AP122
Transfer of pest resistant gene via Agrobacterium into apples and pears

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Agriculture Victoria
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1. Summary

1.1 Industry Summary

Apples and pears are major components of the horticultural industry. A fundamental problem these industries face is a long lead time for the development and introduction of new and improved cultivars. Changing trends in horticultural production, such as the use of insecticides, frequently require rapid responses by industry.

The reliance on conventional breeding in woody perennial crops is not conducive to the requirement for rapid change. Genetic engineering is a valuable adjunct to conventional breeding programs, as it offers the potential to alter an existing character or to add a single characteristic that can originate from a diversity of sources, into both advanced breeders’ lines and presently grown cultivars.

Presently there are several genes available which are suitable for transfer into apple and pear cultivars. These include genes for control of insect pests such as light brown apple moth and codling moth, and genes to regulate ethylene production by fruit, which affects the rate of ripening.

This study aims to investigate the potential to transfer genes into apple scion cultivars Royal Gala, Pink Lady, Fuji and Granny Smith and pear cultivars Packham’s Triumph and Williams’ Bon Chretien (WBC). The cultivars are good targets because they have major economic significance on Australian domestic and export markets.

Tissue culture regeneration systems which are suitable for gene transfer have been developed for apple and pear cultivars. Royal Gala, Pink Lady and Packham’s Triumph are the most regenerable cultivars in tissue culture. In addition, regeneration can also be obtained with apple cultivars Fuji and Granny Smith and pear cultivar Williams’ Bon Chretien, but as yet this is not at a sufficient level to facilitate gene transfer. The cultivars Royal Gala, Pink Lady and Packham’s Triumph have been genetically engineered with gus and npt II genes using Agrobacterium tumefaciens as a gene transfer system.

Sources of genes with the potential to control important insect pests and to regulate fruit ripening have been identified and obtained under a research only agreements. These include a Bacillus thuringiensis (B.t.) gene and a proteinase inhibitor gene to control light brown apple moth and codling moth. It also includes ethylene forming enzyme (EFE) which is important in the production of ethylene.

A proteinase inhibitor gene isolated from Nicotiana alata (NaPI) has been transferred to apple cultivars Royal Gala and Pink Lady and pear cultivar Packham’s Triumph. These plants are now under going biochemical and molecular analysis, multiplication and insect feeding bioassays. The B.t. gene was putatively transferred to apple cultivar Royal Gala, but the shoots were lost due to fungal infection.
This project is one of the first in the world to establish reliable regeneration systems suitable for transformation of important apple and pear scion varieties. In addition it has pinpointed genetic and resolved critical technical issues essential to successful gene transfer.
1.2 Technical Summary

A biotechnological approach has been developed to complement conventional breeding with the specific objective of introducing genes for insect pest control. This approach requires techniques for regeneration and effective genetic transformation.

The apple cultivars Royal Gala and Pink Lady and pear cultivar Packham’s Triumph have been successfully regenerated in tissue culture. The regeneration system developed for these apple and pear cultivars was based on formation of direct adventitious shoots on leaf explants. The most important factors influencing regeneration were:

- a initial 4 week dark incubation period
- the cytokinin TDZ was more effective than the commonly used BAP
- selection of suitable cultivars

Transgenic plants of Royal Gala and Pink Lady and Packham’s Triumph containing the gus and npt II genes were produced using Agrobacterium tumefaciens as a gene transfer system. Important factors for the transfer of genes from Agrobacterium tumefaciens to apple and pear were:

- the strain of disarmed Agrobacterium tumefaciens, with CZ707 being superior to the commonly used LBA 4404,
- the binary vector, pCGP 257 was better than pKIWI 105 and pBI 121,
- an initial dark incubation of culture prior to transfer to light.

The cultivars Royal Gala, Pink Lady and Packham’s Triumph have been genetically engineered with proteinase inhibitor gene which has the potential to control light brown apple moth and codling moth.

The areas recommended for further research are:

- to continue transformation experiments with B.t. gene with Royal Gala and Pink Lady and Packham’s triumph to obtain additional transgenic plants,
- to commence laboratory insect feeding trials with Royal Gala and light brown apple moth
- to prepare a construct with the NaPI gene under the control of a leaf specific promoter,
- to transform Royal Gala, Pink Lady and Packham’s Triumph with NaPI gene controlled by a leaf specific promoter and to continue with the laboratory insect feeding trials,
- to obtain GMAC approval for glasshouse and field trials at IHD, Knoxfield.

