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Transformation of an Australian Variety of *Carica papaya* using Microprojectile Bombardment

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Abstract. We have developed a method for the stable transformation and regeneration of a dioecious Australian cultivar of *Carica papaya* (papaw or papaya) by microprojectile bombardment. This method was developed after investigation of both zygotic and somatic embryos as target tissue and optimisation of a number of parameters using transient expression of the *uid*A reporter gene. The tissue culture regime prior to bombardment was critical in optimisation of transformation. Factors such as age of embryos and various treatments prior to bombardment, increased transient expression by up to 22-fold. Highest *uid*A transient expression results were obtained when somatic embryos 3 weeks since last subculture on solid medium were given a 3 day treatment in liquid medium, and a 2 h osmotic treatment pre- and post-bombardment. Stably transformed plants were obtained 6 months after bombardment using this system. Transformation efficiency was high with two experiments yielding 45% and 37.5% of bombarded plates regenerating plantlets on media containing kanamycin. The presence of both the *uid*A and *aph*A genes, (selectable marker) which code for the enzymes β-glucuronidase (GUS), and neomycin phosphotransferaseII (NPTII) respectively, was confirmed in regenerated plantlets bySouthern hybridisation.

Introduction

Carica papaya L. (papaw or papaya) is an important fruit crop in many tropical and subtropical countries. However, this crop is affected by a range of diseases, the most important of which is caused by papaya ringspot potyvirus type P (PRSV-P). This virus causes a severe mosaic on the leaves, stunting and ringspots on the fruit and is sometimes lethal. PRSV-P has devastated papaya crops in many papaya producing countries and has recently been recorded in Australia (Thomas and Dodman 1993), where it is currently restricted to south-east Queensland, primarily through eradication of infected plants and quarantine measures. However, in many other countries, conventional virus control strategies have been ineffective. Transgenic resistance based on virus-derived transgenes is an alternative control strategy (Gonsalves and Slightom 1993). Significant levels of protection against a Hawaiian isolate of PRSV-P have been reported in papaya (cv. 'Sunrise') expressing the homologous viral coat protein (Fitch *et al.* 1992). However, these plants were not protected against the Australian isolate of PRSV-P (Gonsalves and Slightom 1993; Tennant *et al.* 1994). For implementation of transgenic resistance in the Australian papaya industry, it is therefore necessary to develop resistance in Australian cultivars of papaya against Australian isolates of PRSV-P. An essential component of this strategy is an efficient papaya transformation and regeneration protocol.

Papaya has been previously transformed using *Agrobacterium tumefaciens* (Fitch *et al.* 1993; Yang *et al.* 1996); however, few transgenic plantlet lines were produced and Fitch *et al.* (1993) reported loss of some lines due to *A. tumefaciens* contamination. Transformation using microprojectile bombardment of both somatic and zygotic papaya embryos has been reported (Fitch *et al.* 1990; Cabrera-Ponce *et al.* 1995) and was shown to be more effective for both embryo types than *A. tumefaciens* mediated transformation (Fitch *et al.* 1990). The major limiting factor of these methods was the reliance on availability of green fruit of a specific age as a source of immature embryos and/or the extended periods of time before initiation of somatic embryos.

The induction of somatic embryogenesis prior to transformation has been previously reported from immature zygotic embryos (Fitch *et al.* 1990) and hypocotyl callus (Fitch *et al.* 1993) of four Hawaiian papaya cultivars and petiole callus of a Taiwanese cultivar (Yang *et al.* 1996). The efficiency of induction of somatic embryos differed between experiments and cultivars, possibly related to variation in the age of explant and genotype.

It has also been reported that the biological state of the tissue used for transformation has an effect on

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transformation efficiency and the quality of resultant transgenic plants (De Block 1993; Vain *et al.* 1993; Yibrah *et al.* 1994). Factors such as time of bombardment after subculture (Yibrah *et al.* 1994), osmotic treatment (Perl *et al.* 1992; Vain *et al.* 1993; Livingstone and Birch 1995), and culture regime (Perl *et al.* 1992; Vain *et al.* 1993) have been manipulated prior to transformation to improve the state of the target tissue.

