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**K. V Mullins - D. J. Llewellyn - V. J. Hartney S. Strauss • E. S. Dennis**

# Regeneration and transformation of Eucalyptus *camaldulensis*

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**Abstract** Reliable regeneration protocols for Eucalyptus *camaldulensis using* leaf explants from in vitro-grown Introduction plants have been developed. Out of the 24 clones tested 13 were regenerated and of these, 6 showed regeneration from more than 60% of the explants. Identical protocols were also successful in the regeneration of some clones of E. m*icrotheca, E. ochrophloia, E. grandis and* E. marginata, but at lower frequencies. Co-cultivation of *E. camaldulensis* leaf explants with *Agrobacterium tumefaciens* strains carrying a kanamycin resistance gene and the reporter gene P-glucuronidase (GUS), followed by selection on kanamycin at 9 mg 1-1, allowed the selection of transformed shoots that could be rooted on selective media. Transformation of the plants was verified by staining for the GUS enzyme in various plant tissues, NptII assays and by Southern blotting on isolated DNA using specific probes for both the GUS and selectable marker genes. Transformed tissue was obtained with 5 clones of *E. camaldulensis* tested and a number of *A. tumefaciens strains*. However, only 1 clone regenerated transformed whole plants reliably.

Key words Eucalyptus ~ Regeneration ~ Transformation
**Abbreviations** BA N6-benzyladenine ~ IAA Indole acetic acid ~ NAA naphthalene acetic acid ~ X-Gluc 5-bromo-4chloro-3-indoyl glucuronide

Communicated by J. M. Widholm K.
V. Mullins' ~ V. J. Hartney2 CSIRO Forestry and Forest Products, P.O. Box 4008 Queen Victoria Terrace, ACT 2600, Australia

D. J. Llewellyn, E. S. Dennis S. Strauss 3
CSIRO Plant lndustry, P.O. Box 1600, Canberra City, ACT 2601, Australia
Present *addresses:*
P.O. Box 478, Terrigal, NSW, 2260, Australia
CSIRO Forestry and Forest Products, Locked Bag No. 2, Sandy Bay, Tasmania, 7005 Australia
Department of Forest Science, Oregon State University, Corvallis, Oregon, USA

Eucalypts are the most important plantation hardwoods in the world (Turnbull 1991), yielding industrial wood, fuelwood, essential oils, shade and shelter. *Eucalyptus camaldulensis occupies* a greater geographic range than any other eucalypt species in Australia and is now grown successfully in many countries over a wide range of habitats. Plantations of *E. camaldulensis* exceed 500 000 hectares and are increasing rapidly, especially in tropical areas (Eldridge et al. 1993).

Genetic engineering could assist the genetic improvement of *E. camaldulensis* as it enables specific traits to be added to highly selected genotypes. In contrast to conventional breeding, these traits do not have to reside within the species, but may come from any organism. For example, genes for disease and insect resistance can be transferred between micro-organisms and plants, or between plant species in different genera. As a first step in the development of a genetic engineering technology for eucalypts we have developed a gene transfer system for I clone of *E. camaldulensis based* on an efficient tissue culture procedure and co-cultivation with *Agrobacterium tumefaciens.* A. tumefaciens-mediated gene transfer is preferred over biolistic procedures for a long lived species like trees as it does not generally generate chimeric plants and is to a lesser extent troubled by the insertion of large numbers of gene copies that can lead to gene silencing in the primary transgenic plants.

Commercial applications of genetic engineering to eucalypts will not only require the development of reliable gene transfer systems but also commercial-scale techniques for vegetative propagation of elite transformants. Conventional micropropagation procedures rely on the activation of axillary buds in culture, but shoot organogenesis from leaf explants also has the capacity to generate large numbers of clones and the potential to be automated. However, the potential for tissue culture-induced somaclonal variation (Larkin and Scowcroft 1981) would need to be evaluated before shoot organogenesis procedures could be

used commercially for the micropropagation of elite or transformed clones.

In this paper we describe a method that can be used *to* successfully regenerate plants from leaf explants of *E. camaidulensis* and to transform those plants with genes novel to the eucalypt genome.

