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PLANT TISSUE CULTURE

Transformation system optimization and introduction of a new model *Eucalyptus grandis x urophylla* genotype for transgenic studies

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Abstract

A number of factors that have been reported to affect transformation/regeneration rate in plants were studied in multiple eucalypt genotypes. These include surfactant, acetosyringone, fipexide, basal media, activated charcoal, ascorbic acid, lipoic acid, proline, cytokinins, sugars, spectinomycin selection, Agrobacterium strains and concentrations, forchlorfenuron, PVP, silver nitrate, and explant heating prior to cocultivation. Heating, use of acetosyringone, basal medium, cytokinin type, carbon source, and *Agrobacterium* strain had substantial impacts—whereas the other factors studied had negligible or a negative impact. In search of a model genotype for producing transgenic shoots, 53 diverse genotypes that included *E. grandis, E. grandis* x *E. urophylla*, *E. urophylla* x *E. grandis,* and *E. dunnii,* were screened using a highly infective gall-causing Agrobacterium strain with T-DNA genes isolated from strain C58. One *E. grandis* x *E. urophylla* genotype was identified, Egu45-1, that showed a regeneration rate of transgenic shoots of 4% to 8% (frequency of explants that produced one or more transgenic shoots). This genotype and the associated transformation protocol will be made available to any interested party without restriction.

Keywords Eucalyptus · *Agrobacterium tumefaciens* · Transformation · Organogenesis · Necrosis

Introduction

Eucalyptus species and hybrids are globally significant forest plantation trees, and have been the subject of extensive breeding and genomic studies. However, mechanistic studies of *Eucalyptus* gene function are rare due to their high recalcitrance to genetic transformation. Given their wide economic importance, there is a need for reliable genetic transformation and regeneration protocols, especially for genotypes in the economically significant '*urograndis'* group that is grown widely in the subtropical southern hemisphere.

The genus *Eucalyptus* is of immense ecological and economic importance. It includes hundreds of species that occupy, and often dominate, highly diverse environments in Australia, New Guinea, and nearby islands (Kew Science 2025). Plantations that produce pulp, energy, and solid wood products occupy more than 22 million hectares across the globe (Myburg *et al*. 2014) Girijashankar 2011, Chavan *et al*. 2024).

Transgenic or gene-edited eucalypts have been produced and demonstrated for diverse traits of commercial interest, including herbicide resistance (Wang *et al*. 2025), disease resistance (Ouyang and Li 2016), salt tolerance (Matsunaga *et al*. 2012; Thanananta *et al*. 2018), lignin modification (de la Torre *et al*. 2014), sterility (Elorriaga *et al*. 2021; Nagle *et al*. 2023), and accelerated flowering (Klocko *et al*. 2016). Despite these reports, published research that use transgenic or gene-edited regenerated plants is uncommon. This rarity is particularly striking in comparison to *Populus*, another genus of clonally propagated forest trees, for which several highly transformable genotypes have enabled the extensive use of transgenic methods, including the widely used French clone *P. tremula x alba* 717-1B4 (Mader *et al*. 2016; Zhou *et al*. 2023). Commercial activity has led to the identification of a highly transformable, *E. grandis x E. urophylla,* SP7, clone with a public genome sequence, (Klocko *et al*. 2016, NCBI 2023), however, the transformation method and clone are not available for public use.

Genetic transformation is a multifaceted process influenced by various factors, including host plant genotype, explant type, *Agrobacterium* strain, selection agents, and means for plant regeneration (Pérez-Piñeiro *et al*. 2012).

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Among the available methods, *Agrobacterium*-mediated transformation remains one of the most widely utilized and effective techniques for Eucalyptus; however, there are few established transformation systems for this genus that are considered reliable and repeatable, particularly for clonal rather than seedling derived explants (Wang *et al*. 2024).

A great deal of research has focused on the transformation of *E. camaldulensis* using seedling-derived materials, especially cotyledons and hypocotyls, as explants (Supplemental Table 1). These explants are advantageous for regeneration due to their juvenility but are inherently genetically variable as a result of the outcrossing and high heterozygosity of eucalypts, thus experiments are challenging to replicate (Zhou *et al*. 2022). Protocols are also highly complex, and even clonal plant material is highly variable in its capacity for transformation and regeneration. This is presumably as a result of variation in physiological and epigenetic states in relation to growth environment and propagation history. Thus, replication among laboratories is notoriously difficult (Chauhan *et al*. 2014). There have been several reports of successful transformation of *E. grandis, E. urophylla,* and their various hybrids, including of clonal materials (Supplemental Table 1), yet no single protocol and clone has emerged as a global model for transformation.

Since the development of robust transformation methods in eucalypts is slow, costly, and highly genotype-specific, few researchers undertake such studies. In an attempt to make transgenic studies more widely used in eucalypt biology and biotechnology, particularly for clonal selections, we evaluated a variety of factors reported to improve transformation and/or regeneration. In addition, a method was developed with a clone that we believe has adequate transformation efficiency and reliability for many applications.

Materials and methods

Plant Materials Seedling-derived materials of 53 eucalypt genotypes provided by Sappi were screened; they included a variety of pure and hybrid families, including *E. grandis, E. dunnii, E. urophylla x grandis,* and *E. grandis x urophylla*. The seeds were sterilized by immersion in 70% ethanol for 5 s, followed by a 10 min soak in 20% bleach. They were then rinsed four times with sterile water and placed on a Petri dish with Murashige and Skoog (MS) germination medium (Murashige and Skoog 1962) with 7.0 g L^{-1} agar, pH 5.7). The seeds were incubated in the dark for 7 days, after which they were cultured under cool white fluorescent lights (Philips, Amsterdam, Netherlands) at 25 ± 1˚C, with a light intensity of 40 to 45 µmol m^{-2} s^{-1}, and a 16 h light/8 h dark cycle. The shoot tips of the germinated seedlings were removed and cultured in a Petri dish with shoot multiplication medium (SMM) (Fig. 1*a* and *b*, Table 1). Leaves from 40 to 50-day-old shoots (e.g., Fig. 2*b*) were used for initial regeneration and transformation. Thereafter transformations were repeated under various conditions with micropropagated clonal materials for approximately 10 to 12 cycles of propagation.

Binary vector and agrobacterium strains For the initial screening of transformation rate, a T-DNA was constructed containing the *iaaH*, *iaaM*, and *ipt* genes from *Agrobacterium* strain, C58 (~6 kb region), under native promoter control, alongside a hygromycin resistance gene and DsRed2 fluorescent reporter gene. Some constructs alternatively used a GFP marker expressed using a 35S promoter (e.g. Figure S5). It was then mobilized into a binary vector backbone pTRANS200 (Čermák *et al*. 2017). This binary plasmid was transformed into *Agrobacterium* strain, LBA4404, *via* electroporation that was engineered to be auxotrophic, requiring thymidine for growth in co-culture.

For the transformation experiments intended to produce transgenic shoots, a T-DNA was constructed containing a DsRed2 red fluorescent protein and the selectable marker gene, hygromycin. The DsRed2 gene used the GmScream1 promoter and the hygromycin resistance gene was driven by nos promoter. This T-DNA was also mobilized into the pTRANS200 backbone (Čermák *et al*. 2017) *via* Golden Gate assembly, confirmed by Oxford Nanopore sequencing (Plasmidsaurus, San Francisco, CA) and transformed into *Agrobacterium tumefaciens* strain, AGL1, *via* electroporation (Tu *et al*. 2016). The presence of the plasmid in the antibiotic-resistant bacterial colonies was confirmed by colony PCR using these primer sequences: 5'-gtaatcatggcctcctccga-3' and 5-gtgtagtcctcgttgtggga-3', or by whole plasmid sequencing using Oxford Nanopore technology (Plasmidsaurus).

Plant transformation The *Agrobacterium* strains were inoculated in YEP medium supplemented with appropriate antibiotics (Table 2) and grown overnight at 28˚C on a gyratory shaker set at 250 rpm. The bacterial cells were collected by centrifugation at 4000 rpm for 30 min and resuspended in *Agrobacterium* induction medium (AIM, Table 1 and Table 2) with 50 µM acetosyringone (Sigma Chemical Co., St. Louis, MO) and 0.03% Silwet L77 (PlantMedia.com Dublin, OH) to achieve an optical density (OD, at 595 nm) of approximately 2 to 3.0.

