# FOREIGN GENE TRANSFER INTO *PINUS RADIATA* COTYLEDONS BY *AGROBACTERIUM TUMEFACIENS*

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## ABSTRACT

A wild type *Agrobacterium tumefaciens* strain was used to produce tumours on young *Pinus radiata* D. Don stems *in vitro*, and stable expression of the *uid*A reporter gene in this tumour tissue was confirmed histochemically. The regenerative capacity of detached cotyledons from excised *P. radiata* zygotic embryos was assessed after different treatments involved in *A. tumefaciens* infection. Exposure to acetosyringone or the antibiotic timentin did not have a negative effect on tissue health and regeneration, whereas exposure to the antibiotic cefotaxime resulted in poor tissue health. Meristematic activity and shoot elongation from very small portions of cotyledons was demonstrated. A protocol using *A. tumefaciens* to transfer the *uid*A reporter gene into adventitious-shoot-forming meristems was developed and co-cultivation parameters were improved to maximise transient expression of this reporter gene.

**Keywords**: transformation; organogenesis; *uid*A; transient expression; *Agrobacterium tumefaciens*; *Pinus radiata*.

## INTRODUCTION

With the increase in the worldwide demand for timber and for pulp and paper products, and a growing awareness of the ecological and cultural value of indigenous forests, more emphasis is being directed towards the genetic improvement of trees for plantation forestry. Conventional tree breeding programmes have considerably improved the growth and form of *Pinus radiata* (Shelbourne *et al.* 1989); however, a wide range of commercially desirable traits is not available in the breeding population and the long generation times are also a limiting factor (Carson *et al.* 1990). The development of new techniques, such as genetic engineering, provides additional opportunities for tree improvement complementing traditional breeding programmes.

Major requirements for the production of transgenic trees are, firstly, a reliable tissue culture system for plant regeneration and, secondly, an efficient transformation protocol. Although the transformation of forest tree species has generally lagged behind the genetic engineering of agricultural crops (Walter, Carson, Menzies, Richardson, Carson in press),

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protocols to genetically engineer conifers by biolistic DNA bombardment have been developed (Duchesne & Charest 1991; Stomp *et al.* 1991; Robertson *et al.* 1992; Ellis *et al.* 1993; Walter *et al.* 1994; Rey *et al.* 1996; Häggman *et al.* 1997). *Pinus radiata* embryogenic tissue has been stably transformed using a biolistic technique and transgenic trees have been regenerated (Walter, Grace, Wagner, White, Walden, Donaldson, Hinton, Gardner, Smith in press). Although this technique can be used for routine transformation of *P. radiata*, it has some disadvantages. Firstly, tissue transformed using biolistic techniques often has many copies of the integrated gene (De Block 1993; Hadi *et al.* 1996; Walter, Grace, Wagner, White, Walden, Donaldson, Hinton, Gardner, Smith in press). This may result in tandem and fragmented DNA arrangements, possibly affecting developmental and temporal gene expression (Jorgensen *et al.* 1996), leading to gene silencing (Matzke & Matzke 1995). Secondly, regeneration via embryogenesis is limited to those few *P. radiata* genotypes that form embryogenic tissue. Moreover, not all embryogenic cell lines have the ability to produce mature somatic embryos and thence plants. Therefore, the ability to produce transgenic trees from microprojectile-bombarded *P. radiata* embryogenic tissue will depend on the embryogenic capacity of a cell line prior to bombardment (L.Grace pers. comm.). To overcome these limitations, other tissue culture and transformation systems need to be investigated. Regeneration of *P. radiata* from cotyledons induced to form meristematic tissue and then adventitious shoots (Horgan & Aitken 1981) offers an alternative tissue culture system that many more *P. radiata* genotypes respond to.

*Agrobacterium*-mediated transformation techniques are generally thought to result in more-precise gene integration, with fewer copy numbers (Tinland & Hohn 1995) and less fragmentation of the transgene (Hadi *et al.* 1996). Therefore, this may provide an improved DNA transfer method, compared to biolistic techniques. Currently, the successful transfer of foreign genes into conifers by *Agrobacterium* spp. has been achieved with *Larix decidua* Mill. (Huang *et al.* 1991; Shin *et al.* 1994; Levée *et al.* 1997; M.-A.Lelu pers. comm.), *Picea sitchensis* (Bong.) Carr. (Drake *et al.* 1997; A.Wenck pers. comm.), *Pinus halepensis* Mill. (Tzfira *et al.* 1996), *P. contorta* Loud. (Yibrah *et al.* 1996), and *P. strobus* L. ( V.Levée pers. comm.). *Agrobacterium*-mediated transformation of organogenic tissue of *P. radiata* has not previously been reported despite reports that a range of *Agrobacterium* strains can successfully infect *Pinus* species (Sederoff *et al.* 1986; Ellis *et al.* 1989; Loopstra *et al.* 1990; Bergmann & Stomp 1992).