Drew *et al.* (1994) have previously developed a secondary embryogenesis system for initiation of somatic embryos from immature zygotic embryos from Australian cultivars of papaya which had the potential for use in the development of a transformation system. In this paper, we report the optimisation of a number of parameters using transient expression and the stable transformation of an Australian papaya cultivar using somatic embryos.

Materials and Methods

Initiation of Somatic Embryogenesis

Immature zygotic embryos were excised from green fruit of a high yielding, dioecious, south-east Queensland papaya line (OE) 90–100 days after pollination and were either used directly for transformation or were induced to form somatic embryos. Somatic embryos were induced by placing zygotic embryos in a liquid medium consisting of 1/2 MS (Murashige and Skoog 1962), 2 μM 6-benzylaminopurine (BAP), 0.5 μM α-naphthaleneacetic acid (NAA), 400 μM adenine sulfate and 3% sucrose, pH 5.65 (Drew *et al.* 1994). Cultures were placed on an orbital shaker (70 rpm) at 25°C, with a 16 h photoperiod. Callused cotyledons were removed and discarded after 2–3 weeks. After a further 2 months on the shaker without subculture, the medium was replaced with embryo multiplication (EM) medium (1/2MS, 0.5 μM BAP, 0.05 μM NAA and 3% sucrose). Once secondary embryos were formed on this medium, they were multiplied approximately 10-fold on solid EM medium containing 0.8% Difco Bacto agar (Drew *et al.* 1994). Somatic embryos were maintained on this medium and subcultured monthly.

Culture of Zygotic Embryos Prior to Bombardment

Immature zygotic embryos were excised and placed on 0.8% water agar for 0–8 days before bombardment. The effect of osmotic treatment on transformation efficiency of zygotic embryos was assessed by culturing embryos on a water soaked, sterile filter paper disk lying on the surface of a medium containing 0.25 M mannitol and 0.8% agar in a petri dish (Perl *et al.* 1992). Zygotic embryos were cultured for 2 or 4 h on osmotic medium before and after bombardment. Embryos which were not given an osmotic treatment were bombarded on 0.8% water agar plates.

Culture of Somatic Embryos Prior to Bombardment

Somatic embryos were placed on the same osmotic medium as the zygotic embryos for 2 h before and 2 h after bombardment. Liquid medium pretreatment of somatic embryos was tested by culturing embryos for 0–3 days in 30 mL of liquid EM medium (70 rpm on an orbital shaker). The effect of frequency of subculture on transformation efficiency was tested by culturing embryos for up to 93 days before bombardment. Actively growing embryos (3 weeks from last subculture) were subcultured on EM medium and then left without subculture until bombardment. Embryos were set up using this procedure 8, 15, 24, 35, 46, 56, 66, 72 and 93 days before bombardment; all embryos were given a 3 day liquid medium pretreatment and bombarded on the same day.

Transformation Vector

The transformation vector pCol113 (Fig. 1) was constructed by the excision of an *Eco*RI fragment containing the cauliflower mosaic virus (CaMV) 35S promoter, *uid*A gene and CaMV 35S 3′-untranslated region (UT) from pDHKan^R (gift from Linda Tabe, Division of Plant Industry, CSIRO, Canberra). This fragment was blunt-ended and ligated into the *Sma*I restriction site of pUCpetE(no NcoI)GUSocs118 (Pwee and Gray 1993) (gift from John Gray, Department of Plant Sciences, University of Cambridge). Plasmid DNA for microprojectile bombardment was purified by two cycles of caesium chloride–ethidium bromide density gradient centrifugation (Sambrook *et al.* 1989).