Two types of pre-culture and co-cultivation media were used: PCIM1 (with thymidine) for the C58 oncogene strain, LBA4404, and PCIM2 (without thymidine) for the AGL1 strain (Table 1 and Table 2). Leaf explants were cultured on pre-culture medium for 1 to 2 days, then wounded with a surgical blade dipped in highly concentrated *Agrobacterium* solution with the exception of two experiments which used low OD *Agrobacterium* solutions, as noted (Supplemental

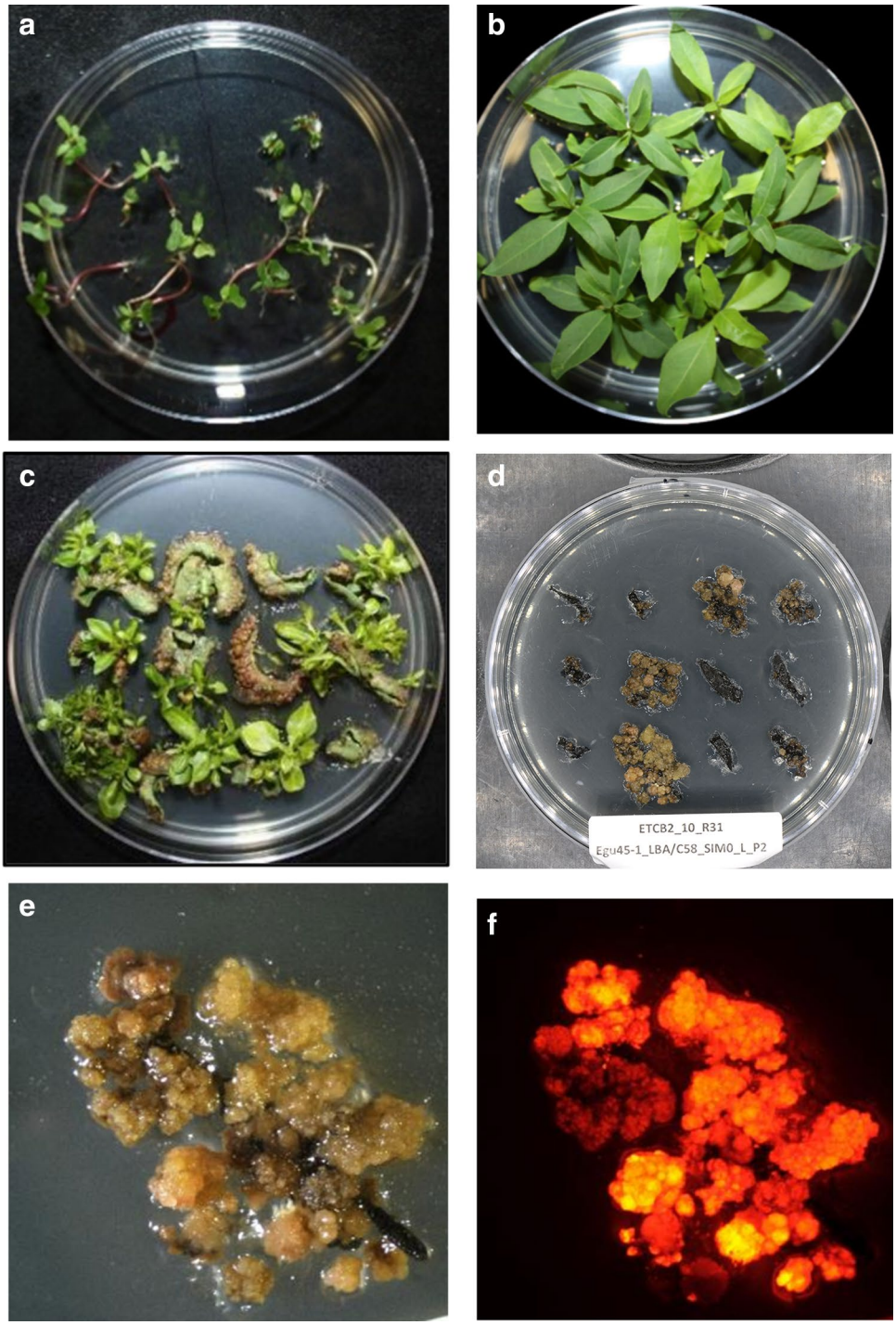

Figure 1. Screening experiments based on regeneration and callus formation as an indicator of *Agrobacterium* transformation. (*a*) Seeds of diverse eucalypt families were germinated in germination medium for 20 days. (*b*) Shoot tips from seedlings were micropropagated for four to ten cycles on multiplication medium, then leaves from 40-d-old shoots were used for regeneration and transformation. (*c*) Shoot formation from direct organogenesis. (*d*) Callus formation from explants infected with wild-type C58 oncogenes after 18 wk of growth on SIM without hormone and selection agents. (*e*) Bright-field and (*f*) dsRed fluorescence from transgenic calluses in (*e*).

Figs. S3 and S4). Following infection, the tissues were co-cultivated on sterile filter paper on PCIM medium for 2 d at 21˚C in the dark.

After co-cultivation, leaf explants infected with C58 oncogene strain do not require washing and can be directly cultured on a thymidine-free, hormone-free, and selection-free medium (Fig. 1*d-f*). However, antibiotics are necessary to control *Agrobacterium* (CIM1, Table 1). Leaf explants infected with AGL1 were washed (Table 2), blotted on sterile filter paper, and transferred to callus induction medium (CIM2, Table 1 and Table 2) for culture in the dark. After 7 days, the explants were moved to shoot induction medium (SIM, Table 1 and Table 2) containing selection agents and antibiotics.

Both types of explants were then cultured in the dark for 30 days to minimize browning that is common in

