THE INTRODUCTION OF TRANSGENES TO CONTROL BLACKHEART IN PINEAPPLE: BIOLISTICS VS AGROBACTERIUM *TRANSFORMATION*

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Abstract. Techniques for the introduction of transgenes to control blackheart by particle bombardment and *Agrobacterium* co-transformation have been developed for pineapple cv. Smooth Cayenne. Polyphenol oxidase (PPO) is the enzyme responsible for blackheart development in pineapple fruit following chilling injury. Sense, anti-sense and hairpin constructs were used as a means to suppress PPO expression in plants. Average transformation efficiency for biolistics was approximately 1% and for *Agrobacterium* was approximately 1.5%. These results were considered acceptable given the high regeneration potential of between 80-90% from callus cultures. Southern blot analysis revealed stable integration of transgenes with lower copy number found in plants transformed with *Agrobacterium* compared to those transformed by biolistics. Over 5000 plants from 55 transgenic lines are now undergoing field evaluation in Australia.

Key words: *Ananas comosus*, genetic transformation, gene silencing, postharvest disorder

INTRODUCTION

Blackheart is a physiological disorder of pineapple fruit occurring when day/ night temperatures drop below 25°/20°C and when combined with low light conditions during fruit development. Injury initially develops as watery spots at the base of individual fruitlets, which subsequently discolours the length of the fruit's core. Efforts to develop new blackheart resistant Smooth Cayenne cultivars via conventional breeding have been hampered by low fertility, long generation time and lack of genetic variability. Therefore genetic engineering is an attractive strategy for developing new cultivars with blackheart resistance for the industry's main processing pineapple.

In pineapple it has been shown that the occurrence of blackheart is directly associated with the oxidation of phenolics by PPO (Stewart *et al.* 2001). Our strategy was based on the development of constructs using conventional anti-sense and co-suppression, as well as more recent RNAi strategies for inactivation of PPO expression. Several groups have made attempts to develop a transformation system as a way to introduce useful genes into pineapple, using either the biolistics or the *Agrobacterium*-mediated technique (Firoozabady and Gutterson, 1998; Graham *et al.*, 2000a; 2000b; Sripaoraya *et al.*, 2001), however specific details of the transformation efficiency and stable incorporation of transgenes is not available. In order to develop an efficient and reliable transformation system for pineapples many parameters need to be optimised both pre- and post transformation. This paper describes successful pineapple transformation techniques based on both biolistics and *Agrobacterium* transformation.

MATERIALS AND METHODS

Preparation of plasmid constructs

Two plasmids expressing the *nptII* selectable marker gene and five plasmids expressing the GUS (β-glucuronidase), GFP (green fluorescent protein) or PPO (polyphenol oxidase) genes were used in these experiments. In plasmid pDH-kan^R expression of the selectable marker gene *nptII*, which confers resistance to the antibiotics kanamycin and geneticin (G418), is controlled by the cauliflower mosaic virus 35S (CaMV35S) promoter and terminator. Plasmid pBS420, the alternative selectable marker, encodes a construct where expression of the *nptII* gene is under control of the Segment 4 promoter and Segment 7 terminator sequences derived from Subclover Stunt Virus (SCSV; Boevink *et al.* 1993).

pART7.35S.GUS expresses the GUS marker gene under control of the CaMV35S constitutive promoter and the *ocs* terminator. In this plasmid GUS coding sequences were cloned into the polylinker site of the expression cassette pART7 (Gleave 1992). To create pBS247.SCSV4.GUS, an expression cassette was constructed where the 35S promoter and *ocs* terminator of pART7 were replaced with the SCSV4 promoter and SCSV5 terminators respectively, GUS coding sequences were cloned into this. The plasmid backbone of pBS247 is identical to that of pART7.35S.GUS. GUS constructs were co-transformed with both selectable gene constructs.

A construct containing the Ubiquitin promoter from maize (*Ubi-1)* linked to GFP as a reporter gene in pGEM (pGEM-Ubi-GFP; Dugdale *et al.* 1998) was co-transformed with pDH-Kan^R as the selectable marker. In this study the synthetic strain of the GFP gene, which is incorporated in the nuclei of the cells was used.

Of the pineapple PPO genes isolated by Stewart *et al*. (2001), constructs were designed containing the PINPPO1 gene in a sense (PPO), anti-sense (OPP) and hairpin (OPP.*i*.PPO) orientation in pART7. The sense construct was controlled by the *Ubi-1* promoter and has an *ocs* terminator. The anti-sense construct was controlled by the CaMV35S promoter and *nos* terminator. The OPP.*i*.PPO construct has the CaMV35S promoter and *nos* terminator driving the OPP gene, and *Ubi-1* promoter and *ocs* terminator controlling the PPO gene. PPO constructs were co-transformed with pBS420.

