B_5 + 3% sucrose, 100 ml L^{-1} coconut milk, 200 mg L^{-1} glutamine, 100 mg L^{-1} casein hydrolysate, + , 1 mg L^{-1} BA, 3 mg L^{-1} NAA	Same as callus induction medium	MS medium (with full-strength micronutrients and vitamins, ½-strength macronutrients) + 1 mg L^{-1} IBA	Rooted plants	Ho et al. 1998
E. globulus	Cotyledon, hypocotyl	WPM + 3% sucrose, 0.8% Bacto agar, 0.6 mg L^{-1} BA, 0.2 mg L^{-1} NAA, and 100.0 mg L^{-1} arginine	-	-	-	Moralejo et al. 1998
E. nitens *E. globulus*	Cotyledon, hypocotyl	MS + 30.0 g L^{-1} sucrose, 5–10% coconut water, 1.0 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, and 0.8% agar	MS + 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP	MS + 20 mg L^{-1} sucrose, 0.7% agar, and 3 mg L^{-1} IBA	Rooting from 30–40% of shoots; successful transfer to soil	Bandyopadhyay et al. 1999
E. grandis x *E. urophylla*	Cotyledon, hypocotyl	Modified MS + 2.0 µM TDZ	Modified MS + BAP (2.5 to 10 µM) + 0.5 µM NAA or zeatin (2.5 to 10 µM) and 0.5 µM NAA Shoot elongation: Modified MS + 1.0 µM BAP, 0.5 µM NAA, and 2.0 µM GA_3	Modified MS + 20 mg L^{-1} sucrose and 2.5µM IBA, incubated for 5 d Modified MS without growth regulators; for 30 d	Medium containing zeatin showed maximum (55%) shoot regeneration Rooted plantlets transferred to greenhouse	Barrueto-Cid et al. 1999

APPENDIX 1. contd....

APPENDIX 1. contd.

Species	Explant source	Callus induction medium	Shoot regeneration medium	Rooting medium	Outcome	Reference
E. camaldulensis	Hypocotyl, cotyledon	Liquid B_5 + 0.2 mg L^{-1} 4-CPPU, 0.02 mg L^{-1} NAA, 10 mg L^{-1} PPT, and 3% sucrose	B_5 + 0.02 mg L^{-1} NAA, 0.2 mg L^{-1} BAP, 1% sucrose, and 0.6% Difco Bacto agar	¼-strength B5 medium + 0.01 NAA, 1% sucrose, and 0.6% agar	Well-established plants in greenhouse	Harcourt et al. 2000
E. camaldulensis	Immature flower, stamen	Seven medias tried: MS + BAP (0.01, 0.1, 0.5 mg L^{-1}), or 0.01 mg L^{-1} 2,4-D, or 0.01 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA, or 0.05 mg L^{-1} 2,4-D and 0.01 mg L^{-1} BA, or 0.05 mg L^{-1} 2,4-D and 0.1 mg L^{-1} BA	-	-	Good callus induction on medium containing combinations of BA and 2,4-D. Callus cultures were used for identification of volatiles.	Giamakis et al. 2001
E. gunnii	Leaf, internode, and node from micropropagated shoot culture	-	MS salts + 3% sucrose, 100 mg L^{-1} myoinositol, 2mg L^{-1} glycine, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} thiamine HCl, 0.25% gelrite, 0.04 µM picloram, and 1 µM BA; incubation for 1 wk Same media but with 2.251 µM BA	-	Nodes showed maximum shoot regeneration, followed by leaf explants. Histological studies revealed that shoots originated from peripheral layers of leaf- and node-derived protuberances	Herve et al. 2001
E. globulus	Cotyledon and hypocotyl from mature embryo and seedling	-	MS + 3% sucrose and 0.2 % phytagel containing various combinations of TDZ, BA, NAA, IBA, 2,4-D, IAA, or dicamba	-	High frequency of adventitious bud formation on media containing either 0.05 µM TDZ+ 0.2 µM 2,4-D or 5µM NAA	Nugent et al. 2001
E. grandis x *E. urophylla*	Seedling	BM [½-strength MS macronutrients, full-strength MS micronutrients, MS B vitamins (Barrueto-Cid et al. 1994), 3% sucrose, and 0.6% Agar] + 2.0 µM TDZ and 2.5 µM NAA	Multiplication medium: BM + 0.006 µM BAP and 0.88 µM NAA Elongation medium: BM + 0.44 µM BAP and 1.0 µM IBA	BM + 4.9 µM IBA	Well-established plants in greenhouse	Gonzalez et al. 2002
E. globulus	Hypocotyl, cotyledon, leaf from *in vitro* clonal plantlet	B_5 + 10% coconut water, BAP (0.05–0.5 mg L^{-1}), and 0.5 mg L^{-1} IBA	B_5 + 0.5 mg L^{-1} BAP	B5 + 0.5 mg L^{-1} IBA and 30 g L^{-1} sucrose	Nodule culture Hypocotyl explants were more responsive	Trindade and Pais 2003
E. phylacis	Shoot culture	MS basal medium + 5.0 µM TDZ	MS + 0.5 µM zeatin and 1.0 µM GA_3 Completed on MS + 1.0 µM zeatin and 0.1 µM BA; in vented culture tubes Shoot elongation: MS medium + 0.5 µM zeatin and 0.2 µM BA	MS + 5.0 µM IBA	Nodular green callus was obtained; low light intensity (PPFD 5–10 µmol $m^{-2}s^{-1}$) was used to minimize blackening of explants	Bunn et al. 2005