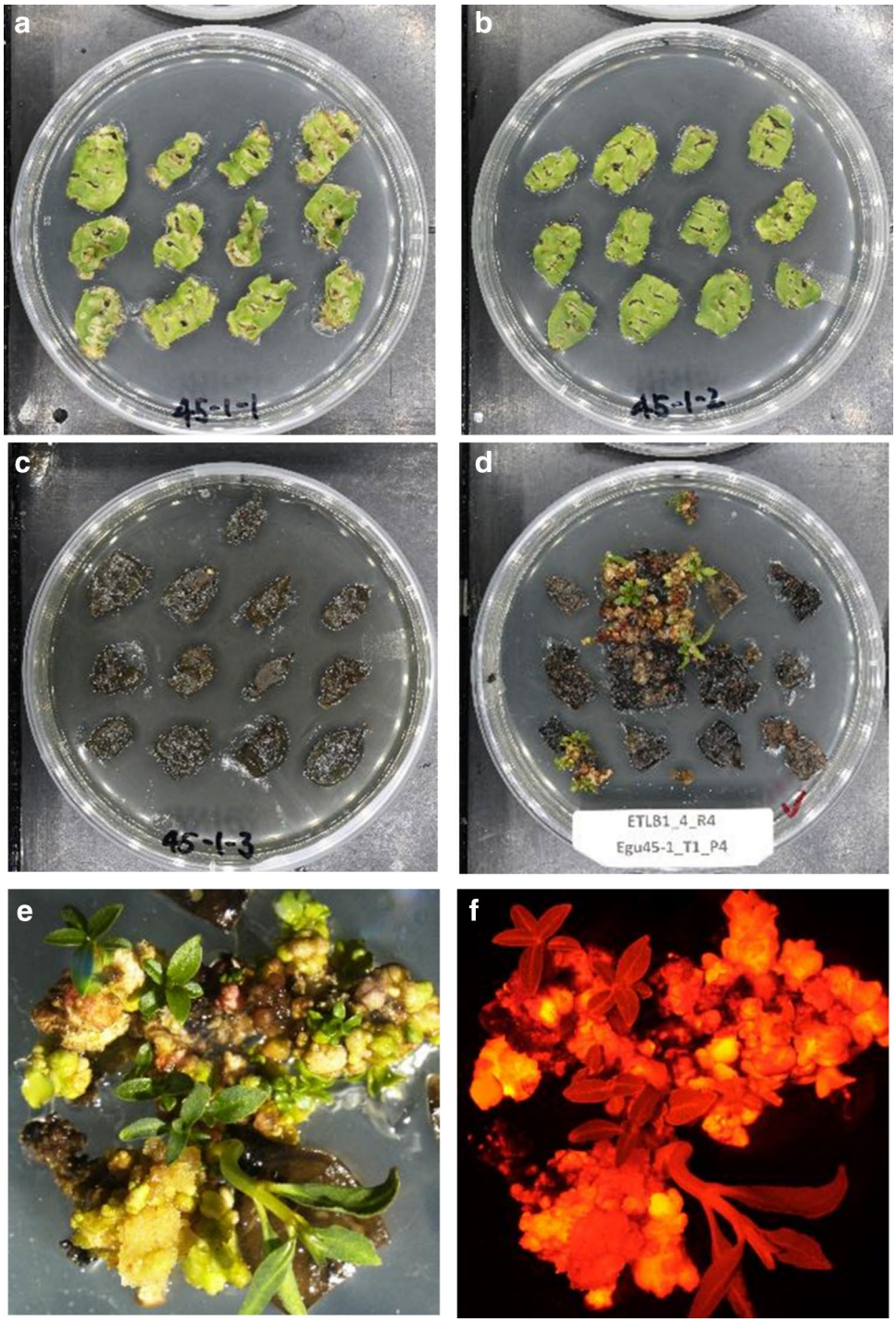

Figure 2. Effect of *Agrobacterium* on tissue necrosis and regeneration of stably transformed plants in clone Egu45-1. (*a*) Regeneration from leaves in SIM (shoot induction medium, composition given in Table 1) in the absence of *Agrobacterium* treatment and plant selection agents led to normal callus development, (*b*). In the absence of *Agrobacterium* infection but with the addition of plant selector, leaves in SIM medium failed to form callus, whereas (*c*, *d*) *Agrobacterium* treated explants showed extensive necrosis after 4 and 20 wk on SIM in the presence of plant selection agents. (*e*, *f*) Transgenic callus and shoots regenerated after 28 wk with multiple transgenic shoots per explant (*e*,) brightfield, (*f*,) DsRed fluorescence)

Eucalyptus following *Agrobacterium* cocultivation (e.g., Fig. 2*a-c*). They were then subcultured every 20–30 d on the same SIM under low light intensity (20μE m-2S-1). Transformed shoots regenerated from AGL1-infected explants were transferred to elongation medium (SEM, Table 1 and Table 2) containing the same selection agents and antibiotics as SIM. DsRed callus and shoots were scored under a microscope every month for up to 8 m (Fig. 2*e-f*). Transformation rates were calculated as the percentage of explants with transgenic callus or shoots. Shoots exhibiting DsRed expression under a fluorescent microscope were placed in root induction medium (RIM, Table 1 and Table 2) for 7 d in the dark, then transferred to root formation medium (RFM, Table 1 and Table 2) and cultured in the light for 4 to 5 wk. Rooted plants were transplanted to soil and grown in a greenhouse.

Table 1. Media used in transformation protocol

Culture medium	Composition
Shoot multiplication medium (SMM)	Murashige and Skoog (MS) medium + 0.01 mg L^{-1} NAA + 0.1 mg L^{-1} BAP + 2.0% sucrose + 0.3% gelrite + 0.2% pytoblend + 0.3% activated charcoal, pH 5.8
Pre- & co-cultivation medium (PCIM1)	Woody Plant Medium (WPM) medium + 1.0 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 3.0% glucose + 1.0 g L^{-1} casein hydrolysate + 2.5 g L^{-1} PVP10 + 2.5 g L^{-1} PVPP + 50.0 mg L^{-1} thymidine + 0.7% agar, pH 5.9
Pre- & co-cultivation medium (PCIM2)	Same as PCIM1, but without thymidine
Agrobacterium induction medium (AIM)	WPM medium + 3.0% glucose + 1.0 g L^{-1} casein hydrolysate, pH 5.0
Callus induction medium (CIM1)	WPM medium + 3.0% glucose + 1.0 g L^{-1} casein hydrolysate + 200.0 mg L^{-1} timentin + 200.0 mg L^{-1} cefotaxime + 0.7% agar, pH 5.9
Callus induction medium (CIM2)	WPM medium + 1.0 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 3.0 g L^{-1} glucose + 1.0 g L^{-1} casein hydrolysate + 2.5 mg L^{-1} hygromycin + 200.0 mg L^{-1} timentin + 200.0 mg L^{-1} cefotaxime + 0.7% agar, pH 5.9
Shoot induction medium (SIM)	WPM medium + 0.02 mg L^{-1} NAA + 0.176 mg L^{-1} TDZ + 3.0 g L^{-1} sucrose + 1.0 g L^{-1} casein hydrolysate + 5.0 mg L^{-1} hygromycin + 200.0 mg L^{-1} timentin + 200.0 mg L^{-1} cefotaxime + 0.7% agar, pH 5.9
Shoot elongation medium (SEM)	WPM medium + 0.02 mg L^{-1} NAA + 5.0 mg L^{-1} Meta-topolin + 3.0 g L^{-1} sucrose + 1.0 g L^{-1} casein hydrolysate + 5.0 mg L^{-1} hygromycin + 200.0 mg L^{-1} timentin + 200.0 mg L^{-1} cefotaxime + 0.7% agar, pH 5.9
Root induction medium (RIM)	½ MS medium + 0.1 mg L^{-1} NAA + 0.3 mg L^{-1} IBA + 2.0% sucrose + 2.5 g L^{-1} gelrite
Root formation medium (RFM)	½ MS medium + 1.5 to 3.0 g L^{-1} activated charcoal + 2.0% sucrose + 2.5 g L^{-1} gelrite

Table 2. Summary of steps in transformation

1. Grow plants: Culture shoots with 3 to 4 leaves on shoot multiplication medium (SMM)

2. Pre-culture

A. Select young, incurved leaves without callus from 40 to 60-day-old shoots

B. Harvest leaves with petioles attached and remove the tip of each leaf in sterile double distilled water. Then put the basal part of leaves with petioles in sterile ddH_2O and shake for 1 h

C. Lay explants with abaxial side in contact with pre-culture media (PCIM1 or PCIM2) and culture in the dark for 1–2 days

3. Transformation

A. Grow *Agrobacterium* in 50.0 mL liquid YEP medium supplemented with 50.0 mg L^{-1} kanamycin, 50.0 mg L^{-1} rifampicin, and 60.0 mg L^{-1} carbenicillin for AGL1 strain and 50.0 mg L^{-1} kanamycin, 50.0 mg L^{-1} gentamycin, and 50.0 mg L^{-1} thymidine for LBA4404 auxotrophic strain at 28˚C overnight

B. Pellet 50 mL bacteria cultures at 4000 rpm for 30 min, resuspend in liquid *Agrobacterium* induction medium (AIM) containing 50.0 µM acetosyringone (AS) and 0.02 to 0.03% silwet L77 to reach a cell density 2–3.0 at OD595

C. Inoculate pre-cultured leaves by cutting them with a scalpel blade (no.10) dipped in very dense *Agrobacterium* solution

D. Apply 3 cuts on the leaf and 1 cut on the petiole, perpendicular to the midrib (place explants on sterile filter paper in the empty dish, then dip-cut with concentrated *Agrobacterium*)

E. Co-cultivate the leaves abaxial side down on sterile filter paper on PCIM1 or PCIM2 medium at 19–21˚C in the dark for 2 days

4. Washing

A. Wash explants 4 times in sterile ddH_2O and once in sterile water with 250.0 mg L^{-1} timentin and 250.0 mg L^{-1} cefotaxime for 1 h

B. Then transfer explants onto CIM1 or CIM2 with 200.0 mg L^{-1} timentin, and 200.0 mg L^{-1} cefotaxime and culture in dark for 7 d

5. Subculture

A. Place the explants on SIM with 5.0 mg L^{-1} hygromycin with 200 mg L^{-1} timentin and 200 mg L^{-1} cefotaxime in dark for 30 days, then under low light intensity (20 µE $m^{-2}s^{-1}$)

B. Subculture explants on SIM (as described above) every 3 to 4 wk

C. Transfer the explants with small green buds onto SEM containing selection agents and antibiotics (as described above) for shoot elongation

6. Multiplication and rooting

A. Transfer the explants with elongated shoots onto SMM with selection agent and antibiotic (as described above) for shoot multiplication

B. Collect shoot tips for transgene confirmation

C. Place PCR positive shoots or GFP or dsRed transgenic shoots in RIM for 5 days in the dark, then move to RFM under the light for 4 to 6 wk

Trial design and data analysis All regeneration and transformation experiments were analyzed by the occurrence of a particular observation (yes/no) on a given explant (transgenic callus), where there were 12 explants per Petri dish (plate). These observations were averaged across all explants on a Petri dish, and those means were used as a

statistical biological replicate. Bar graphs presented are thus means multiple biological replicates (means within petri dishes), with two standard errors shown centered on the mean. As most experiments were not independently repeated, statistical tests were performed only for the graphics shown in the main text, where Tukey tests were done with the 'dplyr' package in R; the letters showing statistical significance of pairwise differences were assigned using the 'multcompView' package. Browning was subjectively scored as present vs. absent for an explant when it had a majority of brown rather than green tissue.