Co-transformation of the selectable marker gene with the reporter gene of interest occurred in a 1:3 molar ratio. The co-transformation technique is reported to be highly effective in incorporating both the selectable marker gene and the gene of interest in the same transformed individual (Birch and Bower 1994).

Development of tissue culture systems

Buds were excised from crowns of selected 'Smooth Cayenne' field grown plants and sterilised in 0.5% NaOCl solution, containing a few drops of the wetting agent Tween #80, for 1 minute. Buds were rinsed three times with sterile distilled water and plated onto a medium containing MS (Murashige & Skoog 1962) basic nutrients and vitamins with the addition of 0.1 mg/L myo-inositol, 20 g/L sucrose, 2.5 mg/L BAP and 3 g/L Phytagel. Plantlets were transferred to basic MS medium for further development. Leaf bases from *in vitro* shoots were cultured and maintained on basic MS medium containing 10 mg/L each of BAP and NAA for callus initiation and proliferation (CAL

medium) (Wakasa 1989). An efficient shoot regeneration system from callus of 'Smooth Cayenne' was established on MS medium containing 5% (v/v) coconut water (Sigma-Aldrich) and 400 mg/L casein hydrolysate (PRR medium) (Rangan 1982). Rooting of shoots did not require any addition of plant growth hormones to the medium. Cultures were incubated at 28°C, under 8 hour light at a photon flux density of 60 μmol m^{-2} s^{-2} (Philips TLD 36W/33 cool white fluorescent tubes).

Bombardment conditions

Bombardment experiments (Graham *et al.* 2000a) were carried out in a particle inflow gun as described by Finer *et al.* (1992). The following bombardment parameters were optimised using the GUS marker gene:

- Helium gas pressure: 1000 kPa. Helium gas pressure of less or more than 1000 kPa did not increase GUS expression significantly.
- Target distance between explants and filter device containing the DNA precipitation: 18.1 cm. Three to five-fold increase of GUS expression (up to 21 blue spots per callus piece) at this setting compared to shorter or longer distances.
- Gold particle (Biorad Laboratories) size: 1.0 μm. Comparing gold particle size of 1.0 μm to that of 1.6 μm for DNA coating showed up to a 4.5-fold increase in GUS transient expression with 1.0 μm particles (and up to approximately 90 spots per transformed explant).
- Pre-treatment with osmoticum in MS medium was found not to be necessary for pineapple callus, contrary to Birch and Bower (1994) who recommend pre-treatment in medium containing 0.2M sorbitol and 0.2M mannitol for 4 hr to overnight in the dark to reduce turgidity and hence damage to cells.
- When DNA coating was performed at room temperature an average of 1.5 – 4 times increase in transformation efficiency was achieved with up to approximately 35 blue spots per transiently transformed callus piece, compared to coating on ice .

For bombardment approximately 20 callus explants, 2-5 mm in size, were arranged in a 2 cm diameter centre of each petridish. By using a baffle to protect the callus explants from the impact during bombardment and for an even dispersal of the DNA, no "zone of death" in the very centre of the dish was created. A vacuum of 26″ Hg was applied for bombardment.

A two-step selection method was employed (Kartha *et al.* 1994), by subjecting the callus pieces to two fortnightly sub-cultures on CAL medium containing 50 μg/mL geneticin (G418), followed by a monthly sub-culture on 100 μg/mL geneticin in the selective culture medium. This selection process was carried out for 6-10 months before transfer of healthy callus to PRR medium.

Agrobacterium *co-cultivation conditions*

Co-cultivation experiments (Graham *et al.* 2000b) involved the use of *Agrobacterium tumefaciens* strains C58(pMP90), EHA101 and LBA4404. The following co-cultivation conditions were optimised using the GUS marker gene:

- *In vitro* leaf bases of approximately 0.5-1.0 cm were trimmed in an *Agrobacterium* slurry with an OD_{550nm} of 1.4-1.6.
- Samples were vacuum infiltrated for 5 min at 27-29.5″ Hg.

- Cultures were incubated on callus initiation medium containing 100 μM acetosyringone in the dark at 25°C for 2 days.
- After co-cultivation for 3 weeks under light, cultures were transferred to CAL medium containing 300 μg/mL of cefotaxime and 50 μg/mL geneticin for 2 monthly subcultures. This was followed by monthly subcultures on CAL medium containing 100 μg/mL geneticin.