APPENDIX 1. contd....

APPENDIX 1. contd.

Species	Explant source	Callus induction medium	Shoot regeneration medium	Rooting medium	Outcome	Reference
			Followed by transfer to MS + 0.5 µM zeatin and 1.0 µM GA_3 (for elongation) or MS + 1.0 µM zeatin + 0.5 µM IAA (for shoot growth)			
E. camaldulensis	Cotyledon	MS + 5.4 µM NAA and 4.44 µM BAP or 2.7 µM NAA and 13.32 µM BAP	MS + 2.7 or 5.4 µM NAA and 4.44 µM BAP	MS (with ½-strength nitrate and ammonium salts) + 0.2% activated charcoal	Rooted plants	Dibax et al. 2005
E. erythronema *E. stricklandii* Hybrid cv. Urrbrae Gem Hybrid 2.5	Apex and leaf explant from seedling and *in vitro* shoot	MS + 30.0 g L^{-1} sucrose, 7.0 g L^{-1} phytagel, and BAP (0–1.0 µM)	Same as callus induction medium	-	Callus formed on all media combinations; shoot buds were obtained only on medium containing 1.0 µM BAP	Glocke et al. 2006
E. camaldulensis	Hypocotyl, cotyledon, mature leaf	B_5 + 3% sucrose, 100 ml L^{-1} coconut water, 100 mg L^{-1} glutamine, 100 mg L^{-1} casein hydrolysate, 1 mg L^{-1} BA, and 3 mg L^{-1} NAA (for seedling explant) or TDZ (0.5 to 1.5 mg L^{-1}) + 3 mg L^{-1} NAA (for mature leaf)	Same as callus induction medium Shoot proliferation: MS + 0.1 mg. L^{-1} BA	Full-strength MS micronutrients and vitamins and ½-strength MS macronutrients + 1 mg L^{-1} IBA	Rooted plants	Chen et al. 2007b
E. grandis *E. grandis x E. urophylla*	Piece of *in vitro* shoot with axillary buds removed	MS + 30.0 g L^{-1} sucrose, 4.0 g L^{-1} gelrite, 5.0 mg L^{-1} IAA, and 0.25 mg L^{-1} BAP	Same as callus induction medium	¼-MS + 15 g L^{-1} sucrose, 0.1 mg L^{-1} biotin, 0.1 mg L^{-1} calcium pantothenate, 0.5 mg L^{-1} IBA, and 4 g L^{-1} gelrite	90% of plantlets were acclimatized under greenhouse conditions	Hajari et al. 2006
E. tereticornis	Cotyledon, hypocotyl	-	MS + 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA Shoot elongation : Same as regeneration medium but without NAA and solidifying agent	MS + 1.0 mg L^{-1} IBA	Rooted plantlets	Prakash and Gurumurthi 2009
E. microtheca F. Muell	Leaf disc, leaf stalk, green twig from 1-yr-old greenhouse-grown seedling	-	Modified MS medium with half-concentrations of KNO_3 and NH_4NO_3 + 1.0 mg L^{-1} NAA, 1.0 mg L^{-1} kinetin, and 0.01 mg L^{-1} TDZ	-	88.09% of explants showed direct shoot regeneration	Mamaghani et al. 2009
E. camaldulensis	Cotyledon	MS N/2 medium (MS medium with half-total nitrogen) + 4.4 µM BAP, 2.7 µM ANA, 2% sucrose, 0.6% agar, and 10% coconut water	Same as callus induction medium	-	-	Quisen et al. 2009