Results and Discussion

Factors affecting transformation A number of factors reported to be important to plant transformation or regeneration were tested using the high-regenerating clones identified below. The factors studied and general results are summarized in Supplemental Table S2, with detailed experimental results given in Supplemental Figures S1–S19.

***Agrobacterium* strain** Initially, the following *Agrobacterium* strains were tested: EHA105, AGL1, and LBA4404. AGL1 was found to be the most suitable for transgene delivery. In a direct comparison, when averaged over rates of transgenic callus for three *Eucalyptus* genotypes, it was found that AGL1 was about 20% more effective compared to an auxotrophic, LBA4404, strain (Fig. S1). AGL1 was also superior for all three genotypes individually. AGL1 is often considered a "supervirulent" strain due to its genetic background (C58 chromosomal background carrying a hypervirulent, Bo542, Ti plasmid). In many plant systems, AGL1 produces high rates of transformation, thus it has been commonly used for eucalypt transformation (e.g., Tournier *et al*. 2003; Fernando *et al*. 2016; Nagle *et al*. 2023).

Basal medium Comparison of a modified MS and WPM (Woody Plant Medium, defined in Table 1) basal media indicated that the low-salt WPM improved the rate of callus formation (Fig. S2*A*). It was also markedly beneficial in reducing explant browning (Fig. S2*C*), although overall it had little effect on the rate of transgenic callus formation (Fig. S2*B*). de França Bettencourt *et al*. (2018) compared WPM and LP medium in *E. urophylla* (LP medium is similar to WPM but has a higher total ionic strength). They found that more transgenic shoots were induced on WPM medium (both media were supplemented with TDZ and NAA).

Surfactant The effect of the commercial surfactant, BREAK-THRU® (S233), was studied as a means to enhance *Agrobacterium* binding to plant cells. Effects were highly genotype specific, but the highest levels tended to reduce regeneration and transformation (Fig. S3). In many studies surfactants are critical for improving transformation, especially for species where transformation is challenging. Huynh *et al*. (2022) compared the efficiency of seven novel surfactants (BREAK-THRU® OE446, S200, S233, S240, S279, S301, SP133) with the commonly used Silwet® L-77 in *Agrobacterium*-mediated floral dip transformation of *Arabidopsis thaliana*. They reported that several of the tested surfactants worked more effectively than Silwet® L-77, suggesting that it would be advisable to screen a greater diversity of surfactants in future efforts to improve Eucalyptus transformation.

***Agrobacterium* density** The concentration of *Agrobacterium* during cocultivation was studied in three Eucalyptus clones (Fig. S4). Surprisingly, the rate of antibiotic resistant callus formation generally declined as *Agrobacterium* density increased above an OD (optical density) of 0.2. Callus formation in clone Eg18-1, however, seemed to benefit from high OD levels, as did the rate of transgenic callus formation, although this varied widely among genotypes and experiments. Nguyen *et al*. (2025) found that low densities (OD_{600} of 0.01 to 0.05) increased callus survival in *Eucalyptus camaldulensis* × *E. urophylla* hybrids. At low densities callus survival rates were highest (70 to 75%). Increasing bacterial density ($OD_{600} = 0.25$ and 1.00) resulted in a significant decrease in survival rates to approximately 30% and 15%, respectively ($p < 0.05$). Most studies of Eucalyptus transformation have used OD_{600} of 0.3 to 0.8 (Aggarwal *et al*. 2011; Oliveira-Cauduro *et al*. 2018; Wang *et al*. 2022).

Acetosyringone (AS) In clone Eug22-1, intermediate levels of AS were most effective at stimulating callus formation under selection (Fig. S5). The rate of GFP marker gene expression in callus followed a similar pattern, although the rates were considerably lower. Very high levels of AS (100.0 µM), during cocultivation, seemed to attenuate the rate of transformation. When effects of 50.0 µM AS were studied in several Eucalyptus clones, four of the seven clones studied showed an increase in transgenic callus formation with use of AS; on average, the rate was increased from 10 to 13% (an improvement of 30%) (Fig. S6). AS acts by stimulating vir gene expression, often improving T-DNA transfer. AS is thus widely used to stimulate T-DNA transfer from Agrobacterium, but its importance for Eucalyptus transformation was not well understood. Most successful Eucalyptus transformation protocols use 50.0 µM AS during co-cultivation (Aggarwal *et al*. 2011; Oliveira-Cauduro *et al*. 2018; Wang *et al*. 2022). For example, Wang *et al*. (2022) used 50 µM AS in shoot-inducing medium to improve the frequency of transformation to 3.8% in *E. urophylla x E. tereticornis*. These findings agree with current results

that 50.0 µM AS usually enhances transformation rate in Eucalyptus.

Fipexide (FPX) The capacity for the growth regulator, FPX, to promote callus or subsequent shoot development was assessed. When considering all callus or transgenic callus rates, there was no clear benefit from FPX. When considering clearly differentiated callus, there was a very small but positive effect in two of the three clones tested (Fig. S7*A*). The transgenic callus also appeared to grow much larger when FPX was added (Fig. S7*B*). Nakano *et al*. (2018) also found that FPX enhanced the rate of Agrobacterium infection in monocots and several tree species, including poplar.

Cytokinins Seven cytokinin treatments were tested for their effect on regeneration of transgenic callus and shoots of two to six Eucalyptus genotypes. TDZ alone or in combination with brassinolide (BR), or metatopolin (MT) at 5.0 mgL^{-1}, gave the highest rates of callus formation. The rate of transgenic callus formation followed a similar trend, except that genotype Eug21-1 had lower rates than Eug22-1 for TDZ with BR or MT alone compared to TDZ alone (Fig. S8*A*-*B*). Only MT at 5.0 mgL^{-1} gave rise to any shoot formation, primarily in Eug21-1 (Fig. S8*C*). There was substantial necrosis in all treatments, with transgenic callus size being largest with TDZ alone (Fig. S8*D*).

Two further treatments tested made use of the growth regulators CPPU and BAP in CIM in varying configurations (Fig. S9). Treatment 1 with BAP was generally superior to T2 with CPPU for both callus and transgenic callus formation for all genotypes (apart from clone Egu45-1 callus, where T1 and T2 were nearly identical). Early transgenic shoot development was observed but was not sufficient to score in this experiment (Fig. S9*B*). T1 gave visibly superior callus development (Fig. S9*C*) and development of transgenic callus (example in Fig. S9*D*). Overall, CPPU was not beneficial at the rates studied.

TDZ appears to be the most common cytokinin for *Eucalyptus* transformation (Gonzalez *et al.* 2002; Tournier *et al*. 2003; Fernando *et al*. 2016). This is likely because TDZ promotes adventitious bud induction and organogenesis, and works well at low concentrations to avoid hyperhydricity (Polivanova and Bedarev 2022). For example, Wang *et al*. (2022) used 0.025 mgL^{-1} TDZ in modified WPM for bud induction, achieving 85.6% shoot formation and a transformation frequency of 3.8% in *E. urophylla x E. tereticornis*. CPPU has not been widely used, but recent protocols show it improves adventitious bud induction in difficult-to-propagate Eucalyptus clones at a common range of 0.2 to 0.3 mgL^{-1} in callus induction or shoot regeneration media, often combined with NAA (auxin) (Wang *et al*. 2025).