Bombardment Conditions

Embryos were bombarded using a particle inflow gun (Finer *et al.* 1992). Gold particles of 1.5–3 μm diameter (Aldrich) were used as microprojectiles; 120 mg of gold was washed three times with 100% ethanol and three times with sterile water before suspension in 1 mL of sterile 50% (v/v) glycerol. For preparation of microprojectiles, 25 μL of the gold suspension was mixed with 0.5 mg plasmid DNA, 25 μL 1 M $CaCl_2$, and 5 μL 0.1 M spermidine-free base. All solutions were kept on ice. The suspension was first sonicated for 10 s, then kept in suspension by occasionally vortexing for 5 min. The suspension was then allowed to settle on ice for 10 min before 22 μL of the supernatant was removed and discarded. The remaining suspension was vortexed immediately before using 4 μL of the mixture for each bombardment. Embryos were arranged without overlapping in an area of approximately 10 mm diameter. A protective baffle of stainless steel mesh with an aperture of 210 μm (Franks and Birch 1991) was placed over the tissue during bombardment. The pressure of the helium blast and the distance of the target embryos from the filter unit containing the coated gold particles were varied to determine the most suitable bombardment conditions for this tissue type, using this particular particle inflow gun. The distance from the target tissue to the filter unit was varied between 9 and 16 cm. The pressure of the helium blast was varied between 500 and 780 kPa.

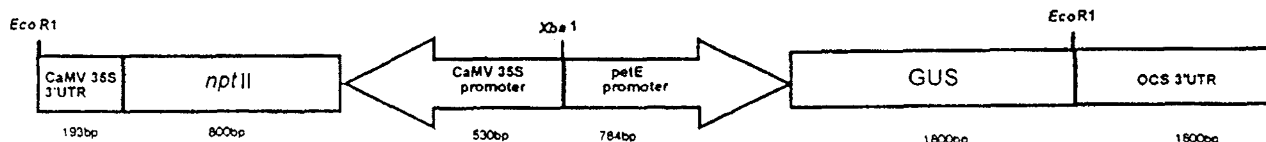

Fig. 1. Transgene expression cassette in pCol113 (8.9 kb).

β-Glucuronidase (GUS) Assay

β-Glucuronidase (GUS) activity was assayed histochemically by incubating embryos or leaf tissue from regenerated plantlets in 8-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37°C (Jefferson 1987). Transient expression was assayed 24–48 h after bombardment and measured as total blue foci count per shot area.

Selection and Regeneration of Transformed Plantlets

Following bombardment, somatic embryos were allowed to recover for 2 days on EM medium, prior to selection on EM medium containing kanamycin monosulfate. The efficiency of selection of non-transformed and bombarded tissue was tested on 75, 100, 150, 200 and 300 mg mL^{-1} kanamycin. Transformed embryos were selected for between 3 and 5 months before they were assayed for GUS activity. For germination, embryos were transferred to a modified de Fossard's medium (de Fossard 1974), containing H minerals and vitamins as described by Drew and Smith (1986) with 25 mg mL^{-1} kanamycin added. Single plantlets were then transferred into vessels containing the same medium without kanamycin.

Southern Blot Analysis of Transgenic Plants

Genomic DNA was extracted from papaya leaf tissue as described by Lassner *et al.* (1989). DNA (10 μg) was digested with *Xba*I and *Eco*RI and electrophoresed on a 0.8% agarose gel. Digested nucleic acids were immobilised on Hybond N nylon membrane (Amersham) after capillary transfer (Southern 1975) in 20 × SSC. Membranes were hybridised with ^{32}P-labelled DNA corresponding to either the *aph*A gene or the *uid*A gene as described by Hardy *et al.* (1985).

Preparation of Probes

DNA fragments corresponding to either the *aph*A or *uid*A gene were excised from pCol113 following digestion with *Xba*I and *Eco*RI and gel purified using a 'Wizard' PCR purification kit (Promega). DNA was ^{32}P-labelled using a 'Ready-to-Go' random prime labelling kit (Pharmacia).

Statistical Analyses

Experiments were designed to determine optimum conditions for transient expression. Data are reported as means ± standard error of the mean. One-way analysis of variance (ANOVA) was used to compare treatment means. A multiple comparisons test using least significant difference (LSD) was used to determine significant difference between means. Data were analysed using the statistical package Statgraphics.

Results

Optimisation of Transient Expression

Following bombardment highest transient expression levels were obtained for zygotic embryos using a helium pressure of 700 kPa with the target tissue at a distance of 13.5 cm from the filter unit containing the particles (data not shown). Somatic embryos had highest transient expression levels when bombarded using 500 kPa helium pressure at a distance of 7.5 or 10 cm from the filter unit containing the particles.