## Materials and methods

In vitro shoot cultures of a number of *Eucalyptus* clones and species were used as the source of explants for regeneration and transformation studies (Hartney and Kabay 1984). Explants were small leaves 3-5 mm long with the petiole removed. The in vitro-grown plants generally had much smaller leaves than those grown in soil. Twenty-three clones of *E. camaldulensis* representing a diverse geographical range of provenances and 12 other eucalypt species (E. *desmondensis, E. diptera, E. globulus, E. grandis, E. marginata, E. melliodora, E. microtheca, E. nitens, E. nitensxglobulus, E. ochrophloia, E. pileata,* and E. *viridis)* were tested (Tables 1, 2).

All cultures were grown under low-light conditions (photon flux density of 10 ltmol m-2 *s* 1 at 20°C). Explants were incubated on a callusing medium for 2-3 weeks, then transferred to a regeneration medium and shoot regeneration assessed after 4 weeks. Media were adapted from those of Muralidharan and Mascarenhas (1987). Callusing Medium consisted of the minerals from Woody Plant Medium (Lloyd and McCown 1981) supplemented with 1.0 g 1-1 casein, 50 g 1-1 sucrose, 0.5% (w/v) Phytagar, pH 5.9, with NAA and BA at 3.0 and 0.1 mg 1-1. respectively (16.1 and 0.45 ltM, respectively) added before autoclaving. Regeneration Medium was Callusing Medium without casein or NAA and with BA increased to 0.3 mg 1-1 (1.35 WO)

**Table 2** Regeneration abilities of different *Eucalyptus* species using the protocols outlined in Materials and methods. Individual seedlings were established in culture, and 30–60 leaf explants were used to assess regeneration ability. Regeneration is the number of explants producing shoot masses/number of explants tested

| Species | Clone | Regeneration ability |
|---|---|---|
| *E. desmondensis* | 1 | 0/30 |
| *E. diptera* | 1 | 0/60 |
| *E. glubulus* | 1 | 0/59 |
| *E. grandis* | 1 | 2/40 |
| *E. grandis* | 2 | 0/40 |
| *E. marginata* | 1 | 5/60 |
| *E. marginata* | 2 | 0/47 |
| *E. marginata* | 3 | 2/67 |
| *E. melliodora* | 1 | 0/90 |
| *E. microtheca* | 1 | 2/40 |
| *E. mircotheca* | 2 | 0/32 |
| *E. microtheca* | 3 | 2/48 |
| *E. microtheca* | 4 | 0/60 |
| *E. nitens* | 1 | 0/60 |
| *E. nitens* | 2 | 0/60 |
| *E. nitens* | 3 | 0/60 |
| *E. nitens* | 4 | 0/60 |
| *E. nitens*×*E. globulus* | 1 | 0/60 |
| *E. nitens*×*E. globulus* | 2 | 0/60 |
| *E. ochrophloia* | 1 | 17/55 |
| *E. ochrophloia* | 2 | 0/34 |
| *E. pileata* | 1 | 0/43 |
| *E. viridis* | 1 | 0/38 |

Table 1 Regeneration of *E. camaldulensis* clones collected from a range of geographical locations. Each clone represents a single seedling clonally multiplied in culture and regeneration tested from at least 30 leaf explants in each of two experiments. Clones with some regeneration ability were given a CML Identification number