Carbon sources Four carbon sources were tested in two genotypes for their effects on necrosis ("browning"), callus formation, and formation of transgenic callus. The genotypes responded to the carbon sources differentially. Carbon source had no effect on browning in genotype Eg18-1 but had a strong effect in Eug22-1, where maltose gave rise to most frequent browning (Fig. S10*A*). Sucrose gave rise to the highest rate of callus and transgenic callus formation in genotype Eg18-1, but (apart from fructose) was similar to the other carbon sources in callus and transgenic callus formation in Eug22-1 (Fig. S10*A*). Despite extensive browning in Eug22-1, maltose gave rise to extensive transgenic callus formation (Fig. S10*B*-*C*). The authors are unaware of comparable studies on carbon source in relation to regeneration or transformation in Eucalyptus.

Spectinomycin selection A range of spectinomycin (spec) levels were tested for their use in selection of transgenic tissues in two Eucalyptus genotypes. Genotype Eug22-1 showed little browning at all spec concentrations, whereas Eug21-1 had extensive browning at all spec levels above zero. Overall callus formation was inhibited by spec in Eug21-1, but had no effect on Eug22-1. In contrast, spec was essential to get significant formation of transgenic callus, but the level at which it was applied had little impact in either genotype (Fig. S11*C*). Higher spec levels gave rise to more prominent transgenic callus loci (Fig. S11*D*). Overall, it appears that spec can be successfully employed at a wide variety of levels. The authors are unaware of other published studies of spectinomycin selection during Eucalyptus transformation.

Resting period Imposition of a one wk "resting period" prior to antibiotic selection was tested on callus formation and transformation rate in one Eucalyptus genotype. A delay in the imposition of selection had no discernible effect on callus formation, and slightly reduced the rate of transgenic callus formation (Fig. S12*A*); although callus formation on plates appeared unchanged (Fig. S12*B*). The higher rate of transgenic callus formation is visible using DsRed fluorescence (Fig. S12*C*). Guo *et al*. (2013) gradually increased antibiotic screening pressure to select transgenic cells and plants in *E. grandis* transformation (7 d without selection, 7 days with 7.5 mg L^{-1} kanamycin, 7 d with 15 mg L^{-1}, and 7 d with 17.5 mg L^{-1} kanamycin). They obtained a low transformation efficiency of 0.26%, and also a high rate of escape non-transgenic tissues.

Explant preheating. Heating explants for 2 to 4 h at 37 °C was studied as a potential means to enhance rate of transgenic callus formation and reduce browning in several genotypes. Browning was not detectably changed by the heating treatments; however, preheating strongly increased

the rate of transgenic callus formation in all four genotypes tested, with the benefit observed for genotypes Egu45-1 and Eug21-1 in two separate experiments (Fig. S13). For Egu45-1 the transformation rate was increased more than two-fold in both experiments, and for Eug21-1 transgenic calluses could only be recovered after heat treatment in both experiments. Likewise, for Eug31-4 and SP7 preheating was required to generate transgenic calluses in these experiments. Preheating appears to be a valuable means to increase transformation rate in Eucalyptus. Though commonly used in some crop plants (Pellegrineschi *et al*. 2002; Kaur *et al*. 2022; Irsyadi *et al*. 2026), published studies on use of preheating in *Eucalyptus* are not evident.

Necrosis mitigation Since browning and necrosis after *Agrobacterium* treatment is problematic with *Eucalyptus* (Chauhan *et al*. 2014), several experiments were conducted to understand its cause and mitigation. Three treatments were implemented to understand its extent: (1) explants without *Agrobacterium* inoculation cultured on CIM and SIM, excluding hygromycin, (2) explants without *Agrobacterium* infection cultured on CIM and SIM containing hygromycin, and (3) explants infected with *Agrobacterium* and cultured on CIM and SIM with hygromycin. After 4 wk, the explants without infection on non-selection medium successfully formed green callus and did not brown (Fig. 2*a*). The explants without infection on selection medium did not form callus, but also did not brown (Fig. 2*b*). In contrast, the explants infected with *Agrobacterium* turned completely brown or black by the end of the culture period (Fig. 2*c*, *d*). These results suggest, unsurprisingly, that *Agrobacterium* infection imposes significant stress on the leaf explants and is a significant obstacle to transformation and regeneration. Several widely used approaches to mitigate necrosis development were therefore studied.

Activated charcoal and ascorbic acid Activated charcoal (AC) and ascorbic acid or vitamin C (VC) was tested in three genotypes as means to reduce oxidative stress and potentially improve growth and transformation. In all genotypes, high levels of AC were strongly inhibitory to callus and transformation rates, and intermediate levels gave a slight reduction in transformation rate in two of three genotypes where any transformation could be detected (Fig. S14). VC likewise had no discernible benefit for callus rate (in fact, a small reduction in one genotype); it also reduced transformation rate in one genotype, while possibly enabling some transformation in another. No transgenic calluses were observed under any treatment for genotype Eg18-1 after 7 m of transformation in this experiment. Wang *et al*. (2024) cited several examples where browning in *Eucalyptus* was mitigated by use of PVP or VC.

Lipoic acid The effects of the antioxidant lipoic acid (LA) was studied for its effects on shoot, and callus transformation rate in three genotypes. Dan *et al*. (2009) tested LA's effects on the transformation of Micro-Tom (tomato cultivar), wheat, soybean, and cotton. They identified LA as a distinctive plant transformation enhancer that was capable of reducing browning in *Agrobacterium*-transformed cells or tissues. This reduction in browning minimized cell or tissue death, enhanced the survivability of transformed cells or tissues and consequently improved both transient expression levels and plant transformation efficiency. In the current study low levels of LA appeared to provide some benefit for callus and transformation rate in Eg18-1, but not in the other genotypes (Fig. S15). Shoots were only obtained for genotype Eug21-1 in this experiment, and LA appeared to repress shoot development, especially at high levels. The authors are unaware of any published studies that employed LA for Eucalyptus transformation.

Proline Proline (PL) was studied as a possible antioxidant in two *Eucalyptus* genotypes. PL is an amino acid which has been recognized for its advantageous role in supporting plants under various stress conditions (Hayat *et al*. 2012). Pawar *et al*. (2015) highlighted the beneficial effects of PL and glutamine on *in vitro* callus induction and subsequent shoot regeneration in rice. Its use in our *Eucalyptus* transformation led to an increase in browning in both genotypes (Fig. S16*A* and *C*) and tended to repress shoot formation in the genotype where shoot formation was observed in this experiment, Eug21-1 (Fig. S16*B*). No published studies appear to have utilized PL for the transformation of Eucalyptus.

PVP Two types of polyvinylpyrrolidone (PVP) was studied as possible antioxidants in one *Eucalyptus* genotype. In both cases no change in browning of explants, and transgenic callus was observed and shoot formation was repressed to different degrees (Fig. S17A-B). The two types of PVP tested do not appear to hold promise for improving Eucalyptus transformation, as is also photographically evident (Fig. S17C-D). Wang *et al*. (2024), in their review of *Eucalyptus* transformation, stated that the addition of anti-browning agents effectively inhibits the occurrence of necrosis. For example, 500 mg L^{-1} PVP was used to prevent the browning of explants *E. tereticornis* callus when used to regenerate buds (Subbaiah and Minocha 1990).

Silver nitrate Silver nitrate (SN) was studied as it has been reported to impair the activity of the wounding/ripening hormone ethylene in plant tissue culture, and facilitate shoot regeneration in some plant species (Songstad *et al*.

1988). Two concentrations were tested in two *Eucalyptus* genotypes for its effect on browning and transgenic callus and shoot formation. There was little effect on browning, although perhaps it was slightly reduced at the highest concentration of SN in Eug22-1 (Fig. S18*A*). The rate of transgenic callus formation declined in both genotypes with the addition of SN (Fig. S18*B*, *F*), however, the rate of shoot formation (not of transgenic shoot formation), increased in genotype Eug22-1. In Egu45-1, the addition of any level of SN eliminated shoot formation, including that of transgenic shoots, entirely (Fig. S18*C*-*E*). The authors are unaware of any published studies that employed SN for Eucalyptus transformation.