Although the initial project proposal included studies on transfer of genes controlling fruit ripening, this work was discontinued due to industry priorities changing, which place higher emphasis on insect pest control and reduced pesticide usage.
2. Technical Report

2.1 Introduction

The apple and pear industries have a farm gate value of $150M, plus $350M in processing. These are major horticultural industries, with approximately 25,000 ha under production. A priority of these industries is the control of pest and disease with reduced chemical inputs. Genetic engineering offers the opportunity for plant improvement in areas such as pest control and food quality that relate to clean agriculture and export.

The genetic transformation of any plant requires four essential and interacting components. Firstly, the identification of genes with useful characters, secondly a procedure for transferring these foreign genes into plant cells, thirdly a protocol for producing whole plants from cells which have received foreign genes, and fourthly the assessment of plants for the presence of genes.

Transgenic apple plants have been produced by using *Agrobacterium tumefaciens* as a vector combined with adventitious shoot regeneration from leaf explants (James et al. 1989; Maheswaran et al. 1992; Norelli and Aldwinkle 1993) and from regenerating callus (Srikandarajah et al. 1993).

Over the past few years there have been significant advances in the genetic manipulation of plants to introduce insect control genes. Three main classes of genes (*B.t.* endotoxin, proteinase inhibitors and lectins) have been developed and proven effective following introduction into plants. *B.t.* has been used as the most effective biological control. This bacterial insect pathogen is sold under several trade names e.g. DIPEL and THURICLE. The toxin encoding genes from several strains of *B.t.* have been cloned and, characterised. *B.t.* protein toxins can be grouped into four major classes. Each of these proteins has its own specificity to an order of insects. These protein toxins kill sensitive insect upon ingestion, beginning with mid gut paralysis and ending up with disruption of midgut cells. The target pests, light brown apple moth and codling moth belong to the insect order Lepidoptera and the protein Cry IAc is lepidopteran-specific (Brunke and Meeusen, 1991). Access to this gene has been negotiated under a research-only contract with Hort. Research Institute, New Zealand.

Proteinase inhibitors (PI) are the proteins produced naturally in plants for defence mechanisms (Ryan, 1990). Different PIs have slightly different target spectra and the basis for those differences are not yet known. PIs inhibit essential digestive proteinases in the insect resulting in abnormal development and death due to a deficiency of essential amino acids. However, it has been shown that a proteinase inhibitor gene isolated from *Nicotiana alata* (NaPI), is likely to have good activity against some Lepidopteran pests including light blown apple moth, as determined in vitro by using gut extract assays (Ms...
Robyn Heath and Dr Marilyn Anderson - pers. comm.). We have obtained a research-only contract to use this gene.

Regeneration and transformation protocols have been optimised for the apple cultivar Royal Gala, and the applicability of this system to other apple and pear cultivars is reported. The transformation system has been used to transfer a proteinase inhibitor into apple cultivars Royal Gala and Pink Lady and pear cultivar Packham's Triumph.

2.2 Materials and Methods

2.2.1 Cultivars

Apple and pear cultivars of interest were identified by J. Cripps (Department of Agriculture, Western Australia), S. Tancred (Department of Primary Industries, Queensland), B. Mancester (ANFIC, Western Australia) and P. Jerie (Department of Agriculture, Victoria). These include the apple cultivars: Royal Gala, Pink Lady, Fuji, Granny Smith, and pear cultivars Packham's Triumph, and W.B.C. Virus tested bud wood was grafted onto appropriate rootstocks and plants were maintained in a glasshouse.

2.2.2 Establishment and maintenance of shoot cultures

The apple cultivas Royal Gala, Pink Lady, Fuji, Granny Smith and pear cultivar Packham's Triumph were successfully introduced into tissue culture and maintained on shoot proliferation medium consisting of MS basal medium (Murashige and Skoog, 1962) supplemented with 5 μM benzylamino purine (BAP) and gelled with 0.7% (w/v) agar (Difco Bacto). The pear shoot cultures were maintained on the same basal medium supplemented with 10 μM BAP plus 1 μM indol-3-butyric acid (IBA). Cultures were incubated at 25°C ± 1°C with 16:8 h photoperiod under photosynthetically active radiation of 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

2.2.3 Regeneration studies on the apple cultivar Royal Gala

2.2.3.1 Preparation of explants

Leaves from micropropagated shoots of Royal Gala were harvested into sterile water. Leaves were grouped into two categories according to their position: young folded leaves, and young expanding leaves. Five leaves of each categories were placed on media after cutting each leaf into five transverse sections. Explants were placed on the media with the adaxial surface uppermost.