| Location of seedlot | | | | Location name | Clone no. (CML no.) | Regeneration ability |
|---|---|---|---|---|---|---|
| Seedlot number | Latitude (°S) | Longitude (°E) | Altitude (m) | | | |
| 15195 | 31 53 | 141 13 | 210 | Umberumberka Creek | 1 (CML20) | +++ |
| 15195 | 31 53 | 141 13 | 210 | Umberumberka Creek | 2 | – |
| 6980 | 31 55 | 141 14 | 230 | Umberumberka Creek | 3 (CML41) | +++ |
| 6980 | 31 55 | 141 14 | 230 | Umberumberka Creek | 4 (CML52) | + |
| 7046 | 26 34 | 120 03 | 490 | Wiluna | 1 (CML42) | +++ |
| 7046 | 26 34 | 120 03 | 490 | Wiluna | 2 | – |
| 90045 | 28 31 | 120 59 | 400 | Victory Creek | 1 (CML83) | +++ |
| 90045 | 28 31 | 120 59 | 400 | Victory Creek | 2 | – |
| 90045 | 28 31 | 120 59 | 400 | Victory Creek | 3 (CML43) | +++ |
| 90037 | 25 45 | 114 16 | 11 | Wooramel River | 1 (CML44) | + |
| 90037 | 25 45 | 114 16 | 11 | Wooramel River | 2 | – |
| 90037 | 25 45 | 114 16 | 11 | Wooramel River | 3 (CML89) | + |
| 90044 | 26 31 | 120 02 | 490 | Gum Creek | 1 (CML95) | +++ |
| 90044 | 26 31 | 120 02 | 490 | Gum Creek | 2 (CML97) | ++ |
| 90034 | 29 16 | 115 00 | 20 | Irwin River | 1 (CML47) | ++ |
| 7792 | 20 11 | 119 11 | 46 | Degrey River | 1 (CML49) | ++ |
| 12342 | 15 48 | 127 53 | 30 | Pentecost River | 1 | – |
| 90030 | 35 07 | 139 17 | 8 | Swanport Bridge | 1 | – |
| 90030 | 35 07 | 139 17 | 8 | Swanport Bridge | 2 | – |
| 90050 | 31 29 | 139 21 | 95 | Wilpena Creek | 1 | – |
| 90050 | 31 29 | 139 21 | 95 | Wilpena Creek | 2 (CML87) | + |
| 10494 | 24 30 | 133 15 | 550 | Finke River | 1 | – |
| 11911 | 35 26 | 141 56 | 55 | Lake Agnes | 1 | – |
| – | 37 24 | 142 02 | 250 | Hamilton | 1 | – |

[a] No shoot regeneration; +, 1–6% of explants regenerated shoot masses; ++, 6–40% regeneration; +++, 40–100% regeneration, averaged over two experiments

Transformation was tested using 5 clones of *E. camaldulensis* and *5* wild-type or disarmed strains *of Agrobacterium tumefaciens* (A6. LBA4404, GV31 11, AGLI and GV3850). Each of the strains contained the same binary transformation vector *(An* et al. *1985)* with the p-glucuronidase (GUS) (Jefferson *1987)* and neomycin phosphotransferase-II (NptII) genes driven by the 35S promoter from cauliflower mosaic virus and the nopaline synthase promoter, respectively. *Agrobacterium* strains were grown overnight in liquid medi*um* containing Rifampicin *(50* mg *I-1)* and Tetracycline *(5* mg t-t), washed three times to remove the antibiotics and resuspended in Callusing Medium at *109* cells/ml prior to co-cultivation. Per experiment 200-300 explants were co-cultivated with *Agrobacterium* for 2 days on a Callusing Medium, washed in sterile distilled water and then transferred to Regeneration Medium containing *9* mg I-t kanamycin *as* the selective agent and 200 mg 1-1 cefotaxime to eliminate the *Agrobacterium.* Shoots formed in 3 months and were rooted on the same medium without hormones but with kanamycin and cefotaxime at the same concentrations used for regeneration of transformed shoots. Transformed plants could be maintained indefinitely in culture by repeated sub-culture to the same medium or transferred to soil. GUS analyses (Janssen and Gardner *1989),* NptII enzyme assays (McDonnell et al. *1987)* and Southern blot hybridisations (Dennis et al. *1985)* were performed using standard procedures. DNA was isolated from transformed plants by the method of Wagner et al. *(1987).*

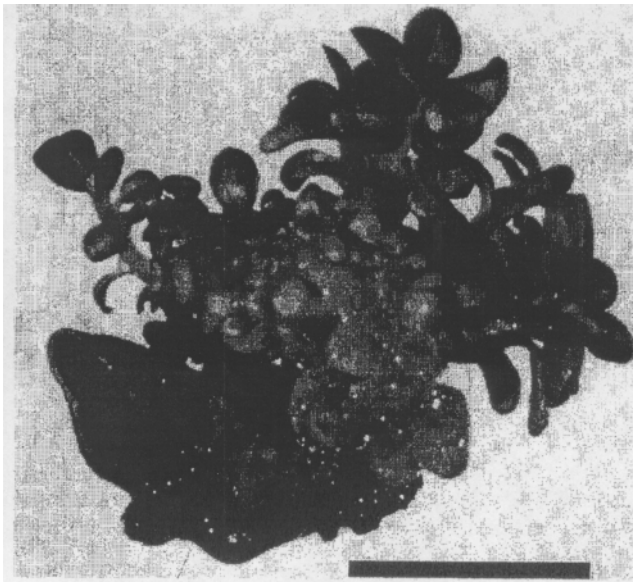

Fig. 1 Shoots regenerating leaf explant of *E. camaldulensis* clone CML43. Multiple shoots were obtained on over *90%* of leaf explants from *vitro* grown shoot cultures treated as described in Materials and methods. Bar: 5 mm