This paper describes the stable expression of the *uid*A reporter gene in crown gall cells induced on *P. radiata* stems *in vitro* after *Agrobacterium tumefaciens* infection. Evidence is presented for the successful transfer and transient expression of the *uid*A gene in *P. radiata* adventitious-shoot-forming meristems transformed with *A. tumefaciens*. Factors which contributed to improving the transformation protocol are also discussed.

## MATERIALS AND METHODS

### Plant Material

Seeds from open-pollinated *P. radiata* were obtained from Proseed, New Zealand. Seeds were surface sterilised, and zygotic embryos aseptically removed (Aitken-Christie *et al.* 1988) and maintained on modified half strength Le Poivre (LP) nutrient medium containing 5 ppm benzylaminopurine ($^1/_2$LP5) (Aitken-Christie *et al.* 1988). After 1, 3, 6, or 9 days,

cotyledons were detached and placed on fresh $^{1}/_{2}$LP5 either intact, or as longitudinally or diagonally cut segments. All the cotyledons from one embryo were grouped together. Cotyledons from six embryos were placed on each plate of media and there were eight plates per treatment. After 21 days, cotyledons were assessed for the proliferation of meristematic tissue using the health scores detailed in Table 1. All the cotyledons from individual embryos were given a collective health score and the average health score was calculated per plate. Surviving cotyledons were then transferred to hormone-free Le Poivre medium (LPO) (Aitken-Christie *et al.* 1988) and shoot elongation was assessed after a further 28 days. The effect on the health and regeneration of intact and cut cotyledons of adding acetosyringone (1 mg/*l*) and the antibiotics, cefotaxime (500 mg/*l*) and timentin (200 mg/*l*), into $^{1}/_{2}$LP5 was assessed visually using the health scores (Table 1). Cultures were maintained at a 16-h photoperiod (80 $\mu\varepsilon/m^2\cdot s$) and a 26°C/15°C day/night temperature regime.

TABLE 1–Health scores for meristematic tissue developing on *Pinus radiata* cotyledons after 21 days on $^{1}/_{2}$LP5 media

| Score | Description |
|---|---|
| 0 | Cotyledons were dead |
| 1 | Cotyledons showed some greening but no meristematic tissue. |
| 2 | Green meristematic tissue proliferation observed on 25% of cotyledons |
| 3 | Green meristematic tissue proliferation observed on 50% of cotyledons |
| 4 | Green meristematic tissue proliferation observed on 75% of cotyledons |
| 5 | Yellow meristematic tissue proliferating all over cotyledons |

Note: All of the detached cotyledons from one embryo were given a collective score

## Bacterial Strains

*Agrobacterium tumefaciens* strains A281 and EHA101 (Hood *et al.* 1986) harbouring the binary vector pKIWI105 (Janssen & Gardner 1989) were used in all experiments. The plasmid pKIWI105 contains the *uid*A gene fused to the CaMV 35S promoter, and the *npt*II gene controlled by the *nos* promoter. The *uid*A gene in this construct was expressed in plant tissue but not in *Agrobacterium* as it lacks a bacterial binding site (Janssen & Gardner 1989). The disarmed strain EHA101 shares the same genetic background as A281 and was used in inoculation experiments as a negative control for tumour induction.

## Inoculation of *Pinus radiata* Seedlings with *Agrobacterium tumefaciens*

The stems of 2-week-old, *in vitro*-germinated, *P. radiata* or tobacco seedlings were inoculated with the two *A. tumefaciens* strains, A281 or EHA101, by making an incision with a sterile scalpel which had been dipped in *A. tumefaciens* cultured on YN agar for 24 h (Janssen & Gardner 1989). Inoculated plants were maintained in the culture conditions previously described. The production of tumours was assessed over a period of 3 months. Tumour tissue was excised from stems and grown aseptically on LPO. After 6 months, tissue was longitudinally sliced and stained for the presence of *uid*A (Jefferson *et al.* 1987) using a modified protocol as described by Walter *et al.* (1994).

### Transformation of Detached Cotyledons of *Pinus radiata*

*Agrobacterium tumefaciens* A281 (pKIWI105) was grown from a single colony in YN broth (pH 7.3) supplemented with kanamycin (at 100 mg /*l*) on a rotary shaker for 48 h at 28°C. An aliquot of this culture was diluted 1:100 in YN broth (pH 5.6) containing kanamycin (100 mg/*l*) and acetosyringone (1 mg/*l*), and grown to an $OD_{550}$ of 0.7–1.1. The pH of the growth media was lowered because acetosyringone is more effective at an acidic pH. In initial experiments, the *A. tumefaciens* suspension was used for inoculating cotyledons but in later experiments it was pelleted by centrifugation at 4000 rpm, resuspended in the original volume of LPO, and acetosyringone (1 mg/*l*) was added.