2.2.3.2 Effect of α-naphthaleneacetic acid (NAA) concentration

MS medium was supplemented with 3 μM thidiazuron (TDZ) and either 0, 0.0625, 1.25,
2.5, 5, 7.5 or 10 μM NAA. All media were gelled with 0.25% (w/v) Gelrite. Three Petri dish replicates were used for each medium. Explants were incubated in the dark at 25°C ± 1°C for 4 weeks before exposure to light. After 6 weeks of culture, the number of explants forming shoots and the number of shoots per responding explant were recorded. Data were processed by analysis of variance (ANOVA) using Genstat 5 statistical package (Lawes Agricultural Trust, Rothamsted, U.K.).

2.2.3.3 Effect of antibiotic concentration

A regeneration medium of MS plus 3 μM TDZ and 5 μM NAA was supplemented either with 0, 25, 50, 100 μg/mL kanamycin sulphate or 300 μg/mL cefotaxime. The effects of antibiotics kanamycin (100 μg/mL) and cefotaxime (300 μg/mL) in combination was also tested. Preparation of leaf explants and statistical design were the same as 2.2.3.2.

2.2.4 Agrobacterium strains and growth

Details of Agrobacterium strains, plasmids and promoter can be found in Tables 1 and 2. Agrobacterium strains were maintained either in LB (Sambrook et al., 1989) or MG/L (Loopstra et al., 1990) medium containing antibiotics (Table 1). The binary vectors pCGP257 in CZ707, EHA101 and K61, pBI 121 and pKIWI 105 in LBA 4404, pNaPI in LBA 4404 and CZ707 and the co-integrate vector pGV1103::3850 were used in the transformation experiments.

2.2.5 Transformation

For transformation, bacterial colonies were grown at 28°C with shaking (200 oscillations/min) until a cell density of 5 x 10⁶ cells/mL was obtained. Leaf explants were prepared as described for regeneration, mixed with the bacterial suspension for 5 min and placed on gelled regeneration medium for 2 days. Explants were then transferred to regeneration medium supplemented with 100 μg/mL kanamycin sulphate and 300 μg/mL cefotaxime. After 4 weeks of dark incubation, explants were transferred to fresh selection medium and exposed to light. Regenerated shoots were isolated and grown in individual tubes containing shoot proliferation medium with 50 μg/mL kanamycin and 300 μg/mL cefotaxime.

2.2.6 Expression assays

Histochemical localisation of GUS activity was determined 2 and 3 weeks after cocultivation. The explants were incubated in 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) solution at 37°C for 3 to 4 h prior to macroscopic examination (Jefferson et al., 1987). GUS activity was quantified using methyl umbelliferyl-β-D-glucuronide (MUG) as a substrate (Jefferson et al., 1987). Florescence was measured using a TKO 100 mini-fluorometer (Hoeffer) and protein concentration was measured according to Bradford.
The activity of NPT II in transformed tissue was assayed according to McDonnell et al. (1987).

2.2.7 DNA extraction and Southern blotting

DNA was extracted from leaves harvested from in-vitro grown shoots using a modified protocol of Delaporta et al. (1983). After precipitation of proteins and polysaccharides with SDS and potassium acetate, the supernatant was filtered into an equal volume of phenol : chloroform (1:1) and extracted before addition of isopropanol, incubation and centrifugation. The pellet was dissolved in 3 mL of TE (10 mM Tris. HCl, 1 mM EDTA, pH 8.0) and the DNA precipitated again by the addition of one volume of 7.5 M ammonium acetate and 4 volumes of ethanol. After a further centrifugation the pellet was resuspended in 3 mL of TE. The concentration of DNA was determined by absorbance at 260 nm. Ten µg of DNA was digested with restriction enzyme and fractionated on 0.8% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The DNA was denatured and neutralised (Sambrook et al., 1989) then transferred overnight to Nylon filters (Amersham Hybond N) with 20 x SSC (1x = 0.15 M NaCl/0.15 M sodium citrate, pH 7.0). The Nylon filters were prehybridized for 1 to 2 h at 42 °C in formamide, 5 x Denharts solution (1 x = 0.02% polyvinylpyrrolidone, 0.02% ficoll, 0.02% BSA), 5 x SSC, 0.05% SDS and 50 mg/L denatured salmon sperm DNA. Hybridization was conducted in the same mixture plus 5 to 10 x 10^6 cpm/ µg DNA of random primed npt II and gus probes (Feinberg and Vogelstein, 1984). The npt II probe was an Eco RI fragment derived from pCGN 1558 (McBride and Summerfelt, 1990) and gus probe was an Xba I/Sac I fragment from pBI 221 (Clonetech Laboratories Inc., Palo Alto, California). Filters were washed in 0.2 x SSC with 1% SDS at 65°C prior to autoradiography. The NaPI probe was an Eco RI fragment from pNaPI (University of Melbourne). DNA from plants transformed with gus and npt II were digested either with Hind III, or Hind III plus Eco RI and those transformed with NaPI were digested either with Eco R1 or Eco RV.