**Results and discussion**

Regeneration

Thirteen clones of *E. camaldulensis* could he reliably regenerated on two or more occasions from among the 24 clones tested (Table I ). Six of these clones exhibited regeneration at a high frequency (more than 60% of explants from these clones regenerated producing multiple shoots (Fig. 1). Regeneration was not confined to particular seedlots or provenances as different clones from within a particular seedlot had marked differences in regeneration ability. Only a limited number of clones were, however, tested within each seedlot or provenance. The clone with the highest regeneration frequency was CML43 (80-90% of explants regenerated shoots in 2 months), and this clone was subsequently used routinely for transformation experiments. Shoots also regenerated from in vitro-grown leaves *of E. grandis, E. marginata, E. microtheca* and *E. ochrophloia* using similar protocols, but in some species regeneration was at a low frequency (less than 10% of explants) (Table 2). A limited number of genotypes were tested for each species, but more extensive screening could probably identify individual clones with much higher regeneration abilities, at least within the species for which some regeneration was noted.

Transformation

A range of kanamycin and cefotaxime concentrations was tested for their effects on the regeneration capacity of a number of E'. *camladulensis* clones, including CML42, CML20 and CML43. Kanamycin concentrations of 9 mg 1-t and above inhibited shoot regeneration from most *E. camaldulensis* explants, but regeneration was not affected by cefotaxime up to 200 mg 1-1. In a typical experiment involving 250 CML43 explants co-cultivated with *Agrobacterium,* approximately 14 explants (3-6% on average) regenerated putative transformants under selection, and 6 of these (30-50% on average) could be rooted in the presence of kanamycin (10 mg 1-t) at levels that inhibited root formation in non-transformed control shoots. Callus or shoot tissue staining blue in X-Gluc solution were obtained for all 5 regenerable clones *of E. camaldulensis* tested and with all the strains of *A. tumefaciens* tested, although the clones that were the most efficient in regeneration (CML43, CML42, CML20) were also the most efficiently transformed with *Agrobacterium.* These 3 highly regenerable lines also regenerated efficiently if cultured immediately on Regeneration Medium without the long intermediate growth on Callusing Medium, although we generally used Callusing Medium for the co-cultivation period during transformation. Rooted transformed plants were only reliably obtained after 6 months with 1 clone (CML43) and with two strains of *A. tumefaciens* (AGLI and GV3850). Over 30 independent rooted transformants of clone CML43 were generated from two experiments involving 600 explants co-cultivated with either AGLI or GV3850 containing the GUS binary vector, and these were analysed biochemically for foreign gene expression. A selected few were analysed in detail at the molecular level. Over half of the plants (17 independent lines) were confirmed as transformed by either the blue staining of roots and leaves with X-gluc (not shown), and/or by NptII enzyme activity in leaves (not shown). Hybridisation with GUS and NptII probes in Southern blots confirmed foreign DNA integration in 4lines analysed (2 of which are shown