Transformation model development - micropropagation media Testing of several shoot multiplication media (SMMs) for *in vitro* propagation of the 53 *Eucalyptus* genotypes (Table 1), indicated that different genotypes required different SMMs. Most genotypes showed green and large leaves in SMM supplemented with 1.5 to 3.0 g L^{-1} activated charcoal (AC) (Fig. 1*b*). However, some genotypes, such as *E. dunnii*, turned brown and eventually died on AC-containing medium (data not shown). Therefore, SMM with or without AC was used for the propagation of different genotypes.

Variation in regeneration Following Zhou *et al.* (2022), the regeneration capacity of various clones was tested to identify possible model genotypes. Both direct organogenesis on shoot induction medium (SIM) in the light (Table 2), and indirect systems [first treatment in the dark on callus induction medium (CIM) then transfer to SIM in the light: Table 2] were tested on nearly a dozen genotypes. These genotypes are those that had exhibited shoot regeneration after the initial screening of 53 seedling-derived genotypes in hypocotyl organogenesis experiments (data not shown). Using the direct method, 95% and 98% shoot regeneration was achieved from leaves of two genotypes of *E. urophylla x E. grandis* (Eug21-1 and Eug22-1), and 100% regeneration from the leaves of one *E. grandis* genotype (Eg18-1) (e.g., Fig. 1*c*). Using the indirect method, 75 to 85% shoot regeneration was obtained from these three clones (data not shown).

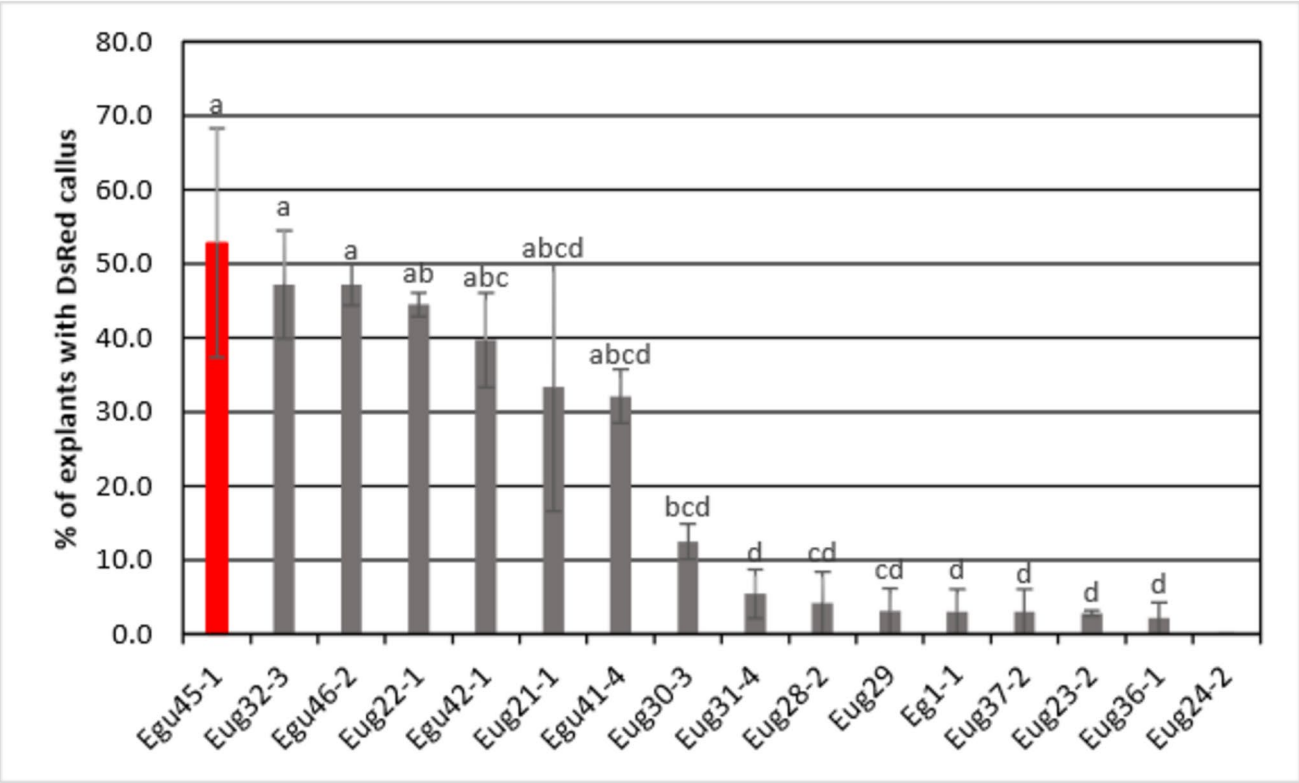

Figure 3. Example of screening for transformation rate with C58 oncogenes for 16 *Eucalyptus* genotypes. Data are percent of explants with transgenic (DsRed fluorescing) cells (callus) after 28 wk on SIM (shoot induction medium, composition given in Table 1) without hormones or selection agents, based on an average of 3 plates per genotype. *Bars* show two standard errors centered on the mean. *Bars* with the same letters above them are not statistically different ($P < 0.05$) based on Tukey multiple comparison tests. The leftmost *bar* (*red*) shows the rate for the proposed Egu45-1 model genotype.

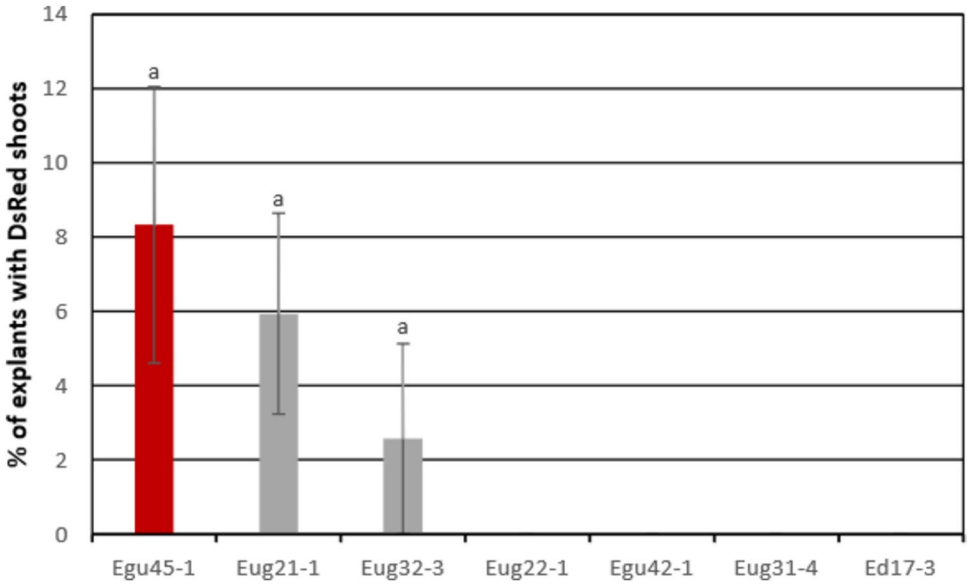

Figure 4. Rate of transgenic shoot production from the proposed Egu45-1 model clone and several comparator clones. The data given are percent of explants producing dsRed fluorescing shoots, based on an average of 5 plates per genotype. *Bars* show two standard errors shown centered on the mean. PVP10 was added to the medium in PCIM1 (pre- & co-cultivation medium, composition given in Table 1) at the rate of 2.5 g L^{-1} for this experiment, though in other work was shown to have a slightly deleterious effect on transformation rate, and no effect on necrosis rate (Supplemental Table S1). *Bars* with the same letters above them are not statistically different ($P < 0.05$) based on Tukey multiple comparison tests (there were no statistically significant differences found). The leftmost *bar* (red) shows the rate for the proposed Egu45-1 model genotype.