2.2.8 Rooting of transgenic shoots

Roots were initiated on transgenic shoots on modified Lepoivre basal medium (Welander, 1988) supplemented with 2.5 µM IBA and 50 mg/L kanamycin sulphate. Shoots on this medium were kept in darkness for 5 days before exposure to light for 2 days and then transferred to hormone free medium.

2.3 Results and Discussions:

2.3.1 Regeneration studies on apple cultivar Royal Gala

2.3.1.1 Effect of NAA concentration

Successful shoot regeneration has been reported for the apple cv. Royal Gala
(Sriskandarajah et al., 1990), and Prof. Skirvin (University of Illinois, USA) who was involved in developing this system, spent his sabbatical in our laboratory. For these reasons Royal Gala was chosen for initial regeneration studies. After 4 weeks of dark incubation, shoots appeared directly on most leaf explants (Fig. 1.B). NAA concentration had no significant effect on the percentage of leaf explants responding for either leaf categories (Table 3). The number of shoots produced per responding explant increased with increasing NAA concentration, with an optimum of 5 µM. Responding explants from folded and expanding leaves produced an average of 12.3 and 13.1 shoots respectively at 5 µM NAA. Callus which developed nearer to the adventitious shoots gave a false appearance to shoots, as if they were originating from callus (Fig. 1A). Callus formation on explants increased with increasing NAA concentration.

2.3.1.2 Effect of antibiotics concentration

Within 4-5 weeks of culture, 93% leaf explants cultured on regeneration medium without any antibiotics, produced adventitious shoots. The addition of 300 µg/mL cefotaxime to the regeneration medium did not have a significant effect on regeneration (Table 4B). In regeneration studies of pear (Predieri et al. 1992) and apple (Maheswaran et al. 1992) a high concentration of cefotaxime (400-500 µg/mL) was reported to decrease regeneration of leaf explants and the number of shoots per explant.

The effect of kanamycin on shoot regeneration is shown in Table 4A. With increase in kanamycin concentration the percentage of explants regenerating shoots decreased (Table 4A). At 25 µg/mL kanamycin a combination of green and pale shoots were observed (Fig. 2). This indicates the green shoots are presumably escapes and 25 µg/mL would not be a good selection to use. At 100 µg/mL kanamycin, no explants responded to form adventitious shoots and this could be a good selection pressure to impose. However the same medium, in the presence of 300 µg/mL cefotaxime, 38% of the explants regenerated shoots. These explants and the shoots were pale in colour at the end of 4-5 weeks of culture.

2.3.2 Transformation studies with gus and npt II genes

The effect of the binary vector pCGP 257 in three strains of Agrobacterium, the binary vector pKIWI 105 and pBI 121 in Agrobacterium strain LBA4404, and the co-integrate vector pGV1103::3850 are shown in Table 5. The binary vectors pCGP 257 and pKIWI 105 were effective in inducing transgenic shoots in Royal Gala. The binary vector pCGP 257 in Agrobacterium strain CZ707 was more effective than in strains EHA101 and K61. This indicates that Agrobacterium strains have different capacities to transfer genes into plant cells and most likely to be contributed by the vir genes, which play an important role in Agrobacterium attachment to the plant cell wall and in the transfer of T-DNA (Zambryski, 1988). This series of experiments have been extremely important, as they have indicated the importance of Agrobacterium strains. Consequently, the Agrobacterium
strains CZ707 and LBA 4404 have been chosen for the subsequent work with economically important genes.