APPENDIX 1. contd....

APPENDIX 1. contd.

Species	Explant source	Callus induction medium	Shoot regeneration medium	Rooting medium	Outcome	Reference
E. tereticornis	Leaf from *in vitro* shoot culture	-	MS + 5 µM BA, 1.0 µM 2,4-D, and 500 mg L^{-1} cefotaxime Shoot elongation: MS + 0.1 µM BA and 0.5 µM NAA	-	44.6% shoot bud organogenesis was achieved	Aggarwal et al. 2010
E. saligna	Leaf	MS medium with half-concentrations of potassium and ammonium nitrates + 0.1 µM NAA and 1.0 µM TDZ	MS medium with half-concentrations of KNO_3 and NH_4NO_3 + 1.45 µM GA_3 Multiplication medium: MS medium with half-concentrations of KNO_3 and NH_4NO_3 + full-strength vitamins and 1.11 µM BAP	½-strength MS medium with full-strength vitamins	Rooted plants	Dibax et al. 2010
E. urophylla	Hypocotyl	Modified MS + 6.6 µM PBU and 0.57 µM IAA	Modified MS + 0.44 µM BA, 0.54 µM NAA, and 0.3 µM GA_3	MS + 2.5 µM IBA	Plantlets successfully established in greenhouse	Huang et al. 2010

APPENDIX 2

Summary of work on somatic embryogenesis in *Eucalyptus* species.

Species	Explant source	Embryogenic callus induction medium	Germination medium	Outcome	Reference
several	*In vitro* seedling	-	-	Complete plant formation	Ouyang et al. 1980, 1981
E. gunnii	Seedling leaf Hypocotyl, cotyledon	Modified MS medium + various growth regulators Modified MS medium/Modified Norstog and Rhamstine (1967) + 1.19 mg L^{-1} NAA and BA (0.11–1.1 mg L^{-1})	-	Embryogenic calli	Boulay 1987
E. camaldulensis	Embryo	B5 medium + 3.0 mg L^{-1} NAA (under darkness) Modified Sussex (1965) salts + MS vitamins, 0.5 mg L^{-1} BA and 10% coconut water (under lights)	½-strength MS medium without growth regulators	Complete plant formation	Muralidharan and Mascarenhas 1987
E. grandis	Shoot from *in vitro* culture	MS + 5.0 mg L^{-1} 2,4-D MS + 5.0 mg L^{-1} kinetin and 0.1 mg L^{-1} NAA MS + 1.0 mg L^{-1} NAA, 1.0 mg L^{-1} kinetin, and 1 mg L^{-1} BA	-	Embryogenic calli	Lakshmi Sita et al. 1986
E. gunnii	Hypocotyl Leaf	MS + 3.7 mg L^{-1} NAA and 1.1 mg L^{-1} BA MS + 9.3 mg L^{-1} NAA and 1.1 mg L^{-1} BA	-	-	Franclet and Boulay 1989
E. citriodora	Cotyledon of germinated seed	B5 + 3.0 mg L^{-1} NAA (under darkness)	B5 medium without growth regulators (under lights)	-	Muralidharan et al. 1989

APPENDIX 2. contd....

APPENDIX 2. contd.