Transformation efficiency

Regeneration of transgenic plants from callus occurred on PRR medium and was achieved from both biolistic and *Agrobacterium* transformed lines over another 3-6 month period. Transformation efficiency was measured as the number of plant lines, verified by PCR analysis of the transgenes, recovered from either the number of calli pieces bombarded or the number of leaf bases co-cultivated with *Agrobacterium*.

Southern blot analysis

Transgenic lines containing the GUS, GFP, PPO and *npt*II constructs, as identified by PCR analysis, were analysed by Southern blot hybridisation (Southern 1975; Sambrook *et al.* 1989) to determine integrated transgenes.

Approximately 4-7 g of fresh leaf material was ground in liquid nitrogen and DNA isolated using a modified CTAB protocol according to Graham *et al*. (1994). Restriction digests were performed overnight using 50 units of restriction enzyme *Bam*HI on 5 μg of each GUS, GFP and PPO sample and *Hind*III for each *nptII* sample. Products were separated on a 1% (w/v) agarose gel (Seakem) in 1x TBE buffer, then stained for 30 min in 1x TBE containing ethidium bromide. The DNA was acid depurinated in 0.25N HCl for 5 min, denatured in 0.5M NaOH/ 1.5M NaCl twice for 15 min each on a shaker. Gels were neutralised in two changes of 1M Tris-HCl (pH 7.5)/ 1.5M NaCl for 15 min on a shaker, then rinsed in water and transferred to 2x SSC (0.3M NaCl/ 30 mM sodium citrate, pH 7.0). DNA fragments were transferred overnight (16-20 hr) onto a nylon membrane (Hybond-N, Amersham Life Sciences, or MagnaProbe, GE Osmonics) by capillary transfer using 20x SSC. The membrane was rinsed in 2x SSC, blotted to remove excess liquid and cross-linked on both sides with 150 mJoule of UV radiation (BioRad GS Gene Linker UV chamber). After drying the membrane was stored at room temperature between sheets of blotting paper wrapped in Al-foil, until used.

To assess the efficiency of DNA transfer the blotted gel was re-stained with ethidium bromide for 30 min. Fragments specific to GUS, GFP and *nptII* respectively, were obtained by PCR amplification of the coding regions using specific primers as previously described, resulting in specific sized fragments for the respective sequences. Template sequence for probing the PPO transgenics was the CaMV 35S promoter, excised from the plasmid pART7 by *Not*I and *Bam*HI digestion, separated on a 0.8% agarose gel run in TAE (tris-acetate/EDTA) electrophoresis buffer and purified by using the UltraClean DNA Purification Kit (Mo Bio Laboratories, CA). The Megaprime DNA labelling system (Amersham Pharmacia Biotech UK Ltd) was used to prepare ^{32}P-labelled GUS, GFP, 35S and *nptII* probes. Pre-hybridisation occurred at 65°C for 4 hr and hybridisation at 65°C overnight ('Super Hyb Kit', Molecular Research Centre Inc.). Hybridisation was detected by autoradiography, using between overnight and 10 days exposure time, with and without intensifying screens, respectively.

RESULTS AND DISCUSSION

Leaf explants cultured on CAL medium took an average of 23-34 days to produce callus visible to the naked eye, with 70-96% producing callus at the cut ends of the explants after 60 days. Callus initiation was not visibly enhanced by culture in darkness; 40-70% of the explants compared to 70-83% in the light after 35 days.

'Smooth Cayenne' plantlets recovered from callus cultures was obtained through organogenesis. On average up to 20-plus healthy shoots per callus piece were produced within two months, although some lines regenerated more profusely than others. Root production occurred within three weeks on 80% of the shoots and consisted of long, thin, single roots. Thick roots are more easily damaged at transplanting to pots, therefore, for root production no growth hormones were required in the media.

Table 1

There was little difference in transformation efficiencies between biolistics and *Agrobacterium* co-transformation. For instance an estimated and acceptable rate of stable incorporation of microprojectile-delivered DNA in plant cells range from 1-9%, but are typically less than 5% (Birch and Bower 1994). This translates to an average range of 0.1-1.0% of bombarded explants yielding a transformed cell line or clone (Bower *et al*. 1996). Our results (Table 1) fall well within this acceptable range.