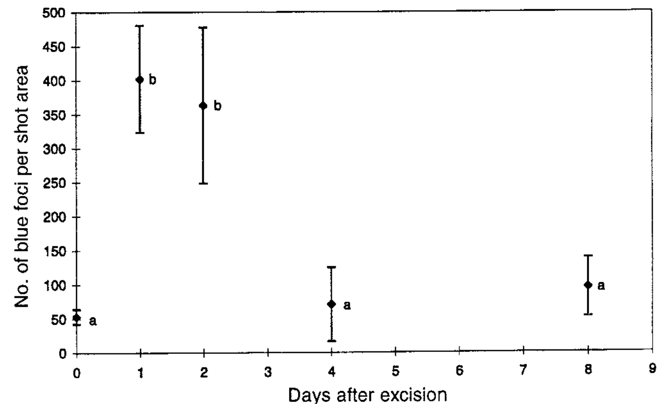

Fig. 2. Transient GUS expression in zygotic embryos following culture on water agar prior to bombardment. a, b are significantly different at $P = 0.05$. Each value is a mean of four replicates. Vertical bars represent standard errors.

Varying the time from excision of zygotic embryos from seed to time of bombardment showed that transient expression was highest (414 blue foci per shot) in zygotic embryos cultured for 1–2 days on water agar before bombardment (Fig. 2). This was approximately four times higher than embryos bombarded on the same day as excision or cultured for 4 or 8 days after excision.

The effect of osmoticum treatment (0.25 M mannitol) pre- and post-bombardment was tested on both zygotic and somatic embryos. Transient expression in zygotic embryos, cultured for 1 day prior to bombardment, was increased threefold by treatment of embryos on a medium of high osmolarity for 2 h before, and 2 h after bombardment

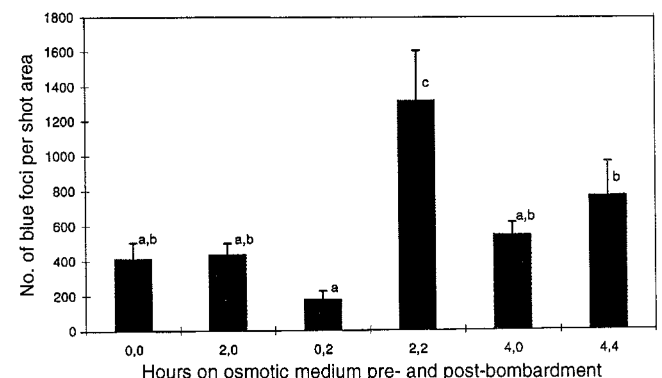

Fig. 3. Effect of osmotic treatment on transient GUS expression in zygotic embryos cultured for 1 day on water agar following excision. a, b, c are significantly different at $P = 0.05$. Each value is a mean of four replicates. Vertical bars represent standard errors.

Table 1. The effect of pretreatment of somatic embryos in liquid medium on transient expression
a, b, c are significantly different at $P = 0.05$. Each value is a mean of four replicates ± the standard error

Days in liquid medium	Number of blue foci
0	364.3 ± 72.60 a
1	698.5 ± 67.28 ab
2	1160.8 ± 182.53 c
3	960.3 ± 117.84 bc

compared to no osmoticum treatment (Fig. 3). None of the other osmotic treatments varied significantly from the untreated control.

Based on the positive effect of osmotic treatment on transient expression in zygotic embryos, somatic embryos were also cultured on high osmolarity medium for 2 h prior to and 2 h after bombardment. Under these conditions, a 16-fold increase in transient expression, from 56 to 938 blue foci per shot area, was observed when compared with no osmotic treatment.

The culturing regime prior to bombardment had a dramatic effect on transient expression. Firstly, somatic embryos cultured in liquid EM for 2–3 days immediately prior to bombardment had up to 3 times higher transient expression levels when compared with embryos maintained on solid EM medium (Table 1). Importantly, the subculture regime prior to bombardment had a significant effect on transient expression. Regular subculturing of somatic embryos, every 8–35 days, prior to bombardment improved transient expression up to 22-fold compared with embryos which had been maintained without subculture for 46 to 93 days before bombardment (Fig. 4).