Species	Explant source	Embryogenic callus induction medium	Germination medium	Outcome	Reference
E. botroides *E. dunnii* *E. rudis*	Hypocotyl, cotyledon, seedling leaf	Modified MS + 1.1 mg L^{-1} 2,4-D	Modified MS + 1.1 mg L^{-1} BA	Formation of embryo-like structure	Qin and Kirby 1990
E. grandis	Leaf from *in vitro* shoot culture	MS + 0.5 mg L^{-1} 2,4-D	MS + 0.05 mg L^{-1} 2,4-D, 0.01 mg L^{-1} NAA, 0.1 mg L^{-1} BA, 0.1 mg L^{-1} GA_3, and activated charcoal	-	Watt et al. 1991
E. dunnii	3-day-old seedling	MS+ 5.5 or 16.5 µM NAA alone or in combination with 4.5 µM 2,4-D and either 10% coconut water or 1.0 g L^{-1} hydrolyzed casein for embryo development	-	Somatic embryos	Termignoni et al. 1996
E. nitens	Hypocotyl, cotyledon, leaf, epicotyl from seedling	Explants kept in liquid medium with high auxin concentration for 3 d Medium containing 0-0.5 µM of both á-NAA and BAP	-	Hypocotyl gave maximum number of somatic embryos per explant Globular somatic embryos on medium containing 0.5 µM BAP and 0.5 µM NAA after 9 wk of culture	Ruaud et al. 1997
E. nitens *E. globulus*	Cotyledon, hypocotyl	MS + 0.5 mg L^{-1} NAA and 1.0 mg L^{-1} BAP	-	Embryogenic calli	Bandyopadhyay et al. 1999
E. tereticornis	Mature zygotic embryo	Callusing: MS + 2.0 mg L^{-1} NAA Embryogenesis :MS + 0.5 mg L^{-1} BA	Plantlet formation: MS+ 50 mg L^{-1} kan and 500 mg L^{-1} cefotaxime Shoot elongation: Liquid MS + 50 mg L^{-1} kan and 500 mg L^{-1} cefotaxime	About 80% of somatic embryos germinated to form complete plantlet within 2–3 wk, which were then acclimatized in soil with 91.1% survival rate	Prakash and Gurumurthi et al. 2005b
E. microtheca f. Muell.	Leaf disc, leaf stalk, green twig from 1-yr-old greenhouse-grown seedling	Modified MS medium with ½-strength KNO_3 and NH_4NO_3 + 4.0 mg L^{-1} NAA, 0.5 mg L^{-1} kinetin, and 1.0 mg L^{-1} TDZ	-	Embryogenic calli obtained from twigs	Mamaghani et al. 2009
E. camaldulensis	Zygotic embryo; cotyledon from 10-day-old seedling Hypocotyl	Callus induction: MS + 1 mg L^{-1} NAA Somatic embryogenesis: MS + 0.5 BA and 0.1 mg L^{-1} NAA Direct somatic embryogenesis: MS + 0.5 mg L^{-1} BA	MS + 0–5 mg L^{-1} ABA or 4% sucrose Dilutions of MS medium were also tried for embryo maturation	Number of mature somatic embryos increased when lower ABA concentrations (0–1 mg L^{-1}) and higher sucrose concentration (4%) were used with diluted MS medium	Prakash and Gurumurthi et al. 2009

APPENDIX 3

Direct DNA transfer in *Eucalyptus* species.

Species	Method	Explant/ tissue	Gene(s) transferred	Outcome	Reference
E. saligna	Electroporation	-	-	-	Kawazu et al. 1990
E. gunnii	Electroporation, chemical treatment	Protoplast	-	-	Teulieres et al. 1990
E. gunnii	Polyethylene glycol (PEG)/ Electropulsation	Protoplast derived from callus/cell suspension	*chloramphenicol acetyl transferase + gus*	PEG-mediated DNA uptake was highly stimulated by heat-shock pre-treatment, and was more efficient than electrical treatment. A promoter corresponding to a protein synthesis elongation factor gene was more effective than the classic 35 S CaMV promoter	Teulieres et al. 1991
E. citriodora Hook	Electroporation	Protoplast	*chloramphenicol acetyl transferase + gus*	Gene expression was enhanced by the addition of 40% (w/v) PEG, in the presence of the carrier DNA, to the protoplasts after electroporation.	Manders et al. 1992
E. globulus	Biolistic gun	Zygotic embryo	*gus*	130 GUS expression events per embryo, with good distribution over the tissue	Rochange et al. 1995
E. globulus	Biolistic gun	Zygotic embryo	*gus + npt II*	Stably transformed calli confirmed by Southern blot analysis	Serrano et al. 1996
E. grandis x *E. urophylla*	Biolistic gun	Cotyledon, hypocotyl	*gus + npt II*	Kan-resistant calli were obtained: 6.8% GUS-positive based on total number of bombarded explants (cotyledon and hypocotyl)	Sertoretto et al. 2002