Prior to inoculation, cotyledons (or in preliminary experiments, whole embryos) were supported on nybolt mesh (Scapa Filtration, 30 μM) or filter paper (Whatman No. 1, 70 mm) and, where stated, desiccated for 15 min by exposure to a sterile airflow in the laminar flow hood. Cotyledons or embryos were inoculated with 500 μl of *A. tumefaciens* by dispensing aliquots of the bacterial solution directly on to the explants and they were co-incubated for 5, 10, 15, 20, 25, or 30 min at room temperature. Alternatively, detached cotyledons were immersed in 500 μl *A. tumefaciens* in 1.5-ml eppendorf tubes for 5 min. Excess *A. tumefaciens* was removed by rinsing the explants on the filters twice with sterile distilled water (with or without timentin at 200 mg/*l*) using a standard filter apparatus (Nalgene). Cotyledons were transferred to ½LP5 solid media containing acetosyringone (1 mg/*l*) for 1, 3, 5, or 7 days' co-cultivation and then transferred from filters to initiation medium (½LP5) containing timentin at 200 mg/*l*. Control cotyledons were inoculated with sterile LPO liquid and transferred in the same way. Four days after *A. tumefaciens* inoculation, explants were stained for *uid*A activity (Jefferson *et al.* 1987) using a modified protocol as described by Walter *et al.* (1994). Expression of *uid*A was estimated by the number of cotyledons or embryos with blue spots and the average number of blue spots per *uid*A-positive explant.

### Data Analysis

Analysis of variance (ANOVA) was carried out to find the effects of day of detachment and media on health scores using the SAS GLM procedure (SAS Institute 1988). The least square means test was adopted to compare the means of treatment where the effect of the treatment was significant from the ANOVA. Each variable was examined and transformed appropriately to satisfy the assumptions of the ANOVA before it was carried out.

## RESULTS AND DISCUSSION

### Susceptibility of *Pinus radiata* Seedlings to *Agrobacterium tumefaciens* Infection

To assess the virulence and DNA transfer of two *A. tumefaciens* strains, the stems of young *P. radiata* seedlings were inoculated with A281 and EHA101 containing pKIWI105. For A281, small tumours (diameter 2–3 mm) were visible 12 weeks after inoculation (Fig. 1). A total of 1500 inoculations were made and 1.3% of infected sites gave rise to tumours. These continued to grow and excised tumour tissue was further propagated on LPO for a period of more than 6 months. Tissue was stained for expression of *uid*A and blue multicellular areas were detected in all 19 tumours (Fig. 2). As expected, inoculation of

*P. radiata* shoots *in vitro* with the disarmed *A. tumefaciens* strain EHA101 containing the vector pKIWI105 did not produce tumours (1500 inoculation sites). Controls with tobacco confirmed that both A281 and EHA101 (both containing pKIWI105) were competent for DNA transfer and were able to transfer the *uid*A gene into plant tissue (data not shown). Our finding that young *P. radiata* seedlings were susceptible to *A. tumefaciens* confirms the previous findings of Stomp *et al.* (1990) and Morris *et al.* (1989). Stomp *et al.* (1990) demonstrated that the degree of gall formation was dependent on the strain of *Agrobacterium*, the degree of woodiness, and the age of the plant. The *Agrobacterium* strain A281 was used in our studies because of the previous findings that related strains were highly infective on *P. radiata* tissue culture shoots (Bergmann & Stomp 1992) and that A281 was useful in transforming recalcitrant species such as *Picea abies* (L.) Karsten (Hood *et al.* 1989) and pines (Loopstra *et al.* 1990; Stomp *et al.* 1990).

## Effect of Acetosyringone, Antibiotics, and Various Handling Procedures on the Regeneration Capacity of Detached Cotyledons

As acetosyringone is effective in inducing *Agrobacterium* virulence genes (Bolton *et al.* 1986), the regeneration potential of cotyledons exposed to this phenolic compound was assessed. There was no significant difference in mean health of cotyledons maintained on $^1/_2$LP5 supplemented with acetosyringone (1 mg/*l*), irrespective of the day on which cotyledons were detached (Fig. 3). The presence of the correct signal molecule (such as acetosyringone or α-hydroxyacetosyringone) is one of the important factors contributing to the virulence of *Agrobacterium* (Stachel *et al.* 1985; Melchers *et al.* 1989). The lack of any detrimental effect on meristem regeneration means that acetosyringone could be utilised in transformation experiments to increase the efficiency of *Agrobacterium*-induced transfer in this species.