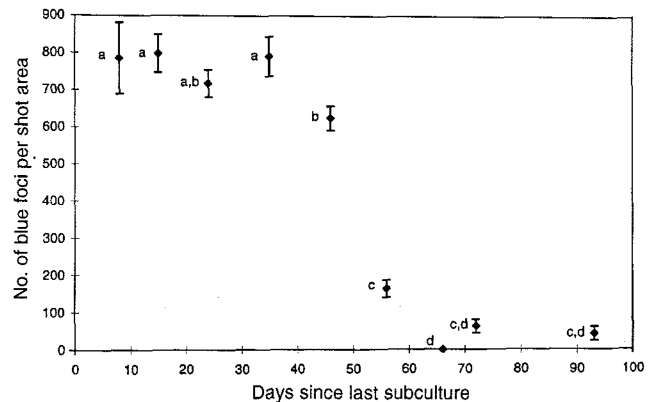

Fig. 4. Effect of subculture regime on transient GUS expression of somatic embryos. a, b, c, d are significantly different at $P = 0.05$. Each value is a mean of five replicates. Vertical bars represent standard errors.

Stable Transformation of Australian Papaya Cultivar OE

Somatic embryos used in stable transformation experiments were cultured and pretreated using optimal conditions determined for transient expression. Non-transformed embryos were able to survive on kanamycin levels of 75 and 100 $\mu g\ mL^{-1}$ but were unable to survive on

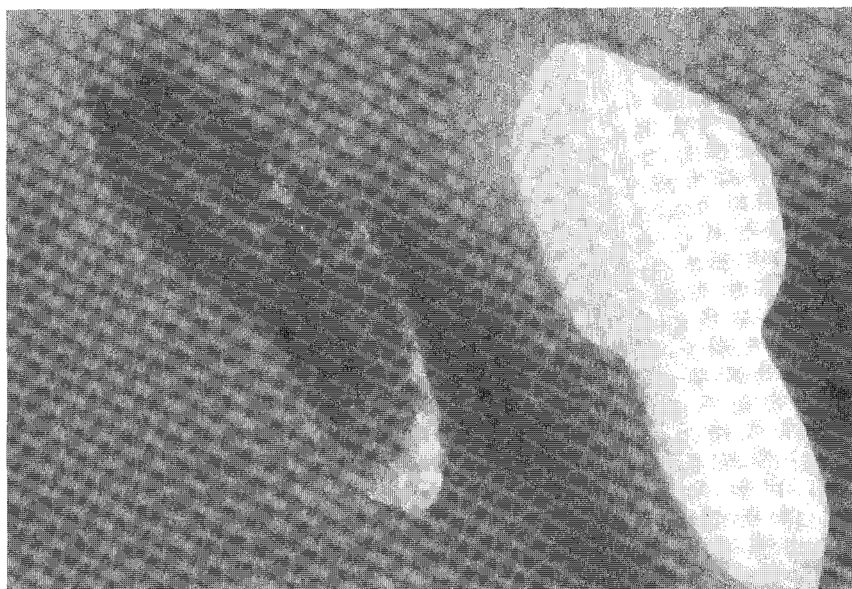

Fig. 5. Somatic embryos assayed histochemically for GUS expression 4 months after bombardment. The transformed embryo expressing GUS is shown on the left, and the untransformed embryo is on the right.

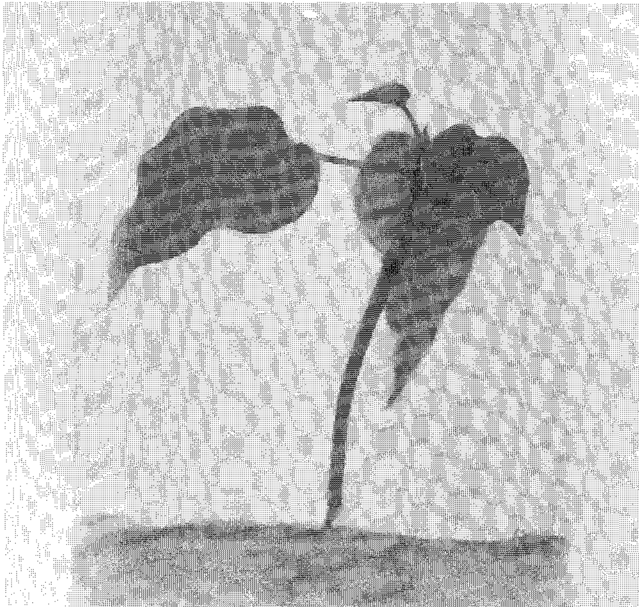

Fig. 6. A transformed plant, line OE13.2, regenerated on media containing 150 μg mL^{-1} kanamycin.