APPENDIX 4

Summary of published studies of *Eucalyptus* transformation.

N.B., transformation efficiency was calculated as the percentage of kan-resistant shoots obtained or PCR-positive plantlets generated.

Species	Explant source	Gene(s) transferred	Marker gene	*Agrobacterium* strain	Regeneration pathway	Final stage	Outcome	Reference
E. gunnii	-	-	-	*A. rhizogenes*	-	-	-	Adam 1987
Several *Eucalyptus* sp.	Hypocotyl	-	-	*A. tumefaciens*	-	-	-	Brackpool and Ward 1990
E. globulus subsp. *globulus*	Seedling, embryo, shoot	-	-	*A. tumefaciens*	-	-	-	Young and Chandler 1990
E. grandis *E. dunnii* *E. nitens*	Seedling (roots removed)	-	-	*A. rhizogenes* LBA9402; R1601; TR8,3	-	-	-	MacRae and van Staden 1993
E. grandis x *E. urophylla*	Leaf from micropropagated plantlet	*uid A*	*npt II*	*A. tumefaciens* C58SZ707, CIB542	Indirect	Calli	-	Chen et al. 1996
E. grandis x *E. urophylla*	-	-	-	wild-type *A. tumefaciens*	-	Shooty tumors	-	Andrade and Brasileiro 1997
E. globulus	Decapitated seedling hypocotyl and stem cuttings from micropropagated plantlet	*uid A*	-	*A. tumefaciens* 82.139, C58	-	Shooty tumors	Inoculation with strain 82.139 overcame the inability of mature organs to regenerate buds, thus solving the problem of recalcitrance	Azmi et al. 1997

APPENDIX 4. contd....

APPENDIX 4. contd.

Species	Explant source	Gene(s) transferred	Marker gene	*Agrobacterium* strain	Regeneration pathway	Final stage	Outcome	Reference
E. grandis x *E. urophylla*	Hypocotyl, cotyledon	-	-	-	-	-	-	Esmeraldo et al. 1997
E. grandis x *E. urophylla*	Seedling shoot	-	-	*A. rhizogenes* A4, R1601,2659, 8196, LBA9402 *A. tumefaciens* T37, 82139, 87170, C58, A208, Ach5, 15955, A66, B653, R10, B0542, A281	-	Hairy roots and tumor formation	Different strains showed various degrees of virulence. Majority of shoots (99%) derived from 82139, T37, C58, and B653 developed normal phenotypes and formed roots.	Machado et al. 1997
E. camaldulensis	Leaf	*uid A*	*npt II*	*A. tumefaciens* A6, LBA 4404, GV31 11, AGL1, GV3850	Indirect	Transgenic plantlet	Over half of the plants (17 independent lines) were confirmed as transformed based on either blue-straining of the leaves and roots or NPT II enzyme activity in leaves. Southern blot confirmed integration of the transgene in 4 analyzed lines.	Mullins et al. 1997
E. camaldulensis	Hypocotyl	*uid A*	*npt II*	*A. tumefaciens* CIB542 (derivative of EHA 101)	Indirect	Transgenic plantlet	Gene expression and transgene integration were confirmed by GUS-staining and Southern hybridization in two putative transgenic plants.	Ho et al. 1998
E. globulus	Cotyledon, hypocotyl	*uid A*	*npt II*	*A. tumefaciens* LBA 4404, C58C1, EHA 105	Shoots from co-cultivated seedling explants	Transgenic plantlet	-	Moralejo et al. 1998
E. nitens *E. globulus*	Cotyledon, hypocotyl	*uid A*	*bar*	*A. rhizogenes* LBA 9402 (hypocotyl) *A. tumefaciens* AGL1 (cotyledon)	Seedling explants were co-cultivated with *Agrobacterium* suspension	Blue-staining of both cotyledon and hypocotyl explants	-	Bandyopadhyay et al. 1999
E. camaldulensis	Leaf from *in vitro* shoot culture	*Populus tremuloides C4H + gus*	*nptII*	*A. tumefaciens* CIB542 (derivative of EHA 101) containing *pSC4H* and *pASC4H*,	Indirect	Transgenic plant	Frequency of producing transgenic lines was 8.1% with C542 (*pSC4H*) and 13.5% with C542 (*pASC4H*), based on the number of transformed shoots obtained per number of leaves co-cultivated. Transgene integration was confirmed in 4 lines via Southern hybridization.	Chen et al. 2001
E. camaldulensis	Hypocotyl, cotyledon	*cry 3A*	*bar*	*A. tumefaciens* AGL1	Indirect	Transgenic plant	Two lines of transformed plants were obtained out of 5 PPT-resistant calli lines. They expressed 0.0025% to 0.01% of total soluble protein, which provided resistance to attack by early instars of the chrysomelid beetle.	Harcourt et al. 2000