Our results also show a consistently high level of stable integration of both transgenes. Hundreds of plants have been regenerated from transgenic callus and have individually been confirmed and screened by PCR analysis. For instance, of the 598 plants recovered from GUS positive callus, 25 plants (4.2%) turned out to be GUS negative, but *nptII* positive. The regeneration of GUS negative shoots from GUS positive callus has previously been observed by Christou *et al*. (1991), describing the survival on selective medium and regeneration of rice plants by non-transformed tissue in close proximity of truly transformed tissue, due to the detoxifying effect on the selective agent by the transformed tissue. Therefore, transformed and non-transformed chimaera sectors can proliferate together and produce a number of non-transformed plants.

Transformation results for both reporter genes (GUS and GFP) are similar, although GFP had a slightly higher transformation efficiency and shoot regeneration level, especially with *Agrobacterium* cultivation. Using two different promoters for the GUS constructs and co-bombarding GUS with two different selective gene constructs resulted in a comparable transformation efficiency.

For blackheart resistance over 5000 callus pieces were bombarded, resulting in an average transformation efficiency of 1.19%, with higher efficiency seen with the hairpin constructs. Further experiments are necessary to confirm these findings with *Agrobacterium*-mediated transgenic lines.

The integration of the GUS, GFP, PPO and *npt*II constructs into the host plant's genome was confirmed by Southern blot analysis, with differing integration patterns verifying that the plants were the result of independent transformation events (Figure 1). Copy numbers of the GUS transgene vary from 5 to 8 between individual lines, 3 to 10 for GFP transgenic lines, 1 to 23 for PPO lines and 3 to13 for *nptII* transgenes from the lines sampled (Table 2). Although multiple integrated copies of transgenes increases the probability of gene silencing, either through homology-dependent promoter silencing,

or co-suppression (Meyer 1995), GUS and GFP were still expressed in those lines that contained up to 10 integrated copies. Southern blot analysis revealed lower copy number found in plants transformed with *Agrobacterium* compared to those transformed by biolistics and supports other studies (Birch and Bower 1994), although there were biolistic transformed lines with 1-2 integrated transgenes.

Given the exceptionally high level of gene silencing achievable with hairpin constructs (Smith *et al*. 2000), gene silencing to achieve blackheart resistance in pineapples is a realistic goal. Over 5000 plants from 55 transgenic lines are now undergoing field evaluation in Australia.

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REFERENCES

Birch, R.G.; Bower, R. Principles of gene transfer using particle bombardment. In: Yang, N.-S.; Christou, P., eds. Particle bombardment technology for gene transfer. New York/ Oxford: Oxford University Press; 1994:3-37.

Boevink, P.; Chu, P.W.G.; Keese, P. Sequence of subterranean stunt virus DNA: affinities with the geminii virus. Virology 207:354-361; 1993.

Bower, R.; Elliott, A.R.; Potier, B.A.M.; Birch, R.G. High-efficiency, microprojectile-mediated co-transformation of sugarcane, using visible or selectable markers. Mol. Breeding 2:239-249; 1996.

Christou, P.; Ford, T.L.; Kofron, M. Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957-962; 1991.

Dugdale, B.; Beetham, P.R.; Becker, D.K.; Harding, R.M.; Dale, J.L. Promoter activity associated with the intergenic regions of banana bunchy top virus DNA-1 to -6 in transgenic tobacco and banana cells. J Gen Virology 79:2301-2311; 1998.

Finer, J.J.; Vain, P.; Jones, M.W.; McMullen, M.D. Development of the particle inflow gun for DNA delivery to plant cells. Plant Cell Rep. 11:323-328; 1992.

Firoozabady, E.; Gutterson, N. Genetically transformed pineapple plants and methods for their production. International Patent Application number PCT/US98/03681; 1998.

Gleave, A.P. A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Molec Biol 20:203-1207; 1992.

Graham, G.C.; Mayers, P.; Henry, R.J. A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. BioTechniques 16:48-50; 1994.

Graham, M.; Ko, L.; Hardy, V.; Robinson, S.; Sawyer, B.; O'Hare, T.; Jobin, M.; Dahler, J.; Underhill, S.; Smith, M. The development of blackheart resistant pineapple through genetic engineering. Acta Hort. 529**:**133-136; 2000a.

Graham, M.; Smith, M.; Hardy, V.; Ko, L. A method of transformation. International Patent Application number PCT/AU00/01350; 2000b.

Kartha, K.K.; Nehra, N.S.; Chibbar, R.N. Genetic engineering of wheat and barley. In: Henry, R.J.; Ronalds, J.A., eds. Improvement of cereal quality by genetic engineering. New York and London: Plenum Press; 1994:21-30.