Genotypic variation During transformation protocol development, it is routine to experimentally validate a regeneration procedure and then develop transformation and selection procedures on top of that method. Given the clear negative responses to *Agrobacterium* treatment in many Eucalypt genotypes, whether a given clone was susceptible to *Agrobacterium* and became highly necrotic was first tested, rather than if it is regenerable in the absence of Agrobacterium. To do this, 53 genotypes were screened for *Agrobacterium* susceptibility based on callus production using T-DNA genes isolated from wild *Agrobacterium* strain C58 (*ipt*, *iaaH*, and *iaaM* under native promoter control), and a DsRed gene. There was extreme variation in transgenic callus formation rate in the absence of hormones (Fig. S19*A*-*C*; Fig. 3). The addition of a small amount of cytokinin to a subset of genotypes, mostly, did not affect ranking of transformability, except for one genotype tested, Ed15-3 (Fig. S19*D*). The variation seen among callus clusters for this genotype is shown in Fig. S19*E*. After eight wk of culture after transformation with LBA4404-thy in the absence of transgenic cell antibiotic selection or hormones, 53% of the explants from Egu45-1 produced extensive transgenic callus (Fig. 3). In contrast, 44%, 33%, and 0% explants from three high-regenerating clones identified in prior work (Eug22-1, Eug21-1, and Eg18-1, respectively) formed small or no DsRed callus (Fig. 3, Fig. S19*A*, *B* and *D*). Although some other genotypes produced 60 to 90% transgenic callus, they failed to produce transgenic shoots in subsequent transformation experiments and thus were not explored further. Egu45-1 also had the highest rate of transgenic shoot formation in a subsequent experiment where it was compared to other promising genotypes; in the presence of PVP10, Eug45-1 had the highest transformation rate, 8.3%, among seven clones tested (Fig. 4). It was therefore chosen as the best model genotype for experimental studies, with a detailed transformation protocol given in Tables 1 and 2.

Conclusion

Rather than first screening for regenerability—whose rates are often high among many clones—it was found that initially screening for susceptibility to *Agrobacterium* infection using oncogenes from the C58 strain of *Agrobacterium*, without the addition of plant hormones to the medium, led to the rapid identification of clones suitable for transgenic studies. The transformation protocol (Tables 1 and 2) with clone Egu45-1 gave transformation efficiencies of up to 8.3%, with transgenic shoot emergence within 7–8 months. The authors hope this method will be effective in other laboratories, and inspire use of Eucalyptus for scientific and biotechnology

studies in more laboratories. Efforts in our laboratory will continue to try and improve, and post to our web site, method improvements. Of highest priority for this work are efforts to mitigate necrosis, and to seek genotypes that might transform and regenerate faster and at higher rates.

In addition to the amendments to improve regeneration or transformation reported here, a number of developmental regulator genes were also tested in our laboratory in anticipation of improved production of transgenic shoots. None of the genes tested, which included overexpression of various forms of *GROWTH REGULATORY FACTOR 4 (GRF4)* (Ryan 2022), and also of *WUSCHEL (WUS), LEAFY COTYLEDON 1 (LEC1), EARLY BUDBURST1 (EEB1),* and *WUSCHEL RELATED HOMEOBOX11 (WOX11)*, gave consistent or useful results. As detailed above, it was found that most of the amendments commonly used to mitigate necrosis and improve transformation were ineffective in Eucalyptus. However, some limited benefits were seen, in some genotypes, from variations in AS, basal medium, cytokinin type, carbon source, *Agrobacterium* strain, and heating prior to cocultivation. Eucalyptus truly deserves its well-earned reputation for high recalcitrance to transformation!

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Data availability Original data, and the 45-1 eucalypt genotype, is available upon request from the corresponding author.

Declarations

Competing Interests The authors report no competing personal interests.

References

Aggarwal D, Kumar A, Sudhakara Reddy M (2011) *Agrobacterium tumefaciens* mediated genetic transformation of selected elite clone(s) of *Eucalyptus tereticornis*. Acta Physiol Plant 33:1603–1611. https://doi.org/10.1007/s11738-010-0695-3

Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono T (2017) A multipurpose toolkit to enable advanced genome engineering in plants. Plant Cell 29:1196–1217. https://doi.org/10.1105/tpc.16.00922

Chauhan RD, Veale A, Ma C, Strauss SH, Myburg AA (2014) Genetic transformation of eucalyptus - challenges and future prospects. Tree biotechnology. Edited by Kishan Gopal Ramawat, Jean-Michel Mérillon, M. R. Ahuja. CRC Press (Taylor & Francis), pp 392–425

Chavan SB, Dhillon RS, Sirohi C, Saleh IA, Uthappa AR, Keerthika A, Jinger D, Halli HM, Pradhan A, Kakade V, Morade A, Chichaghare AR, Rawale GB, Okla MK, Alaraidh IA, AbdElgawad H, Fahad S, Nandgude S, Singh R (2024) Optimizing planting geometries in eucalyptus-based food production systems for enhanced yield and carbon sequestration. Front Sustain Food Syst 8:1386035. https://doi.org/10.3389/fsufs.2024.1386035

Dan Y, Armstrong CL, Dong J, Feng X, Fry JE (2009) Lipoic acid—an unique plant transformation enhancer. In Vitro Cell Dev Biol - Plant 45:630–638. https://doi.org/10.1007/s11627-009-9227-5

de França Bettencourt GM, Soccol CR, Giovanella TS, Franciscon L, Kestring DR, Gerhardt IR, Degenhardt-Goldbach J (2018) *Agrobacterium tumefaciens*-mediated transformation of *Eucalyptus urophylla* clone BRS07-01. J Forestry Res 31:507–519. https://doi.org/10.1007/s11676-018-0777-4

de la Torre F, Rodríguez R, Jorge G, Villar B, Álvarez-Otero R, Grima-Pettenati J, Gallego PP (2014) Genetic transformation of *Eucalyptus globulus* using the vascular-specific EgCCR as an alternative to the constitutive CaMV35S promoter. Plant Cell Tiss Org Cult 117:77–84. https://doi.org/10.1007/s11240-013-0422-z

Elorriaga E, Klocko AL, Ma C, de Plessis M, An X, Myburg AA, Straus SH (2021) Genetic containment in vegetatively propagated forest trees: CRISPR disruption of LEAFY function in *Eucalyptus* gives sterile indeterminate inflorescences and normal juvenile development. Plant Biotechnol J 19:1743–1755. https://doi.org/10.1111/pbi.13588

NCBI (2023) Eucalyptus grandis x Eucalyptus urophylla genome assembly ASM2883001v1. https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_028830015.1/. Accessed 12 Jun 2025

Kew Science (2025) Eucalyptus L'Hér. Plants of the World Online. In: Plants of the World Online. http://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:27534-1. Accessed 12 June 2025

Fernando SC, Goodger JQD, Gutierrez SS, Johnson AAT, Woodrow IE (2016) Plant regeneration through indirect organogenesis and genetic transformation of *Eucalyptus polybractea* R.T. Baker. Ind Crops Prod 86:73–78. https://doi.org/10.1016/j.indcrop.2016.03.025

GIT Forestry Consulting - Consultoría y Servicios de Ingeniería Agroforestal - Eucalyptologics (n.d.) https://www.git-forestry.com/. Accessed 9 Dec 2025

Girijashankar V (2011) Genetic transformation of eucalyptus. Physiol Mol Biol Plants 17:9–23. https://doi.org/10.1007/s12298-010-0048-0