A successful transformation protocol relies on the effective removal of *Agrobacterium*, which is usually achieved by the application of antibiotics. Cotyledons maintained on media containing timentin (200 mg/*l*) showed no decrease in health compared to those maintained without an antibiotic (Fig. 4A). Indeed, at one time point (when cotyledons were detached and cultured on $^1/_2$LP5 one day after dissection ), explant health was significantly ($p< 0.005$) better on timentin than on media containing cefotaxime (500 mg/*l*) or on an antibiotic-free medium (Fig. 4A). After tissue had been maintained on LPO for a further 28 days, shoot elongation and mean health were significantly better ($p<0.01$) when cotyledons were maintained either on antibiotic-free media or on media containing timentin (Fig. 4B). Overall, cefotaxime appeared detrimental to health and subsequent shoot elongation was poor (Fig. 4B); therefore, in subsequent experiments timentin was used as a decontaminating agent. The toxic effect of cefotaxime on plant regeneration has also been observed with other plant species. For example, cefotaxime inhibited bud formation and elongation in *Picea glauca* (Moench) Voss embryos and leaf disk regeneration in tomato (Ellis *et al.* 1989); however, no toxic effect was observed with either *Populus nigra* L. (Confalonieri *et al.* 1994) or hybrid larch (Levée *et al.* 1997).

The health of detached cotyledons was assessed after different treatments involved in the *Agrobacterium* transformation protocol (Table 2) to ensure that cotyledons would survive additional handling procedures. Plant health and regeneration were improved when cotyledons

TABLE 2–Effect of different treatments involved in *Agrobacterium* transformation on regeneration

| Factor assessed | Treatments* | Outcome |
|---|---|---|
| Filter support (on ½LP5 media) | Nybolt<br>Whatman filter | Nybolt is preferred as cotyledons regenerated poorly on filter paper. Filter paper also harboured *Agrobacterium,* causing overgrowth |
| Virulence-inducing agent agent | Acetosyringone (1 mg/*l*) | No significant effect on health and regeneration (Fig. 3) |
| Resuspension media for *Agrobacterium* | YN or LPO | Regeneration improved if *Agrobacterium* was resuspended in plant tissue culture media (LPO) |
| Incubation time | Incubate cotyledons in *Agrobacterium* for 5, 10, 15, 20, 25, or 30 min. | Longer incubation did not lead to increased overgrowth |
| Washing solution | Sterile water<br>Sterile water + timentin (200 mg/*l*) | Both equally effective in preventing *Agrobacterium* overgrowth—timentin not necessary |
| Antibiotic-containing medium | Cefotaxime (500 mg/*l*)<br>Timentin (200 mg/*l*) | Cefotaxime generally detrimental to health and regeneration while timentin has no significant effect on regeneration (Fig. 4) |

* A "no treatment" control was included in all experiments

were supported on nybolt mesh rather than filter paper. Filter paper harboured *A. tumefaciens,* which resulted in overgrowth and tissue damage (data not shown). It appeared that nybolt allowed better diffusion of nutrients and antibiotics, which resulted in more efficient meristematic activity and the prevention of *A. tumefaciens* overgrowth. Explant health and regeneration improved if cotyledons were inoculated with *A. tumefaciens* which had been resuspended in LPO, rather than in YN. Cotyledons may have been sensitive to one or more of the bacterial growth agents present in YN. This result confirmed an earlier observation that YN was detrimental to *P. radiata* tissue health (J.Gemmell unpubl. data).

Incubation in *A. tumefaciens* cultures for increasing lengths of time (5, 10, 15, 20, 25, or 30 min) did not result in overgrowth of *A. tumefaciens,* allowing cotyledons to be incubated longer if required by the dictates of the experiment. In general, the length of co-incubation does not affect transient expression (Drake *et al.* 1997; R.Gardner pers. comm.), and so this was assumed to be true for *P. radiata*. Various washing procedures to remove *A. tumefaciens* after co-cultivation were also evaluated. Cotyledons were washed in sterile water either with or without timentin at 200 mg/*l*. The sterile water alone was as effective in controlling overgrowth of *A. tumefaciens* as sterile water plus timentin. The addition of the antibiotic timentin (200 mg/*l*) to ½LP5 medium successfully prevented *A. tumefaciens* overgrowth, but cefotaxime (500 mg/*l*) was not effective.

From these experiments, an improved procedure was adopted, whereby *A. tumefaciens* was resuspended in LPO and cotyledons were incubated for up to 30 min, washed in water, co-cultivated for 3 days on nybolt on ½LP5 containing acetosyringone (1 mg/*l*), and then transferred to ½LP5 containing timentin (200 mg/*l*).

FIG. 1–*Agrobacterium* induces tumours on *in vitro* shoots of *P. radiata*. Tumours were induced on stems by inoculating *P. radiata* seedlings *in vitro* with *A. tumefaciens* A281 containing the binary vector pKIWI105. After 3 months, 1.3% of infected sites gave rise to tumours similar to that shown.

FIG. 2–The *uid*A gene is expressed in tumour tissue of *P. radiata*. Tumours excised from infected *P. radiata* seedlings were maintained in cell culture for 6 months, sliced longitudinally, and stained for *uidA* expression.