The binary vectors pCGP 257 and pKWI 105 contain gus and npt II genes. The gus gene in transformed tissues was detected by histochemical and fluorimetric assays. Histochemical GUS expression in a developing shoot primordia was detected 2 weeks after inoculation (Fig. 3). Figure 4 shows a developing shoot from an explant 4 weeks after inoculation. Histochemical GUS expression in leaves showed that 11 transformed shoots (from 2.7% of all explants) were recovered after inoculation with pCGP257:CZ707 and 2 shoots (0.6%) with pKWI 105:LBA 4404 (Table 5). GUS activity was quantified in leaf tissue from in vitro grown plants. Specific activity of GUS varied between individual transgenic shoots (Fig. 5). Differential expression might be attributable to positional effects when a gene is incorporated in the genome (Jones et al., 1985), copy number effects (Sanders et al., 1987), or to incomplete gene transfer (Parrott et al., 1989).

Leaves harvested from all putative transgenic shoots, showed NPT II activity (Fig. 6).

Integration of the T-DNA containing npt II and gus genes was confirmed by southern blot analysis for all putative transformants. Extracted DNA was digested with Hind III and Hind III plus Eco RI, and Southern blots were probed with npt II and gus fragments. Integration patterns could be predicted from the T-DNA restriction map (Fig. 7). This combination of enzyme (Hind III plus Eco RI), could be expected to hybridise to fragments of a known size, based on the T-DNA map. In other case (Hind III) variable size fragments would be anticipated, confirming integration into high molecular weight DNA and providing an estimate of the integrated T-DNA copy number. The southern blot data shown in Fig. 8 A, B & C were derived from leaves harvested from transgenic lines which had single shoot origin. The anticipated variable Hind III fragments were also observed, and their size differed between individual transformed plants (Fig. 8A). DNA from these plants digested with Hind III plus Eco RI hybridised with the gus probe resulted in the expected 2.9 kb band (Fig. 8B) and with the npt II probe giving the expected 1.5 kb fragment (Fig. 8C).

Four explants co-cultivated with pCGP 257:CZ707 produced a cluster of shoots. Shoots recovered from each of these clusters were different in number (11, 5, 3 and 3). A cluster of shoots could have resulted either from branching of a single shoot, or a number of shoots could have developed from closely placed cells and could have given a cluster appearance on development of shoots. If the shoots recovered from a cluster were the result of branching of a single shoot, then they would be expected to have the same pattern of both gene integration and level of protein expression. In contrast, shoots originating from closely placed cells cannot be treated as a uniform population of shoots. In order to maintain pure transgenic lines, shoots recovered from a cluster were treated as individual lines and were maintained on shoot multiplication medium under selection (50 mg/L kanamycin).
Specific activity of GUS in shoots recovered from 3 of the clusters (Fig. 9 - Clusters 2, 3 and 4) was almost the same within each cluster except in cluster 1. Differential activity observed in cluster 1 might be attributed to the different cell origin of each shoot. To confirm this observation, a Southern blot assay was performed on all shoots recovered. The Southern blot data on shoots originated from 4 clusters are presented in Fig. 10 A, B, C & D. Shoots from clusters 2, 3 and 4, showed almost the same size Hind III fragment with gus probe indicating shoots from these clusters could have resulted from a single transformation event in each of these clusters (Fig. 10B). Shoots from cluster 1 showed variable Hind III fragments (Fig. 10A) indicating that the shoots originated from different transformation events. As expected, DNA isolated from shoots from all 4 clusters hybridised to 2.2 kb Hind III fragment (Fig. 10 C&D).

2.3.3 Transformation of other cultivars of apple and pear

Pink Lady and Packham's Triumph were selected for transformation with pCGP257:CZ707 using the same protocol developed for Royal Gala. Shoots of both cultivars have been regenerated in the presence of kanamycin and are GUS positive. Further biochemical and molecular analysis will be done on this material.

2.3.4 Transformation studies with potential pest control gene (Nicotiana alata Proteinase Inhibitor)

Leaf explants of Royal Gala and Pink Lady inoculated with pNaPI:CZ707 regenerated shoots in the presence of kanamycin. The transgenic nature of these shoots was confirmed by Southern blot analysis (Fig. 12). Integration pattern could be predicted from the T-DNA map (Fig. 11). DNA digested with Eco RV resulted in variable size fragments (Fig. 12A). For Royal Gala, regenerated plants contained 3-5 copies of the NaPI gene where transgenic plants of Pink Lady contained 1-4 copies. DNA digested with Eco RI resulted in the expected fragment of 2.0 kb (Fig. 12B). This confirmed the integration of NaPI gene into apple genome of cultivars Royal Gala and Pink Lady.

