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Use of the GUS gene as a selectable marker for *Agrobacterium*-mediated transformation of *Rubus*

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Abstract

A transformation system was established for red raspberry, blackberry and blackberry × raspberry hybrids, utilizing the binary vector system of *Agrobacterium tumefaciens*. Leaf discs or internodal stem segments were inoculated with *Agrobacterium* strain LBA4404 containing the binary vectors PBI121.X, which has the β-glucuronidase (GUS) marker gene, or Bin 19, which has the neomycin phosphotransferase II (NPT II) gene. Regenerants were produced on media containing MS salts, 20 gl^{-1} sucrose, 7 gl^{-1} agar, 100 mgl^{-1} inositol, 0.5 mgl^{-1} nicotinic acid, 0.5 mgl^{-1} pyridoxine-HCl, 0.1 mgl^{-1} thiamine, and either 0.1 mgl^{-1} IBA and 2 mgl^{-1} BAP for leaf discs, or 0.2 mgl^{-1} BAP and 0.2 mgl^{-1} 2,4-D for stem segments. Kanamycin sulphate, which was used as a selective agent for the NPT II gene, inhibited organogenesis at 50 mgl^{-1} and was therefore unsuitable for use as a selectable marker gene in *Rubus*. All regenerants were assayed utilizing the fluorogenic assay procedure to determine if the GUS gene had been transferred into the material and could therefore cleave the substrate 4-methyl-umbelliferyl-β-D-glucuronide. Seven GUS-positive plantlets were obtained which confirmed that this marker gene had been transferred into *Rubus*. A 'dot blot' assay was carried out on GUS-positive plant material to establish if the NPT II gene had also been transferred to the plant material.

Introduction

Considerable progress has been made in the development of techniques for introducing new genes into plants. Most emphasis has been on annuals and crops of major importance or those that are amenable to manipulation in tissue culture. Little has been done with perennial crops such as fruit.

The highly heterozygous nature of fruit cultivars precludes the incorporation of a single gene through hybridization without altering the essential characters of the cultivar or proceeding through many generations of inbreeding. In soft fruit, a cultivar can be widely grown and highly successful even though it lacks a desirable characteristic which may be controlled by a single major gene.

Techniques which allow the introduction of a single gene without disturbing the remainder of the genome would therefore be particularly valuable for these crops. In some instances it could be used to introduce a totally new characteristic and in others it would provide a more advantageous means of incorporating an improvement that can only be achieved with difficulty by conventional breeding methods. The introduced genes may be from other genera, usually microorganisms, or they may have been isolated and cloned from the same genera.

The simplest and most successful techniques have used the gene vector system of *Agrobacterium tumefaciens* to insert foreign genes into plants. The system is based upon the ability of *Agrobacterium* to facilitate the transfer of DNA into its host plant and is used to introduce the novel genes which are

then expressed as if they were part of the host genotype [6,7,8,13,14,24,25].

The role of *Agrobacterium*-mediated gene transfer in the genetic improvement of fruit trees has been highlighted recently [9] and morphologically normal transgenic plants have been produced in a number of species, including apple [10], using binary vectors [1,7,24]. A binary Ti-plasmid vector system in *Agrobacterium* consists of two plasmids. The engineered binary vector utilizes the *trans* acting functions of the *vir* region of a co-resident Ti-plasmid to transfer sequences bordered by left and right T-DNA sequences into the nuclear genome of plants.

We have recently described in vitro regeneration techniques for *Rubus* using either leaf discs or internodal segments [19] and have also assessed the infectivity of various *Agrobacterium* isolates for *Rubus* hosts. We now describe the use of the binary vector PBI121.X [12] for the insertion of a foreign gene into *Rubus*, and the expression of this gene in regenerated plantlets. This vector contains the β-glucuronidase (GUS) gene which is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronidases. Hence the gene codes for a stable enzyme with desirable properties that serves as a marker of successful transformation [11] because it induces changes in the transformants that can be assessed quickly and simply.

Material and methods

Plant material

Micropropagated plantlets of the red raspberry SCRI 8242E6, the blackberry × raspberry hybrids cvs. Tayberry and Sunberry and the blackberry cv. Loch Ness were maintained in vitro on Murashige & Skoog medium (Flow Laboratories Ltd., Irvine, Scotland, Cat. No. 26-100-200) with agar ($7\,g\,l^{-1}$), sucrose ($30\,g\,l^{-1}$), activated charcoal ($20\,g\,l^{-1}$) and the following additions: $100\,mg\,l^{-1}$ inositol, $1\,mg\,l^{-1}$ thiamine-HCl, $2\,mg\,l^{-1}$ glycine, $200\,mg\,l^{-1}$glutamine, $0.01\,mg\,l^{-1}$ biotin, $1\,mg\,l^{-1}$ nicotinic acid, $1\,mg\,l^{-1}$ pyridoxine-HCl, $1\,mg\,l^{-1}$ calcium pantothenate and $1\,mg\,l^{-1}$ cysteine-HCl.