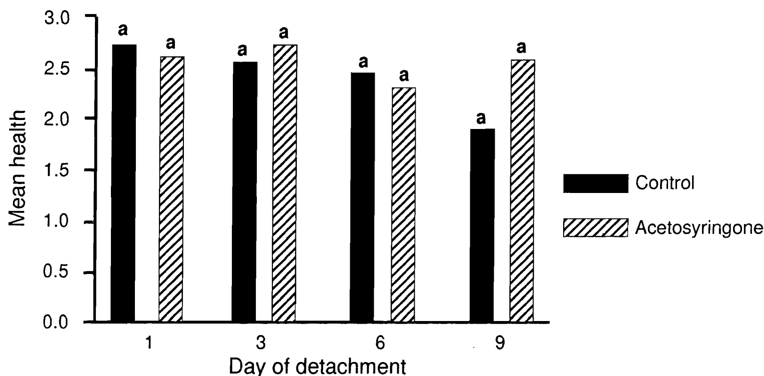


FIG. 3–Acetosyringone in the media does not affect tissue health. Health scores of cotyledons detached after 1, 3, 6, and 9 days and grown for 21 days on ½LP5 containing acetosyringone. Each bar represents the average mean health (*see* Table 1) of 120–150 cotyledons, and treatments not bearing the same letter are different at the 0.5% level of significance.

## Transformation of Detached Cotyledons and Factors Influencing Transient Expression

A protocol to transform detached *P. radiata* cotyledons was developed using transient expression of the *uid*A reporter gene as an assay. Blue areas on *uid*A-positive cotyledons frequently occurred close to the detached end or wound site (Fig. 5). Both single cells and multicellular areas were stained blue. Control cotyledons, which had not been inoculated, did not display any *uid*A expression as assayed histochemically.

Preliminary experiments using either whole embryos or detached cotyledons identified a number of different factors that influenced the level of transient expression of *uid*A.

(1) Resuspending *A. tumefaciens* cultures in LPO rather than YN increased transient *uid*A expression (Fig. 6A). This effect may simply reflect the improved survival and health of the explant (Table 2), or may indicate a separate effect on gene transfer.

(2) In three independent experiments transient expression also increased if embryos were desiccated in a sterile airflow in a laminar flow hood for 15 min prior to *A. tumefaciens* infection (Fig. 6B). We presume that desiccation made the explants more receptive to liquid absorption, and therefore promoted *A. tumefaciens* uptake.

(3) Media supplemented with acetosyringone (1 mg/*l*) increased transient expression (Fig. 6C) while having no detrimental effect on health (Fig. 3). Acetosyringone has been demonstrated to promote *Agrobacterium* transformation of *Arabidopsis* (Sheikholeslam & Weeks 1987) and recently was found to increase transient *uid*A expression in embryogenic cells of *Picea sitchensis*, although the effect was not proportional to acetosyringone concentration (Drake *et al.* 1997). Similarly, an increase in gene transfer frequency has been described for *Agrobacterium*-mediated transformation of *Picea abies* (A.Wenck pers. comm.) and *Pinus strobus* (V.Levée pers. comm.) if acetosyringone was used. Conversely for *Agrobacterium*-mediated transformation of hybrid larch, the