Table 6 shows that both strains of disarmed Agrobacterium, LBA 4404, and CZ707 were effective in producing putative transgenic shoots in Royal Gala and Pink Lady. In both cases, Royal Gala produced higher numbers of transgenic shoots than Pink Lady (Table 6). Leaf explants co-cultivated with LBA 4404 produced higher numbers of transgenic shoots than when co-cultivated with CZ707. Preliminary experiments with Packham's Triumph have resulted in one transgenic shoot.

The above results indicate that the transformation protocol developed for apple cultivar Royal Gala using gus and nptII genes is applicable to other apple (Pink Lady) and Pear (Packham's Triumph) cultivars. Application of the protocol was extended to transferring the potential pest control gene (NaPI) into the apple cultivars Royal Gala and Pink Lady and pear cultivar Packham's Triumph.
Acknowledgments:

This work was funded by Apple and Pear Australian Corporation and HRDC. I wish to thank Mr G. Darcy for the technical support given during this work.


2.5 Extension/adaptation by industry:

Field trials will be conducted under the guidelines of GMAC before the commercial release of genetically modified apple and pear cultivars to growers can occur. With respect to commercialisation of genetically engineered food products, it should be noted that the transgenic tomatoes has been released onto the market in the USA. This is the first release of transgenic plant material onto a food market. We expect this commercial release to pave the way for the acceptance of genetically modified food by the public in general. GMAC has received request for field trials of the following genetically engineered plants: potato, tomato, sugarcane, lupene, subclover and apple. GMAC has now approved some of these applications. CSIRO is conducting a wide ranging public education program to help facilitate the acceptance of genetically modified products. Results from this research are suitable for publication in scientific journals, trade journals, and scientific and industry forums. It is anticipated that grower organisations will wish to be informed of this new and rapidly expanding area of science, the potential of which is immense.

2.6 Direction of future research:

Future research will be confined to laboratory and glasshouse studies. Transgenic plants with pest resistance genes \textit{NaPI} will be assayed. Transformation experiments will be continued with \textit{B. t.} gene with Royal Gala, Pink Lady and Packham's Triumph to obtain additional transgenic plants. Putative transgenic plants will be multiplied \textit{in vitro} to provide material for the confirmation of gene transfer using techniques of polymerase chain reaction (PCR) and Southern blot analysis. Gene products will be quantified in individual transgenic plants using immuno-blot techniques. Techniques to measure the level of proteinase inhibitor gene expression have already been developed by our collaborators at University of Melbourne and are now available for use in the assessment of transgenic plants. Colonies of light brown apple moth and codling moth have been established in the insectary at IHD and will provide a continuous supply of test insect larvae for bioassay. Feeding bioassays, measuring larval growth rate and mortality, will initially screen transgenic shoots to assess the level of expression of PI and \textit{B. t.} endotoxin. For more advanced lines, bioassay techniques will be developed for leaf and fruiting parts to determine tissue specific expression. Feeding bioassays with different larval ages will fully define the level of control that may be expected from the transgenic plants. Genes will be reconstructed under a tissue specific promoter (e.g. green tissue-specific). The effect of different promoters will be included in future transformation studies. The transgenic plants carrying the genes under the control of either constitutive or green tissue
specific promoters will also be transferred to glasshouse conditions in order to conduct insect feeding bioassays and to initiate field evaluation.
Table 1: Media and antibiotics used in maintaining the *Agrobacterium* strains.

<table>
<thead>
<tr>
<th>Plasmid/Strain</th>
<th>Medium + Antibiotics</th>
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<tbody>
<tr>
<td>pCGP 257:CZ707</td>
<td>LB + 100 µg/mL gentamycin</td>
</tr>
<tr>
<td>pCGP 257:K61</td>
<td>LB + 100 µg/mL gentamycin</td>
</tr>
<tr>
<td>pCGP 257:EHA101</td>
<td>LB + 100 µg/mL gentamycin</td>
</tr>
<tr>
<td>pKIWI 105:LBA 4404</td>
<td>MG/L + 100 µg/mL kanamycin + 30 µg/mL chloramphenicol</td>
</tr>
<tr>
<td>pBI 121:LBA 4404</td>
<td>LB + 25 µg/mL kanamycin + 50 µg/mL</td>
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<tr>
<td>pNaPI:LBA 4404</td>
<td>LB + 5 µg/mL tetracycline</td>
</tr>
<tr>
<td>pGV1103::3850</td>
<td>LB + 25 µg/mL kanamycin + 50 µg/mL rifampicin</td>
</tr>
<tr>
<td>pNaPI:CZ707</td>
<td>LB + 5 µg/mL tetracycline</td>
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Table 2: Promoters driving *gus*, *npt II*, and *NaPI* genes in binary vectors pCGP 257, pBI 121, pKIWI 105, and pNaPI and co-integrated vector pGV1103::.