APPENDIX 4. contd....

APPENDIX 4. contd.

Species	Explant source	Gene(s) transferred	Marker gene	*Agrobacterium* strain	Regeneration pathway	Final stage	Outcome	Reference
E. grandis x *E. urophylla*	Seedling	*uid A* *Lhcb1*2*	*npt II* *nptII*	*A. tumefaciens* LBA 4404	Seedlings were germinated and sonicated before *Agrobacterium* infection Indirect regeneration using cotyledon from sonicated and co-cultivated seedling after 15 d	Transient GUS expression using seedlings Transgenic plant	Approximately 17.6% of explants showed blue-staining. In all, 27 plantlets were obtained from 500 seeds. Genomic DNA hybridizations revealed the presence of 4 independent transformation events from 1 callus.	Gonzalez et al. 2002
E. urophylla	Leaf	Cecropind gene		*A. tumefaciens*	-	-	-	Shao et al. 2002
E. grandis x *E. urophylla*	Leaf from seedling-derived shoot culture	CAD antisense cDNA	*npt II*	*A. tumefaciens* AGL1	Direct	Transgenic plant	About 120 positive lines were selected, CAD activity was down-regulated in 58% of the PCR-positive transformants. Southern blot analysis showed low transgenic copy numbers (1–4).	Tournier et al. 2003
E. camaldulensis	Young leaf of regenerated plantlet from individual seedling	CAD	*npt II*	*A. tumefaciens* EHA 105	Direct	Shoot clusters from explants	Transformation efficiencies of 19% and 24% were obtained for TCAD and ECAD construct, respectively, which were calculated as the number of kan-resistant plantlets regenerated per 100 initial explants. Using an homologous construct, up to 17% of residual CAD activity was down-regulated for the endogenous CAD gene.	Valerio et al. 2003
E. camaldulensis	-	*codA*	-	-	-	-	-	Yamada Watanabe et al. 2003
E. tereticornis	Mature zygotic embryo	*uid A*	*npt II*	*A. tumefaciens*	Embryogenesis	Transgenic plant	The transformation status was analyzed by growth of plantlets in the presence of kanamycin, as well as by conducting GUS assays and PCR-amplifications of *gus* and *npt II*.	Prakash and Gurumurthi et al. 2005a

APPENDIX 4. contd....

APPENDIX 4. contd.