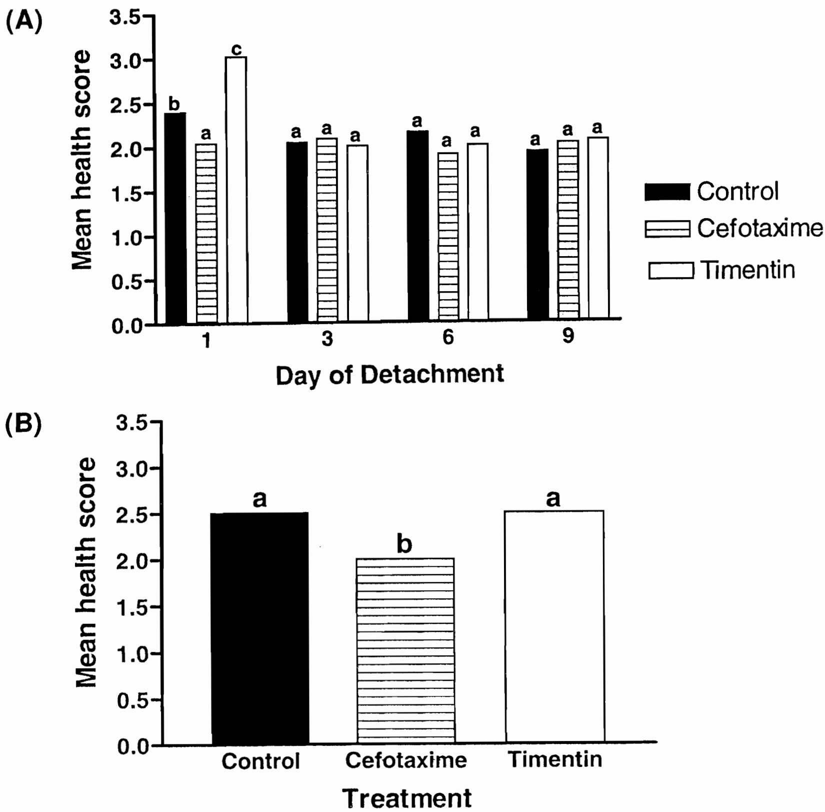


FIG 4—Mean health of cotyledons detached after 1, 3, 6, and 9 days, grown on media containing cefotaxime and timentin.

(A) Assessment of meristematic activity of cotyledons treated with cefotaxime or timentin after 21 days on 1/2LP5. Treatments were significantly different ($p<0.005$) only 1 day after detachment. Each bar represents the average mean health of 120–150 cotyledons, and treatments not bearing the same letter are different at the 0.5% level of significance.

(B) Progressive development of cotyledons in (A)—health scores of shoots which developed on cotyledons treated with cefotaxime or timentin, after 28 days on LPO. Treatments not bearing the same letter are different at the 1% level of significance.

addition of acetosyringone did not correlate with an increase in transformation efficiency (Levée *et al.* 1997).

(4) Increasing the length of co-cultivation time (on media containing acetosyringone) showed that transient *uid*A expression was highest after a 3-day co-cultivation period (Fig. 6D). Tzfira *et al.* (1996) demonstrated that *uid*A transient expression in embryos or seedling explants of *Pinus halepensis* infected with *A. rhizogenes* increased with longer co-cultivation periods. However, they also found that increasing co-cultivation time contributed to a considerable decrease in explant survival, which was due mainly to *Agrobacterium* overgrowth. For *Picea sitchensis*, Drake *et al.* (1997) found that transient expression of *uid*A in embryogenic tissue inoculated with *A. tumefaciens* was 15-fold higher after 3 days' co-cultivation than after 2 days.

FIG. 5—*Uid*A expression in cells of detached cotyledons. *Pinus radiata* cotyledons transformed with *A. tumefaciens* A281 (pKIWI105) had several blue spots which frequently occurred close to the detached end or wound site.

Based on the results detailed above, a protocol was developed that reliably gave 10% of cotyledons which expressed *uid*A (average of seven experiments, each with at least 500 cotyledons). To further improve the transformation protocol, detached cotyledons were immersed in *A. tumefaciens*, rather than dispensing the bacterial suspension directly on to the cotyledons. As a result, in two replicate experiments, about a two-fold increase in transient expression was observed (Fig. 6E) with an average of 16% and 24% of cotyledons expressing *uid*A, respectively. It is possible that *A. tumefaciens* was more likely to attach to the cotyledons and therefore integrate DNA when in continuous contact with the explants. In contrast to this result, Levée *et al.* (1997) found no difference in the amount of kanamycin-resistant embryogenic tissue recovered after transformation with *A. tumefaciens*, regardless of whether tissue was co-cultivated in diluted bacterial solution, or dispersed directly on to the embryogenic masses.

## Health and Regeneration of Cut *v.* Intact Cotyledons

Finally, the regenerative capacity of detached cotyledons which had been diagonally or longitudinally cut was assessed relative to intact cotyledons. The purpose of these additional cuts was to improve access of *Agrobacterium* and also to create an area of wounding so that plant cells would produce phenolic compounds to induce the *Agrobacterium vir* genes (Hooykaas & Beijersbergen 1994). The health of intact and cut cotyledons (detached after 1, 3, 6, or 9 days) was significantly ($p<0.005$) affected by the type of cut and the day of detachment (Fig. 7A, B), respectively. There was also a significant interaction between the