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Promoter driving <em>gus</em> gene</th>
<th>Promoter driving <em>npt II</em> gene</th>
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<tr>
<td>pCGP 257</td>
<td>CaMV 35S</td>
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Table 3: Effect of NAA concentration on adventitious shoot regeneration of folded and expanding leaf segments of apple cultivar Royal Gala cultured on basal medium MS supplemented with 3 μM TDZ.

<table>
<thead>
<tr>
<th>NAA Conc.(μM)</th>
<th>Folded</th>
<th>Expanding</th>
<th>Folded</th>
<th>Expanding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86.7</td>
<td>82.3</td>
<td>6.2</td>
<td>6.4</td>
</tr>
<tr>
<td>0.063</td>
<td>88.7</td>
<td>91.0</td>
<td>6.3</td>
<td>6.1</td>
</tr>
<tr>
<td>1.25</td>
<td>82.3</td>
<td>93.0</td>
<td>8.1</td>
<td>7.0</td>
</tr>
<tr>
<td>2.5</td>
<td>82.0</td>
<td>93.3</td>
<td>4.2</td>
<td>6.8</td>
</tr>
<tr>
<td>5.0</td>
<td>93.3</td>
<td>97.7</td>
<td>12.3</td>
<td>13.1</td>
</tr>
<tr>
<td>7.5</td>
<td>64.3</td>
<td>91.0</td>
<td>5.7</td>
<td>4.4</td>
</tr>
<tr>
<td>10</td>
<td>72.0</td>
<td>91.0</td>
<td>6.3</td>
<td>9.4</td>
</tr>
</tbody>
</table>
**Table 4A: Response of leaf explants on shoot regeneration medium supplemented with kanamycin.**

<table>
<thead>
<tr>
<th>Concentration of kanamycin (µg/mL)</th>
<th>% explants forming shoots</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>93 (green)</td>
</tr>
<tr>
<td>25</td>
<td>69 (38% green; 31% pale)</td>
</tr>
<tr>
<td>50</td>
<td>27 (pale)</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 4B: Effect of antibiotics on leaf explants placed on shoot regeneration medium supplemented with kanamycin and cefotaxime.**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Number of explants forming shoots (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin (µg/mL)</td>
<td>Cefotaxime (µg/mL)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>300</td>
</tr>
<tr>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 5: Effect of vectors and *Agrobacterium* strains on transformation of apple cv. Royal Gala.

<table>
<thead>
<tr>
<th>Vector: <em>Agrobacterium</em> strain</th>
<th>No. of explants</th>
<th>Explants with green shoots</th>
<th>Explants with transformed shoots (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCGP 257:CZ707</td>
<td>404</td>
<td>11</td>
<td>2.7</td>
</tr>
<tr>
<td>pCGP 257:K61</td>
<td>258</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pCGP 257:EHA101</td>
<td>312</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pKIWI 105:LBA 4404</td>
<td>325</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
<td>pBI 121:LBA 4404</td>
<td>259</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pGV1103::3850</td>
<td>270</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 6: Efficacy of *Agrobacterium* strains in transferring *NaPI* gene into apple cultivars Royal Gala and Pink Lady.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Royal Gala</th>
<th>Pink Lady</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of explants cultured</td>
<td>Explants with green shoots</td>
</tr>
<tr>
<td>pNaPI:LBA 4404</td>
<td>908</td>
<td>26</td>
</tr>
<tr>
<td>pNapI:CZ707</td>
<td>539</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 1A Effect of NAA concentration on number of shoots formed and callus production. Leaf cultured on basal medium (Murashige and Skoog, 1962) supplemented with (A) - 3 μM TDZ; (B) - 3 μM TDZ plus 1.25 μM NAA; (C) - 3 μM TDZ plus 2.5 μM NAA and (D) - 3 μM TDZ plus 5 μM NAA.