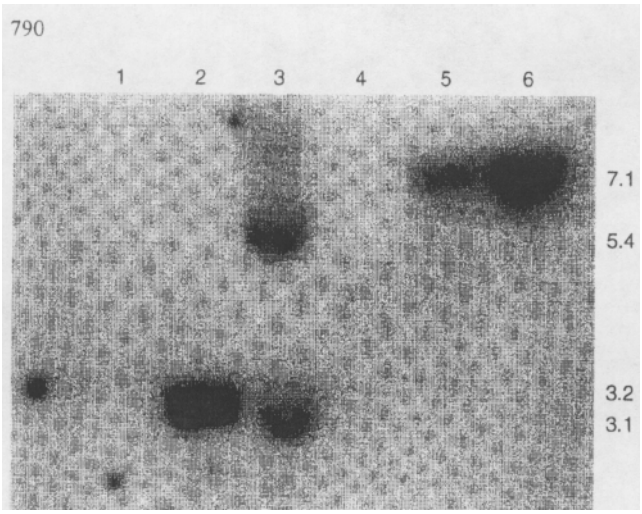


Fig. 2 Southern blot analysts of transformed *E. canaaldulensis* plants. DNA 10-Etg aliquots from two independent transformed plants *(Lanes 2, 5* and *lanes 3, 6)* and a control non-transformed plant *(lanes 1, 4)* was digested with either EcoRl *(lanes 1-3)* or *HindlII (lanes* 4-6) and run out on a *0.8%* agarose gel, depurinated, denatured and blotted to Hybond-N membrane. The membrane was probed with a nick-translated GUS fragment probe and exposed overnight to X-ray film. Molecular weights are in kilobase pairs

in Fig. 2) Southern blots on these lines using different restriction enzymes and probes (not shown) indicated that one of the transformants had an intact single-copy insertion of the T-DNA and the other had two copies, one of which had rearranged during transfer, as some of the fragments observed were not of the size expected for a complete T-DNA. We have not yet demonstrated inheritance of the transgene in any of the progeny of the primary transformants as eucalypts do not flower and set seed for 5 or more years, but clonally propagated cuttings or shoot cultures of transformants continued to express the. GUS gene for at least 2 years and were easily hardened off and transferred to soil,

## Conclusions

Although there were clearly differences among clones in their susceptibility to A. *tumefaciens,* shoot cultures of a number of clones of *E. camaldulensis* grown in vitro were amenable to transformation mediated by this plant pathogen. Using a kanamycin selection regime and a sensitive reporter gene assay we have been able to demonstrate gene transfer into *E. camaldulensis* and have produced transformed whole plants in soil with the most efficiently regenerated clone. Southern blot analysis was used to demonstrate the integration of both the NptII and GUS gene constructs in four independent plants (Fig. 2).

While inany of the parameters in the transformation protocol remain to be optimised we have developed one of the first workable systems for gene introduction into a Eucalyptus species. Even though untransformed tissues of *E. camaldulensis* can survive in the presence of kanamycin, regeneration was particularly sensitive to this antibiotic at levels as low as 9 tng 1-1, making it an effective se lective agent. When combined with a further selection for the ability of regenerated shoots to root on a similar level of kanamycin, we were able to eliminate many of the shoots :hat had "escaped" the selection. These escapes were presumably the result of cross-protection where transformed tissues degrade sufficient of the antibiotic in and around non-transformed tissues so that they can both continue to grow and regenerate shoots. Some shoots that survived both selections were subsequently shown not to be transformed when assessed for expression of the GUS gene and by Southern blots for the GUS and NptII gene, suggesting that there is still a need for further improvements in the protocol to increase the efficiency and extend it to other regenerable clones and species.

*E. camaldulensis will* offer a useful system in which to develop genetic engineering in eucalypts and in establishing the protocols for release of such transgenic trees into plantations. Useful plant traits such as tolerance to beetle and caterpillar pests or to specific herbicides are currently being introduced into this species to allow us to validate their commercial potential on a plantation scale. *E. camaldulensis* also provides a suitable model species in which to test aspects of gene function and regulation in a transgenie eucalypt.

The production of primary transformants is only one component in the package to develop transgenic trees for plantations. Of critical importance are methods to expand the individual transgenic clones into the thousands or millions of trees needed for a commercial plantation. Vegetative propagation is the preferred means of delivering these genetically improved trees to plantations because breeding and selection are slow and genes may be lost or their effects diluted through sexual reproduction. Clonal plantations of *E. camaldulensis* already exist in Nepal (White 1988), Morocco (Marien 1993) and the USA, and selected clones are also grown in Australia and overseas to ameliorate salt-affected land (Morris 1991; Marcar 1989; Midgley et al. 1986). The regeneration protocols described here may provide a useful starting point for the development of an effective micropropagation system for this species. Since *E. camaldulensis is* probably the world's most widely planted tree species in arid and semi-arid areas (Eldridge et al. 1993), it seems likely that genetic engineering will be used commercially for the genetic improvement of this species in the near future.

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