Species	Explant source	Gene(s) transferred	Marker gene	*Agrobacterium* strain	Regeneration pathway	Final stage	Outcome	Reference
E. globulus	Stem segment	*uid A*	-	*A. tumefaciens* AGL1, LBA 4404	-	Transient GUS expression in stem segments	A promising protocol was developed for transforming wood-producing stem segments in eucalypts. It has the potential to become standard practice for functional analysis of genes involved at various stages in the wood formation process because it focuses directly on the tissue of interest and can be accomplished within a reasonable time frame.	Spokevicius et al. 2005
E. camaldulensis	Hypocotyl, cotyledon, mature leaf	*uid A*	*npt II*	*A. tumefaciens* CIB542 (derivative of EHA 101)	Indirect	Transgenic plant	-	Chen et al. 2007b
Several *Eucalyptus* sp.	-	Glucose oxidase	-	-	Indirect	Transgenic plant	-	Chen et al. 2007a
E. saligna	Leaf	*P5CSF129A + gus*	*npt II*	*A. tumefaciens* EHA105	Indirect	Transgenic plant	Transformation efficiency of 0.5% (1/200 explants) was obtained; transformants were confirmed by PCR and Southern blot analysis.	Dibax 2007
E. occidentalis	Cotyledon, hypocotyl	*uid A*	*npt II*	*A. tumefaciens* AGL1	Direct	Transgenic plant	Transformation efficiency per explant was 1.6%, based on GUS expression in the shoots.	Southerton 2007
E. camaldulensis	Leaf, cotyledon	*uid A*	*npt II*	*A. tumefaciens* C58C1	Indirect	Putative transgenic plant	In all 45 putative transgenic plants were obtained from leaf explants and 178 from cotyledon explants	Quisen 2007
Several *Eucalyptus* sp.	-	*hrap* from sweet pepper	-	*A. tumefaciens*	-	Transgenic plant	-	Wang et al. 2007
E. tereticornis	Cotyledon, hypocotyl	*uid A*	*npt II*	*A. tumefaciens* LBA 4404	Direct	Transgenic plant	Transformation frequency averaged 21.29% for cotyledons and 14.43% for hypocotyls. PCR results indicated that 8 randomly selected transgenic plants carried *uid A* and *nptII*, which was further confirmed by Southern blot analysis.	Prakash and Gurumurthi 2009
E. camaldulensis	Cotyledon	*uid A*	*npt II*	*A. tumefaciens* C58C1	Indirect	Putative transgenic plant	The largest (average) number of regenerated shoots from an explant (5,4) was observed in the presence of 300 mg L^{-1} of Augmentin® for 15 d, followed by 150 mg L^{-1} for 15 d and 100 mg L^{-1} for 30 d.	Quisen et al. 2009

APPENDIX 4. contd....

APPENDIX 4. contd.

Species	Explant source	Gene(s) transferred	Marker gene	*Agrobacterium* strain	Regeneration pathway	Final stage	Outcome	Reference
E. saligna	Leaf	*P5CSF129A + gus*	*npt II*	*A. tumefaciens* EHA105	Indirect	Transgenic plant	Transformation efficiency was 0.5%	Dibax et al. 2010
E. grandis x *E. urophylla*	Kawazu et al. 2003 (patented method)	Rs*PIP1;1* and Rs*PIP2;1*	Kawazu et al. 2003 (patented method)	*A. tumefaciens* EHA 101	Indirect	Transgenic plant	Down-regulation of PIP & PIP 2 caused severe damage, but up-regulation of PIP2 improved photosynthetic activity in eucalypts.	Tsuchihira et al. 2010
E. tereticornis	Leaf from shoot culture	*uid A*	*nptII*	*A. tumefaciens* EHA105	Direct	Transgenic plant	Preculturing and co cultivation durations of 2 days each using 0.8 OD_{600} of *Agrobacterium* for inoculation recommended. Efficient transgenic shoots regeneration achieved by replacing nitrate with sulphate in MS medium (potassium nitrate replaced with 990 mg L^{-1} potassium sulphate and ammonium nitrate with 392 mg L^{-1} ammonium sulphate) and increasing the mesoinositol concentration from 100 to 200 mg L^{-1}). Transgenic plants were confirmed by GUS expression assays and PCR.	Aggarwal et al. 2011
E. grandis x *E. urophylla*	Leaf from micro-cutting shoot culture	*EguCBF1a/b*	*npt II + gfp*	*A. tumefaciens* AGL1	Direct (Tournier et al. 2003)	Transgenic plant	Transgenic lines over-expressing *CBF* had decreased stomatal densities and an over-accumulation of anthocyanins. One line showed a reduced leaf area, higher density of oil glands, and a wax deposition on the cuticle.	Navarro et al. 2011