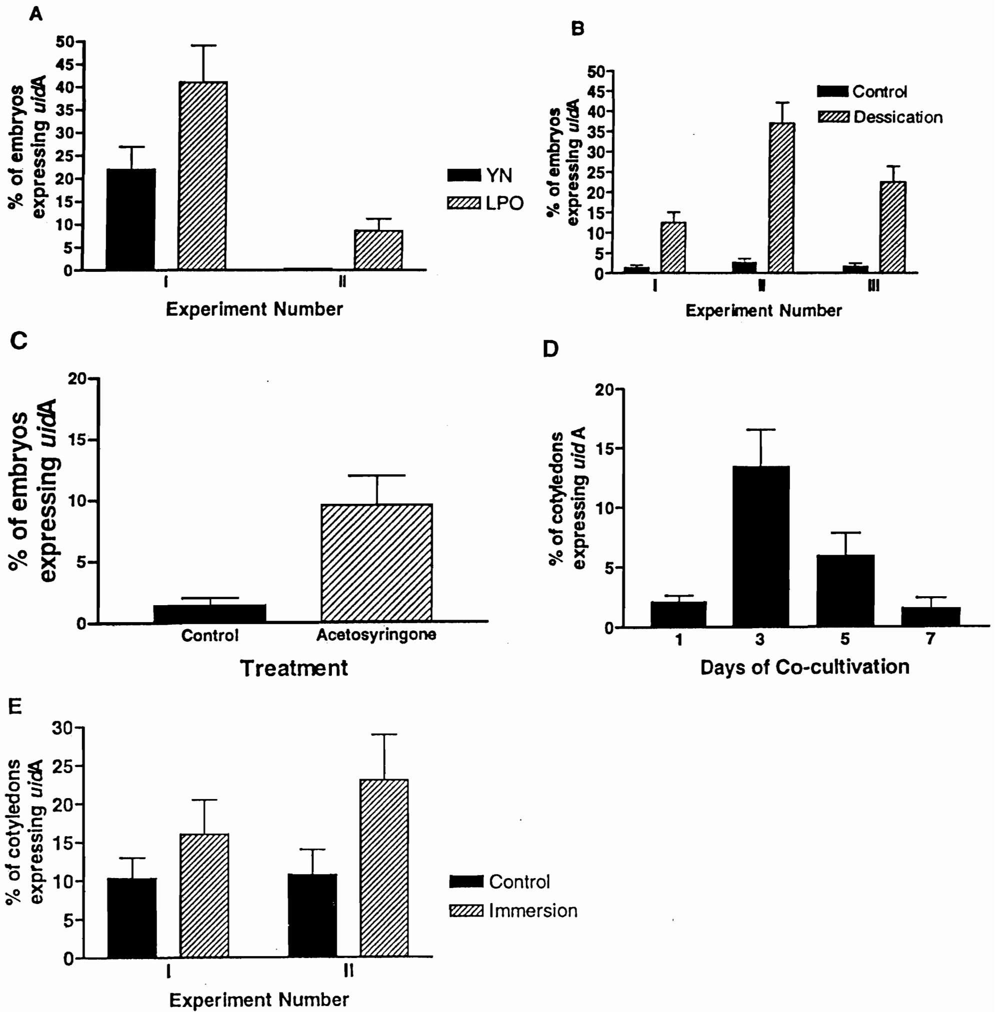


FIG. 6–Effect of co-cultivation parameters on transient expression of *uid*A. All graphs show the percentage of whole embryos or detached cotyledons expressing *uid*A transiently, after inoculation with *A. tumefaciens* A281 containing pKIWI105. Where appropriate, data from at least two experiments are presented. The shaded bars represent the average of at least 36 embryos or 150 cotyledons and the vertical bars are the standard error of the mean for each treatment.

(A) Resuspending *Agrobacterium* in LPO instead of YN liquid increased transformation efficiency of 1-day-old embryos.

(B) Desiccating embryos prior to inoculation increased *uid*A expression.

(C) Addition of acetosyringone to *Agrobacterium* inoculum and co-cultivation media increased *uid*A expression.

(D) The percentage of cotyledons expressing *uid*A transiently peaked 3 days after co-cultivation.

(E) Transient *uid*A expression increased if cotyledons were immersed in *Agrobacterium*.