Meyer, P. Variation of transgene expression in plants. Euphytica 85:359-366; 1995.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum 15**:**473-497; 1962.

Rangan, T.S. Pineapple. In: Evan, D.A.; Sharp, W.R.; Ammirato, P.V.; Yamada, Y., eds. Application of plant tissue culture: methods for crop improvement. New York: Macmillan; 1982:373-382.

Sambrook, J.; Fritsch, E.F.; Maniatis, T. Molecular cloning - a laboratory manual. New York: Cold Spring Harbor Laboratory Press; 1989.

Smith, N.A.; Singh, S.P.; Wang, M.B.; Stoutjesdijk, P.A.; Green, A.G.; Waterhouse, P.M. Total silencing by intron-spliced hairpin RNAs. Nature 407:319-320; 2000.

Southern, E.M. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517; 1975.

Sripaoraya, S.; Marchant, R.; Power, J.B.; Davey, M.R. Herbicide-tolerant transgenic pineapple (*Ananas comosus*) produced by microprojectile bombardment. Annals Bot. 88**:**597-603; 2001.

Stewart, R.J.; Sawyer, B.J.B.; Bucheli, C.S.; Robinson, S.P. Polyphenol oxidase is induced by chilling and wounding in pineapple. Aust J Plant Physiol 28:181-191; 2001.

Wakasa, K. Pineapples. In: Bajaj, Y.P.S., ed. Biotechnology in Agriculture and Forestry 5: Trees II. London: Springer-Verlag; 1989:13-29.

TABLE 1

TRANSFORMATION EFFICIENCIES OF VARIOUS CONSTRUCTS BETWEEN A BIOLISTICS AND *AGROBACTERIUM* CO-TRANSFORMATION METHOD

Method	Construct	No experiments	No transgenic lines	Transformation efficiency (%)
Biolistics	GUS	4	15	0.59
	GFP	4	15	1.21
	PPO (sense)	7	14	0.56
	PPO (anti-sense)	4	8	1.20
	PPO (hairpin)	5	21	1.66
Agrobacterium	GUS	2	7	1.59
	GFP	3	6	4.98
	PPO (sense)	3	7	0.38
	PPO (hairpin)	1	4	0.32

TABLE 2

NUMBER OF TRANSGENE COPIES INTEGRATED INTO PLANTS TRANSFORMED BY BIOLISTICS OR *AGROBACTERIUM*

Method	Line No., Transgene	GUS	GFP	PPO	*npt*II
Control		0	0	0	0
Biolistics	1, $SCSV_4$.GUS	7			8
	2, $SCSV_4$.GUS	5			6
	1, 35S.GUS	6			10
	2, 35S.GUS	8			7
	1, Ubi-GFP		10		13
	2, Ubi-GFP		3		3
	3, Ubi-GFP		4		9
	4, Ubi-GFP		5		6
	1, PPO			2	
	2, PPO			10	
	3, PPO			5	
	4, PPO			1	
	5, PPO			4	
	6. PPO			23	
Agrobacterium	1, PPO			5	
	2, PPO			2	
	3, PPO			4	
	4, PPO			4	

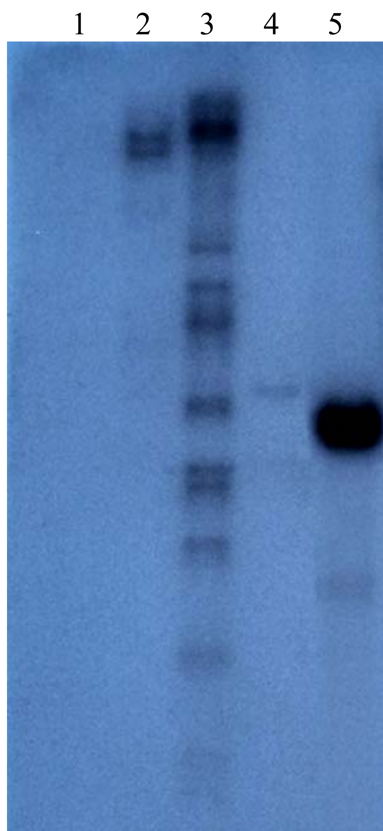

FIG. 1. Southern blot analysis of transgenic PPO pineapple lines demonstrating variation in copy number of integrated PPO gene: lane 1- untransformed control line; lanes 2 to 4- transgenic PPO (sense) lines with lane 2 with 2 bands, lane 3 with 23 bands and lane 4 with 1 band; lane 5- 200 pg of BamHI digested pART7.35S.PPO.