González E, Andrade A, Bertolo A, Lacerda G, Carneiro R, Defávari V, Labate M, Labate C (2002) Production of transgenic Eucalyptus

grandis × E. urophylla using the sonication-assisted Agrobacterium transformation (SAAT) system. Funct Plant Biol 29:97–102
Guo L, Zeng B, Liu Y (2013) Agrobacterium-mediated High-efficient transformation of Eucalyptus grandis Clone Eg5. Chinese Bull Bot 48:87–93. https://doi.org/10.3724/SP.J.1259.2013.00087
Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: A review. Plant Signal Behav 7:1456–1466. https://doi.org/10.4161/psb.21949
Huynh J, Hotton SK, Chan R, Syed Y, Thomson J (2022) Evaluation of novel surfactants for plant transformation. BMC Res Notes 15:360. https://doi.org/10.1186/s13104-022-06251-5
Irsyadi MB, Sari SK, Oktiastuti E, Rineksane IA, Handayani E, Aisyah SN (2026) Potential of pre-treatment in enhancing of transformation efficiency *in-planta Agrobacterium*-mediated genetic transformation on soybean *Glycine max* L.)sSeeds. Intl J Hortic Sci Technol 13. https://doi.org/10.22059/ijhst.2025.380607.904
Kaur A, Reddy MS, Kumar A (2022) Heat shock enhanced Agrobacterium tumefaciens mediated T-DNA delivery to potato (Solanum tuberosum L.). J Plant Biochem Biotechnol 31:853–863. https://doi.org/10.1007/s13562-021-00762-1
Klocko AL, Ma C, Robertson S, Esfandiari E, Nilsson O, Strauss SH (2016) FT overexpression induces precocious flowering and normal reproductive development in Eucalyptus. Plant Biotechnol J 14:808–819. https://doi.org/10.1111/pbi.12431
Mader M, Le Paslier M-C, Bounon R, Bérard A, Rampant PF, Fladung M, Leplé JC, Kersten B (2016) Whole-genome draft assembly of Populus tremula x P. alba clone INRA 717-1B4. Silvae Genet 65:74–79. https://doi.org/10.1515/sg-2016-0019
Matsunaga E, Nanto K, Oishi M, Ebinuma H, Morishita Y, Sakruai N, Suzuki H, Shibata D, Shimada T (2012) *Agrobacterium*-mediated transformation of *Eucalyptus globulus* using explants with shoot apex with introduction of bacterial choline oxidase gene to enhance salt tolerance. Plant Cell Rep 31:225–235. https://doi.org/10.1007/s00299-011-1159-y
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
Myburg A, Grattapaglia D, Tuskan G et al (2014) The genome of Eucalyptus grandis. Nature 510:356–362. https://doi.org/10.1038/nature13308
Nagle MF, Nahata SS, Zahl B, Niño de Rivera A, Tacker XV, Elorriaga E, Ma C, Goralogia GS, Klocko AL, Gordon M, Joshi S, Strauss SH (2023) Knockout of floral and meiosis genes using CRISPR/Cas9 produces male-sterility in *Eucalyptus* without impacts on vegetative growth. Plant Direct 7:e507. https://doi.org/10.1002/pld3.507
Nakano T, Tanaka S, Ohtani M, Yamagami A, Takeno S, Hara N, Mori A, Nakano A, Hirose S, Himuro Y, Kobayashi M, Kushiro T, Demura T, Asami T, Osada H, Shinozaki K (2018) FPX is a novel chemical inducer that promotes callus formation and shoot regeneration in plants. Plant Cell Physiol 59:1555–1567. https://doi.org/10.1093/pcp/pcy139
Nguyen PD, Ngo VAT, Pham VT, Pham XH (2025) Factors influencing the efficiency of *Agrobacterium*-mediated transformation in *Eucalyptus camaldulensis* × *Eucalyptus urophylla* hybrid variety CT4. Aust J Crop Sci 19:987–993. https://doi.org/10.21475/ajcs.25.19.10.p05
Oliveira-Cauduro Y de, Adamuchio LG, Bespalhok JC, Gerhardt IR, Degenhardt-Goldbach J, Bernardes M, Quoirin M (2018) Optimization of factors affecting the *Agrobacterium tumefaciens*- mediated transformation of *Eucalyptus saligna*. Rev Árvore 41. https://doi.org/10.1590/1806-90882017000300015
Ouyang LJ, Li LM (2016) Effects of an inducible aiiA gene on disease resistance in *Eucalyptus urophylla* × *Eucalyptus grandis*. Transgenic Res 25:441–452. https://doi.org/10.1007/s11248-016-9940-x
Pawar B, Kale P, Bahurupe J, Jadhav A, Kale A, Pawar S (2015) Proline and glutamine improve *in vitro* callus induction and subsequent shooting in rice. Rice Sci 22:283–289. https://doi.org/10.1016/j.rsci.2015.11.001
Pellegrineschi A, Brito R, Velazquez L, Noguera L, Pfeiffer W, McLean S, Hoisington D (2002) The effect of pretreatment with mild heat and drought stresses on the explant and biolistic transformation frequency of three durum wheat cultivars. Plant Cell Rep 20:955–960. https://doi.org/10.1007/s00299-002-0438-z
Pérez-Piñeiro P, Gago J, Landín M, Gallego PP (2012) Agrobacterium-mediated transformation of, transgenic plants - Advances and Limitations. In: PhD. Yelda Ozden Çiftçi (ed) InTech, Available from: http://www.intechopen.com/books/transgenic-plants-advances-andlimitations/agrobacterium-mediated-transformation-of. Accessed 27 Apr 2026
Polivanova O, Bedarev V (2022) Hyperhydricity in plant tissue culture. Plants 11:3313. https://doi.org/10.3390/plants11233313
Ryan NW (2022) Overexpression of the GROWTH REGULATING FACTOR 4-GRF-INTERACTING FACTOR 1 Transcription Factor Chimera Modifies Transformation and Regeneration Efficiency in Populus and Eucalyptus. MS Thesis, Oregon State University. https://ir.library.oregonstate.edu/concern/graduate_thesis_or_dissertations/s4655q40j. Accessed 27 Apr 2026
Songstad DD, Duncan DR, Widholm JM (1988) Effect of l-aminocyclopropane-l-carboxylic acid, silver nitrate, and norbornadiene on plant regeneration from maize callus cultures. Plant Cell Rep 7:262–265. https://doi.org/10.1007/BF00272538
Subbaiah MM, Minocha SC (1990) Shoot regeneration from stem and leaf callus of Eucalyptus tereticornis. Plant Cell Rep 9:370–373. https://doi.org/10.1007/BF00232401
Thanananta N, Vuttipongchaikij S, Apisitwanich S (2018) *Agrobacterium*-mediated transformation of a *Eucalyptus camaldulensis* × *E. tereticornis* hybrid using peeled nodal-stem segments with yeast HAL2 for improving salt tolerance. New for 49:311–327. https://doi.org/10.1007/s11056-017-9621-5
Tournier V, Grat S, Marque C, El Kayal W, Penchel R, de Andra G, Boudet AM, Teulières C (2003) An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* × *Eucalyptus urophylla*). Transgenic Res 12:403–411. https://doi.org/10.1023/A:1024217910354
Tu Q, Yin J, Fu J, Herrmann J, Li Y, Yin Y, Stewart AF, Müller R, Zhang Y (2016) Room temperature electrocompetent bacterial cells improve DNA transformation and recombineering efficiency. Sci Rep 6:24648. https://doi.org/10.1038/srep24648
Wang X, Chen S, Zhang H, Luo P, Zhou F, Zeng B, Xu J, Fan C (2022) *Agrobacterium*-mediated genetic transformation of the most widely cultivated superior clone *Eucalyptus urophylla* × *Eucalyptus grandis* DH32-29 in Southern China. Front Plant Sci. https://doi.org/10.3389/fpls.2022.1011245
Wang X, Wei H, Zhang S, Gao J, Zeng B, Fan C (2025) Transgenic *Eucalyptus urophylla* × *Eucalyptus grandis* superior clone DH3229: achieving glyphosate resistance through genetic modification. Plant Cell Tiss Org Cult 160:43. https://doi.org/10.1007/s11240-025-02967-x

Wang Y, Wu Z, Li X, Shang X (2024) Regeneration and genetic transformation in Eucalyptus species, Current research and future perspectives. Plants 13:2843. https://doi.org/10.3390/plants13202843

Zhou T, Lin Y, Lin Y, Luo J, Ding J (2022) Regeneration and *Agrobacterium*-mediated genetic transformation of twelve *Eucalyptus* species. For Res. https://doi.org/10.48130/FR-2022-0015

Zhou R, Jenkins JW, Zeng Y, Shu S, Jang H, Harding SA, Williams M, Plott C, Barry KW, Koriabine M, Amirebrahimi M, Talag J, Rajasekar S, Grimwood J, Schmitz RJ, Dawe RK, Schmutz J, Tsai CJ (2023) Haplotype-resolved genome assembly of *Populus tremula* × *P. alba* reveals aspen-specific megabase satellite DNA. Plant J 116:1003–1017. https://doi.org/10.1111/tpj.16454