Leaf discs and internodal segments were prepared and regenerants were obtained as described by McNicol & Graham [19], except the cultures were inoculated with a bacterial suspension immediately after leaf discs and stem segments were excised from the micropropagated stock cultures.

Baterial strain and binary vectors

A. tumefaciens strain LBA4404 which was disarmed of its ability to induce galls on inoculated plants and carried the binary vectors PBI121.X [12] or Bin19 [1] was maintained at 4 °C on LB agar [15]. A loopful of bacteria was inoculated into a vial containing 10 ml of LB broth [15] with $50\,mg\,l^{-1}$ each of the antibiotics kanamycin sulphate and streptomycin for PBI121.X or kanamycin sulphate and rifamycin also at $50\,mg\,l^{-1}$ for Bin19. The vials were placed in a shaking water bath at 28 °C overnight and the bacterial suspensions were then centrifuged at 14000 rpm for 10 min. The resulting pellets were resuspended in 10 ml aliquots of liquid Murashige & Skoog basal medium (Flow Laboratory Cat. No. 26-100-200) which were poured into the 9 cm plastic Petri plates to be used for inoculation.

Inoculation of explants

Leaf discs and internodal stem segments for inoculation were placed asceptically into the bacterial suspensions. After 20 min they were moved to sterilized filter paper discs and placed on the surface of solidified regenerating medium [19]. This contained MS medium (Flow Laboratories), $7\,g\,l^{-1}$ agar, $20\,g\,l^{-1}$ sucrose, and the hormones 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) both at $0.2\,mg\,l^{-1}$ for internodal stems, and indolkebutyric acid (IBA) at $0.1\,mg\,l^{-1}$ and BAP at $2\,mg\,l^{-1}$ for leaf discs.

The inoculated explants were co-incubated for 24 h at 24°C under continuous warm white fluorescent tubes ($70\,\mu mol\,m^{-2}\,s^{-1}$). They were then removed from the filter paper discs, dipped in a solution of the antibiotic carbenicillin ($400\,mg\,l^{-1}$), dipped into sterile distilled water and blotted on sterile filter paper before being placed on a fresh plate of the appropriate regeneration medium. The leaf discs were always placed with their abaxial surface uppermost but in other respects the ex-

plants were maintained under the conditions previously described. The procedure of dipping into carbenicillin, sterile distilled water and blotting was repeated at two-day intervals until plantlet regeneration occurred. The plants were subsequently placed onto micropropagation media containing 400 mg l^{-1} carbenicillin.

Explant material was not placed directly onto antibiotic-containing media after co-incubation as we have previously observed that regeneration of *Rubus* sp. was greatly inhibited in the presence of as little as 50 mg l^{-1} of the antibiotics kanamycin, carbenicillin and cefotaxime.

When *A. tumefaciens* LBA4404 containing the binary vector Bin19 was used for inoculation the explants were placed on regenerating media containing the selection agent kanamycin sulphate at 50 mg l^{-1} to identify the transformants. The results indicated that this antibiotic severely inhibited plantlet regeneration. This was confirmed in the absence of *Agrobacterium* by placing 28 internodal segments each of the berry hybrids cvs. Tayberry and Sunberry on a regeneration medium containing one of three levels of kanamycin sulphate (0, 50 and 75 mg l^{-1}). The antibiotic clearly inhibited plantlet regeneration and largely precluded the use of this marker to select transformed plants containing the neomycin phosphotransferase II (NPT II) gene of Bin19. The GUS marker of PBI121.X was therefore used in all further experiments to identify transformants.

GUS assay

The fluorogenic assay procedure [11] which uses 4-methyl-umbelliferyl-β-D-glucuronide as the substrate was used to identify plant material containing the GUS gene. Tissue was prepared for assay by grinding it in GUS extraction buffer [11] and centrifuging at 14 000 rpm. The supernatant (360 μl) of the liquid obtained was added to 40 μl of substrate to start the reaction. After 0, 15, 30, 45 and 60 min, 80 μl of the reaction mixture were removed and the reaction terminated by the addition of 900 μl of Na_2CO_3 (0.2 M). Non-inoculated plant material and the *A. tumefaciens* strain LBA4404 containing the PBI121.X vector were used as negative and positive controls, respectively.

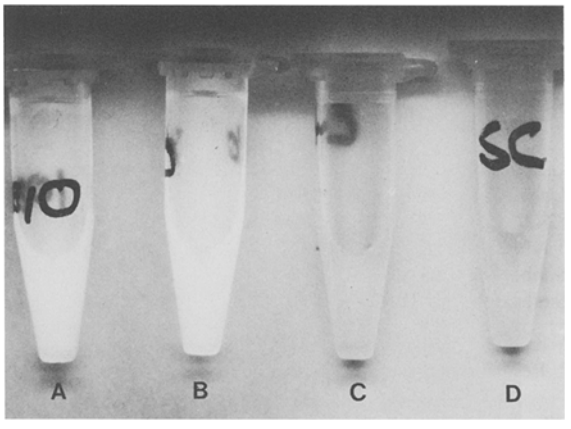

Fig. 1. GUS assay showing the strong fluorescence (tubes A and B) from blackberry cv. Loch Ness plantlets derived from an explant inoculated with *Agrobacterium tumefaciens* strain LBA4404 carrying the vector PBI121.X, and non-fluorescence (tube D) from a non-inoculated plantlet of cv. Loch Ness. Tube C shows slight fluorescence and was derived from a bacterial suspension of LBA4404 carrying PBI121.X. Reaction times: tubes A and C 15 min, B 30 min and D 24 h.