levels above 100 μg mL^{-1} so potentially transformed somatic embryos were selected on media containing between 150 and 300 μg mL^{-1} kanamycin.

Embryos selected on kanamycin levels of between 150 and 300 μg mL^{-1} grew slowly and therefore, after 5 months on selection, kanamycin levels were reduced to 25 μg mL^{-1} allowing embryos to increase growth rate. Although embryos developed into plantlets (Fig. 6), growth rates of these plants were still slow when compared to non-transgenic plants on kanamycin free medium.

Most of the surviving embryos (Fig. 5) and regenerated plantlets expressed *uid*A in leaves, roots and shoots. In the first experiment, 3/5 regenerated lines were positive for *uid*A expression and in the second experiment all three lines regenerated expressed *uid*A. Southern analysis indicated that at least one complete copy of each gene was present in both GUS expressing and non-expressing plants tested (Fig. 7.).

In our papaya secondary somatic embryo cultures, the embryos grew in clumps, budding from each other, and thus the embryos were at various stages of development and varied in size. It was therefore impossible to count individual embryos prior to transformation. During the 3-day liquid medium pretreatment stage, the secondary embryo clumps broke apart into individual embryos. Therefore, we standardised our transformation experiments using a uniform area (10 mm diameter circle) covered with somatic embryos, without overlapping. We measured transformation efficiency as a percentage of bombarded plates that produced transformed clones.

The transformation and regeneration protocol described here for transformation of somatic embryos was rapid and reproducible with two experiments yielding efficiencies of 45% (5/11) and 37.5% (3/8) of bombarded plates regenerating plantlets on media containing kanamycin. Embryos germinated into plantlets 5–6 months after bombardment.

Discussion

We have developed an efficient transformation and regeneration system for secondary somatic embryos of an Australian cultivar of papaya. Fitch *et al.* (1992) reported transformation efficiency of somatic embryos to be 6-fold lower than zygotic embryos. However we observed higher transient expression in somatic embryos as was also reported by Cabrera-Ponce *et al.* (1995).

Using our system, an average of 41% of bombarded plates produced kanamycin resistant plantlets. In more recent experiments using similar constructs with the same selection cassette, up to 67.5% (27/40) of bombarded plates produced kanamycin resistant plantlets (results not shown). This is in contrast to Fitch *et al.* (1992), who reported an efficiency of only 17% of bombarded plates of embryogenic callus producing transformants. In addition, we consistently

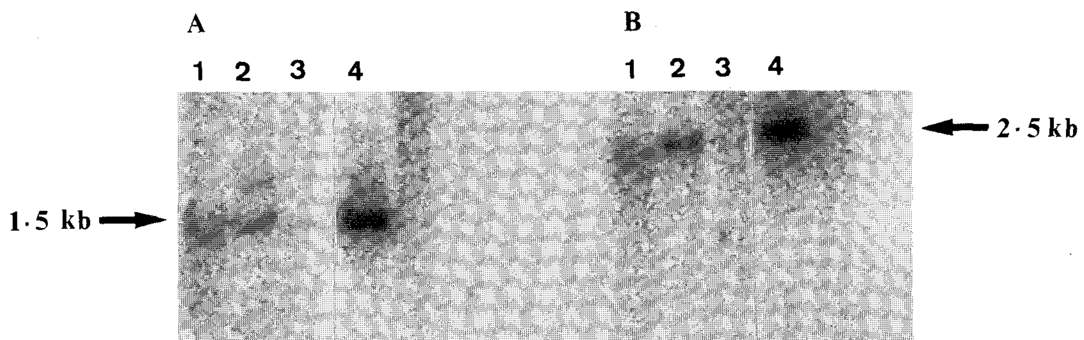

Fig. 7. Detection of *npt*II gene (A) and *uid*A gene (B) in genomic DNA from transgenic and untransformed papaya following digestion with *Eco*RI and *Xba*I. Lane 1, transgenic line OE2.1 (expresses GUS); Lane 2, transgenic line OA13.1 (doesn't express GUS); Lane 3, untransformed papaya; Lane 4, plasmid pCol113 digested with *Eco*RI and *Xba*I.