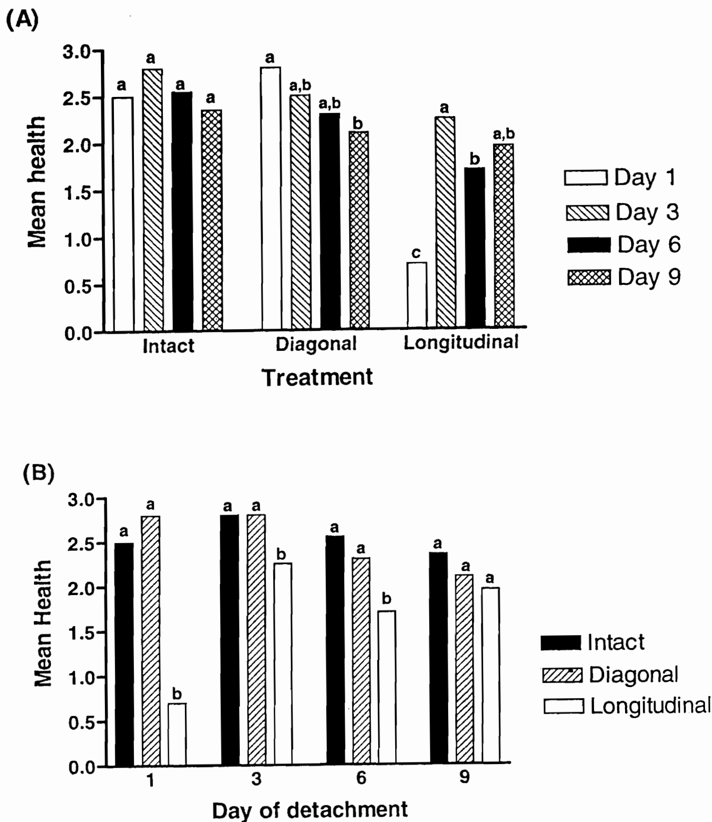


FIG. 7–Timing and manner of cutting affect health of excised cotyledons.
(A) Effect of increasing detachment time on tissue health of cotyledons which had been left intact or cut diagonally or longitudinally.
(B) The same data replotted to compare different cutting treatments 1, 3, 6, or 9 days after detachment. For both graphs, each bar represents the average mean health score of 120–150 cotyledons. Treatments not bearing the same letter are different at the 0.5% level of significance.

two variables. When cotyledons remained intact, health was not dependent on the day of detachment (Fig. 7A). When cotyledons were cut diagonally, those detached and cut at day 1 were significantly healthier than those detached at day 9 (Fig. 7A). The health score of cotyledons which were cut longitudinally was significantly better when cotyledons were detached at days 3, 6, and 9 than after only 1 day (Fig. 7A). Regardless of which day cotyledons were detached, a diagonal cut did not significantly affect the formation of adventitious-shoot-forming meristems compared to those left intact (Fig. 7B). Diagonal cuts had the least effect on health, possibly because they generated a smaller wound site whereas cutting longitudinally may have caused more damage to cells capable of regeneration. Cutting cotyledons longitudinally significantly decreased ($p<0.005$) mean health on all days of detachment except day 9 (Fig. 7B) and it was also technically more difficult and time consuming.

After 28 days on shoot elongation medium, the mean health of shoots on intact cotyledons and diagonally cut cotyledons was significantly better than the mean health of shoots on longitudinally cut cotyledons on all days of detachment except day 9 (data not shown).

Our results demonstrated that very small pieces of *Pinus radiata* cotyledons were capable of regeneration and they developed a high proportion of meristematic tissue. Meristematic tissue is likely to be transformed more efficiently than non-meristematic tissue (Grimsley *et al.* 1988) which may be related to the fact that rapidly dividing tissue is more amenable to transformation (Stomp *et al.* 1990). *Agrobacterium* co-cultivation of smaller explants may minimise the ratio of untransformed tissue to transformed tissue, thus reducing the production of toxins from the death of untransformed tissue during regeneration. In addition, *Agrobacterium* may have better access to cells and selection procedures may prove more effective with smaller explants. In preliminary experiments using the small explant system described above and an improved transformation protocol, transient *uid*A expression was higher in diagonally cut cotyledons than in those left intact (J.Charity *et al.* unpubl. data).

Although expression of *uid*A was sufficient in the experiments presented here to determine transformation parameters, it could possibly be further improved if the CaMV 35S promoter, which has been found to give low *uid*A expression in *P. radiata* embryogenic tissue (Walter *et al.* 1994), was replaced with a more efficient promoter. For example, in some conifers, the expression of *uid*A under the control of a sunflower ubiquitin gene (Rey *et al.* 1996; Häggman *et al.* 1997) or a double 35S (+ Kozak) promoter (Walter *et al.* 1994) increased compared to *uid*A expression driven by a CaMV 35S promoter. Besides promoters, other factors which may further improve the transformation protocol are currently being assessed. For example, we are assessing the performance of different *A. tumefaciens* strains with various chromosome backgrounds. Some of these have been shown to influence transient *uid*A expression (Janssen & Gardner 1989; Drake *et al.* 1997; A.Wenck pers. comm.), although for hybrid larch embryogenic tissue, transient expression was not strain-dependent (Levée *et al.* 1997).

## CONCLUSIONS

The detection of *uid*A in cultured gall cells, 6 months after inoculation, demonstrated that *A. tumefaciens* can be used successfully to insert foreign genes into *P. radiata.* After co-cultivation with *A. tumefaciens*, transient expression of the *uid*A reporter gene in *P. radiata* cotyledons was achieved. Transient expression increased, in order of importance, if:

(1) *Agrobacterium tumefaciens* was resuspended in plant media rather than bacterial growth media;

(2) Cotyledons were desiccated prior to inoculation;

(3) Acetosyringone was present in the media.

Transient *uid*A expression peaked after 3 days of co-cultivation and increased if explants were immersed directly in the *A. tumefaciens* suspension. Regeneration was not affected by addition of the virulence-inducing agent acetosyringone or by the antibiotic timentin. Very small pieces of *Pinus radiata* cotyledons were capable of regeneration, rendering them suitable as explants for *Agrobacterium*-mediated transformation. Future experiments are aimed at further optimisation of the transformation system and the development of selection protocols to regenerate stably transformed tissue and plants.

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