Results and discussions

There is little or no detectable β-glucuronidase activity in almost any higher plant [11], and since none was found in the *Rubus* genotypes used in these transformation experiments prior to inoculation untreated *Rubus* material can be used as negative controls. The GUS gene can therefore be used conclusively to identify successful transformation provided care has been taken to ensure no *Agrobacterium* contamination is present on the plant material.

A fluorogenic assay [11, 12] was used to detect the presence of GUS using the commercially available substrate 4-methyl-umbelliferyl glucuronide (Sigma, Cat. No. M-9130). This does not fluoresce until cleaved by the β-glucuronidase enzyme to release 4-methyl-umbelliferone. In plant species, where the GUS gene on the PBI121.X vector has been inserted into the genome, the substrate will have cleaved and fluoresce. In our experiments the positive control (LBA4404 containing PBI121.X) and stem and leaf sections from inoculated plant material changed from non-fluorescent purple to a very strong fluorescent blue under UV light within one hour (Fig. 1). The negative controls remained purple, even after 24 hours of assay. Transformed

Table 1. Effect of inoculation and kanamycin sulphate on plantlet regeneration.

Genotype	Explant source[a]	Percentage of explants regenerating shoots				
		− *Agrobacterium* − kanamycin	+ *Agrobacterium* − kanamycin	− *Agrobacterium* + kanamycin 50 mg l^{-1}	− *Agrobacterium* + kanamycin 75 mg l^{-1}	+ *Agrobacterium* + kanamycin 50 mg l^{-1}
Loch Ness	S	38-64[b]	23			
8242E6	S	38-64[b]	10			
Tayberry	S	50	3	0	0	
Sunberry	S	54	-	4	0	4
Sunberry	LD	46[b]	13			

[a] S = internodal stem segments; LD = leaf disc

[b] Results from similar experiments reported by McNicol & Graham [18]

plant material produced a strong blue fluorescence after only 15 minutes whereas the positive control took 30 minutes to give the same strength of colour.

Positive GUS assays were obtained from four Tayberry, one Sunberry, two 8242E6 and seven Loch Ness regenerants arising from 110 explants that had been inoculated with LBA4404 carrying the binary vector PBI121.X. This confirms that the marker gene β-glucuronidase had been incorporated into the genomes of the red raspberry, blackberry and hybrid berry.

The absence of *Agrobacterium* contamination was shown by plating out the GUS-positive plant material onto nutrient broth.

Kanamycin sulphate was used to select those plants transformed with LBA4404 and containing the binary vector Bin19 and therefore the neomycin phosphotransferase II gene. Plants containing this gene remain green and those without it turn white when grown on media containing the antibiotic [2, 3, 5, 21], but kanamycin sulphate almost totally inhibited organogenesis at levels sufficient to cause whitening of *Rubus* leaves (50 mg l^{-1}) [18] (Table 1). The presence of *Agrobacterium* also reduced the numbers of regenerants (Table 1) and no regenerants were formed when the antibiotic and bacteria were present together. A further disadvantage of the kanamycin selection system is that it is unreliable as a method of detecting the neomycin phosphotransferase II gene in plants [23].

Of the two binary vector systems used, PBI121.X carrying both the NPT II and β-glucuronidase genes provided a more efficient identification system for the occurrence of transformation. The GUS assay can be carried out quickly and simply on a large number of regenerants at one time to identify transformed plants.

Once regenerated and assayed utilizing the GUS system, a small number of plantlets were placed onto micropropagation media containing 75 mg l^{-1} kanamycin to establish if the NPT II gene had also been transferred. All GUS-positive plantlets remained green (non-transformed plant material whitened) and a 'dot blot' assay [17] was carried out confirming the presence of this gene.

When the techniques described for inoculation and incubation are combined with those for plantlet regeneration [19], the insertion of exogenous DNA into *Rubus* plants becomes feasible for the first time. This will provide a new spectrum of germplasm for *Rubus* breeders and facilitate the introduction of genes for pest and disease resistance into established cultivars. Hopefully this will be accomplished without otherwise changing other characteristics of the cultivar as would happen with traditional breeding techniques involving a sexual process. The behaviour of inserted exogenous genes in mature plants of *Rubus* and their stability during development and in the germ cell has still to be determined. However, the indications from other plant species are favourable [16,22,23,24].

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