Fig. 1B: Adventitious shoot developing directly from leaf explant after 4 weeks on Murashige and Skoog medium containing 3 μM TDZ and 5 μM NAA.
Fig. 2. Response of leaf explants on shoot regeneration medium supplemented with (A), 0 mg/L kanamycin and 0 mg/L cefotaxime; (B), 300 mg/L cefotaxime; (C), 100 mg/L kanamycin and 300 mg/L cefotaxime; (D), 25 mg/L kanamycin; (E), 50 mg/L kanamycin and (F), 100 mg/L kanamycin.
Fig. 3: Shoot primordia developing on leaf explant 2 weeks after co-cultivation with a primordia showing GUS activity.

Fig. 4: Leaf explant 4 weeks after co-cultivation with a well defined shoot with GUS activity.
Fig. 5: GUS activity in leaf tissue of transformed plants. Columns 1 and 2 were inoculated with pKIWI105:LBA4404 and columns 3 to 8 with pCGP 257:CZ707. C shows the activity in un-transformed (negative) Royal Gala plant. TO was leaf extract from a GUS-positive transformed tobacco plant and was included for comparison.
Fig. 6: Dot blot showing NPT II activity in leaf tissue. NPT II activity is indicated by dark spots.
Fig. 7: Diagrammatic representation of binary vector pCGP 257. The various components of the vector are represented by boxes, and the positions of the Eco RI and Hind III restriction sites within the T-DNA are indicated by arrows. Shaded boxes below the map represent npt II and gus probes. Lines below these probes denote the anticipated size of Hind III and Hind III/Eco RI restriction fragments. Solid lines indicate internal fragments and broken lines indicates fragments generated from sites outside the border sequences. LB = left border; RB = right border; 5'nos = nopaline synthase (NOS) promoter; 3'nos = NOS terminator.
Fig. 8: Southern blot analysis of plants transformed with pCGP 257. Lanes 1 to 11 are individual transformants. Lane 12 is un-transformed Royal Gala. Lane 13 is positive M26 transformed with pCGP 257. A and B were probed with gus and C with npt II. Fig. 8A shows results from Hind III digestion; 8B and 8C results from Hind III/Eco RI digestion. The size of some hybridizing fragments (kb) is indicated.
Fig. 9: GUS activity in leaf tissue of transformed plants with pCGP257. Each column shows activity in a single shoot. Columns 1 to 11 shows the activity in shoots recovered from cluster 1, columns 1 to 5 in shoots recovered from cluster 2, columns 1 to 3 in shoots recovered from cluster 3 and columns 1 to 3 in shoots recovered from cluster 4. C shows the activity in un-transformed Royal Gala plant. TO was extracted from a GUS positive transformed tobacco plant and was included for comparison.
Fig. 10: Southern blot analysis of plants transformed with pCGP257 following Hind III digest. In fig. 10A, lanes 1 to 11 are from shoots recovered from cluster 1 and lane 12 is positive Royal Gala transformed with pCGP 257, and of single shoot origin. In fig. 10B, lanes 1 to 5 are from shoots from cluster 2; lanes 6 to 8 are from shoots from cluster 3; and lanes 9 to 11 are shoots from cluster 4. Lane 12 is positive M26 transformed with pCGP 257. A and B were probed with gus and C and D with npt II.
Fig. 11: Diagrammatic representation of binary vector pNaPI. The various components of the vectors are represented by boxes, and the positions of the Eco RI and Eco RV restriction sites within the T-DNA are indicated by arrows. Boxes below the map represent npt II and NaPI probes. Below these probes, fragment sizes expected after restriction enzymes digest of the plant DNA with Eco RI and Eco RV are shown. LB = left border; RB = right border; 5'nos = nopaline synthase (NOS) promoter; 3'nos = NOS terminator; s2 = self incompatibility gene terminator.
Fig. 12: Southern blot analysis of apple plants transformed with pNaPI. In Fig. 12A and B lanes 1 to 3 are DNA isolated from transgenic lines of Royal Gala and 4 to 6 are from transgenic lines of Pink Lady. Fig. 12A shows the fragment size after digesting with \textit{Eco RV} and probing with \textit{NaPI}. Fig. 12B shows the fragment size after digesting with \textit{Eco RI} and probing with \textit{NaPI}. 