obtained transformed plantlets within 6 months of bombardment which was considerably faster than previously reported selection regimes which extended to 23 months (Fitch *et al.* 1992). Yang *et al.* (1996) recently reported *A. tumefaciens*-mediated transformation of petioles and subsequent regeneration of papaya via somatic embryogenesis. They obtained 13 transformed lines from 415 petiole fragments, with selection and regeneration taking 10–11 months. Cabrera-Ponce *et al.* (1995) transformed both zygotic and somatic embryos of papaya using microprojectile bombardment. They measured transformation efficiency as percentage of *uid*A positive zygotic embryos or as number of *uid*A positive clones/mg fresh weight of somatic embryos. Cabrera-Ponce *et al.* (1995) reported a transformation efficiency of 60 transgenic clones per gram of fresh weight and plantlets were obtained 7–8 months after bombardment.

Results from our experiments indicated that the quality or physiological state of the papaya embryos had a significant effect on transformation efficiency. Transient expression results were greatly improved in both somatic and zygotic embryos when treated osmotically before bombardment. High osmotic medium reportedly reduces turgor pressure in target cells potentially minimising damage following bombardment (Perl *et al.* 1992). Similar treatments have been used in other transformation systems (Perl *et al.* 1992; Christou 1993; Vain *et al.* 1993; Livingstone and Birch 1995). We found that zygotic embryos were a suitable explant for transformation. However, excision of immature zygotic embryos was time consuming and labour intensive and was limited by the availability of green fruit of a suitable age. Importantly, the secondary embryogenesis system (Drew *et al.* 1994) provided a continuous supply of reproducible, uniform tissue suitable for transformation. This system has produced somatic embryos for more than 2 years without the need for re-initiation from immature zygotic embryos and without loss of embryogenicity. Although somaclonal variation has not been investigated for our transgenic plants, when 80 non-transgenic plants were regenerated from the somatic embryo cultures of equivalent age and planted in the field, only one plant displayed morphological variation: it had slightly larger flowers than normal.

Tissue culture conditions prior to bombardment such as frequency of subculture and pre-treatment of tissue also had a significant effect on transformation efficiency. Ellis *et al* (1993) suggested that *Picea glauca* cells were more competent for transformation at different growth stages and Okada *et al.* (1986) and Iida *et al.* (1991) found that transformation of tobacco cells that were undergoing cell division resulted in increased transformation frequencies. De Block (1993) suggested that tissue pre-treatment induces DNA replication resulting in a higher level of insertion of DNA into the genome. We found that subculture onto fresh medium and pre-treatment in liquid medium prior to bombardment improved expression of the introduced DNA in the cells which may also indicate an improved competence for transformation.

A proportion of plants which regenerated and were tested histochemically did not express *uid*A. In a previous report of microprojectile bombardment of papaya (Fitch *et al.* 1990), a higher proportion of transformed lines from somatic embryos (3/4) were positive for both *aph*A and *uid*A expression, compared with zygotic embryos (2/11). Using our system, both *uid*A expressors and non-expressors were obtained; however, there were more expressors than non-expressors. Similar results have been previously reported and it has been suggested that lack of expression of a non-selected transgene could be due to factors such as incomplete gene transfer, gene rearrangement, integration position, gene copy number or methylation (Fitch *et al.* 1990; Chee and Slightom 1992; Finnegan and McElroy 1994; Register *et al.* 1994).

We found transformation of secondary somatic embryos to be convenient, reproducible and a rapid means of obtaining transgenic plants, with stably transformed *uid*A expressing plantlets obtained within 6 months after bombardment. This system is currently being used to transform papaya with potential resistance genes derived from PRSV-P.

Acknowledgment

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