Feasibility Studies Using β-Glucuronidase as a Gene Fusion Marker in Apple, Peach, and Radish

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Abstract. The udiA gene encoding the enzyme β-glucuronidase (GUS) appears promising as a genetic marker for early confirmation of successful plant cell transformation. Two strains of Agrobacterium rhizogenes and eight strains of A. tumefaciens were selected as hosts to carry a binary plasmid (pBI121) containing the marker gene encoding the GUS marker that is controlled by the CaMV35S promoter. Presence of plasmid pBI121 in the bacteria was confirmed by resistance to kanamycin, plasmid re-isolation, and restriction enzyme analysis. When the GUS enzyme was expressed in transformed plant cells, reaction with the histochemical substrate 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) lead to blue pigment development. Expression of GUS in viable bacteria that had not been eliminated from transformed tissue caused problems with the early transformation detection in radish, peach, and apple stem sections by also producing a positive X-gluc color reaction. Putative transformation of apple xylem parenchyma callus was accomplished, as judged by resistance to kanamycin, opine analysis, GUS marker gene expression, and presence of the APH(3')11 enzyme. In this system, elimination of bacterial contamination was accomplished during multiple culture transfers on selective media. To be more useful as a marker, the GUS gene should be coupled with a promoter that will not be expressed by Agrobacterium. Parenchyma callus may serve as a primary screen to provide an efficient way of determining the ideal strain for transformation of deciduous tree fruit genera. In our studies, strain A281 consistently proved to be a vector superior to others tested.

Techniques in molecular biology provide a more-rapid approach for cultivar improvement than conventional breeding. Using these techniques, an undesirable characteristic of an established cultivar might be corrected without resorting to the time-consuming tactics common in standard plant breeding procedures. If the trait to be corrected is under single-gene control, then gene insertion methods may be possible. Examples include tolerance to herbicides (Shah et al., 1986; Fillatti et al., 1987a), insects (Vaeck et al., 1987; Fischhoff et al., 1987), and viruses (Powell et al., 1986). Although the previous citations concern work with annual plants, the principles used should hold true for perennial deciduous tree genera. Still, one cannot be certain of successful phenotypic change until the gene has been expressed. If the response concerns flowering, years will elapse before evaluation is possible in deciduous genera.

Marker genes can ameliorate some of the problems mentioned and accelerate transformation evaluation. These inserted mark-
ers are genes, foreign to the plant’s genome, that can be detected with sensitive molecular biological techniques. The \textit{kan} gene encoding a bacterial kanamycin phosphotransferase [APH(3’)] and genes encoding opine synthesis have been used successfully in various woody plants (Dandekar et al., 1987, 1988, 1990, Fillatti et al., 1987b; Martin et al., 1989; Hammerschlag et al., 1989; James et al., 1989). The system used in our current work exploits the \textit{udiA} gene found in \textit{Escherichia coli} encoding the GUS enzyme (Jefferson et al., 1986). The advantages of GUS assays are their sensitivity, ease of administration, and low relative cost.

The goals of our research were as follows: to insert a GUS-containing plasmid into two strains of \textit{Agrobacterium rhizogenes} and eight strains of \textit{A. tumefaciens}, to verify the presence of GUS-containing plasmids in those strains, and to test the usefulness of the \textit{Agrobacterium} strains containing the GUS gene marker system as a quick probe to determine whether radish, peach, and apple could be transformed.

\section*{Materials and Methods}

\textbf{Bacterial strains and plasmids.} Sixty strains of \textit{Agrobacterium} were previously tested for tumor formation on four forest tree species and a range of pathogenicity was observed (Morris et al., 1989). Eight strains were selected, covering the pathogenicity range, for introduction of the GUS marker-containing plasmid (Table 1). Two other strains were selected as nononcogenic controls—NT1, which has no Ti plasmid (“cured”), and LBA4404, which is disarmed of oncogenic properties; thus, the vir region of the Ti resident plasmid will only transfer the plasmid T-DNA that contains the gene encoding the GUS marker controlled by a CaMV35S promoter.

\textit{Agrobacterium transformation and analysis.} The \textit{udiA} gene encoding the GUS gene product and NPT II gene encoding kanamycin resistance is contained within the T-DNA left and right borders on the binary plasmid pBI121 (Jefferson et al., 1986). The binary plasmid pBI121 was introduced into the tumorigenic \textit{A. tumefaciens} strain A6 by triparental mating using procedures described by Ditta et al. (1980). The recipient A6, donor HB101(pBI121), and helper HB101(pRK2013) were cultured overnight in liquid 523 medium (Kado and Heskett, 1970). The cells were centrifuged and resuspended in minimal media broth (Miller, 1972), and aliquots of each were mixed on standard 523 plates and incubated for 4 hr at 37°C. The mixture was washed with 1 ml of minimal medium and spread on minimal medium plates containing 50 µg kanamycin/ml for incubation at 28°C until colonies appeared (usually 2 days). The transconjugants were maintained on type 523 plates containing 50 µg kanamycin/ml.

The remaining strains were transformed by the direct method of Ebert et al. (1987). pBI121 DNA was prepared as described by Birnboim and Doly (1979), mixed directly with the recipient strains, and cold- and heat-shocked. Transformants were plated on 523 medium containing 50 µg kanamycin/ml and incubated at 28°C until colonies appeared.

Hereafter, the strains containing pBI121 will be referred to as A6(pBI121), A281(pBI121), etc. Strains of the tumorigenic bacteria carrying the binary plasmid pBI121 should be capable of 1) forming tumors by transfer of their endogenous T-DNA or 2) transfer of the gene encoding GUS by transfer of T-DNA contained on pBI121. Transformed bacterial strains were maintained on medium containing 50 µg kanamycin/ml. Only the \textit{Agrobacterium} strains with the pBI121 insert survived on kanamycin-containing plates. The 10 wild-type \textit{Agrobacterium} strains (without pBI121) did not survive on the kanamycin-containing medium.

All pBI121-containing \textit{Agrobacterium} strains were analyzed by the plasmid quick-screen method (Ebert et al., 1987). Aliquots of the plasmid DNA were restricted with HindIII and BglII for structural analysis and were compared to similar digests of purified pBI121 DNA. The strains were tested for GUS activity using the substrate X-gluc (Jefferson et al., 1986).

\textbf{Plant material.} Radish roots used in these studies were obtained from local commercial sources, surface-sterilized, and free-hand cross-sections (=2 mm thick) were made for treatment. Peach and apple plants were grown from seeds in the greenhouse. When =25 cm high, 4-mm-long stem segments were cut and surface sterilized for treatment. The specific manner of plant treatment is described where appropriate.

\textbf{Tissue inoculation.} Radish roots were submerged in 70% ethanol for 10 sec, 0.5% sodium hypochlorite solution (10% Clorox) for 5 min, washed with water, and then sectioned. Overnight cultures of \textit{Agrobacterium} strains were prepared in 1 ml of liquid 523 medium; of which 50 µl was applied to the radish root sections and incubated at 29°C on standard type 523 agar plates for 2 to 3 days. Incubated sections were washed with H2O and sodium phosphate buffer before submersion in X-gluc solution.

Seedling apple and peach stems were surface sterilized as

\begin{table}[h]
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\begin{tabular}{|l|l|l|l|l|}
\hline
\textbf{Bacteria} & \textbf{Strain} & \textbf{Plasmid} & \textbf{Opine} & \textbf{Reference} \\
\hline
\textit{Agrobacterium rhizogenes} & A4 & pRIa4 & Agropine & Moore et al., 1979 \\
 & K47 & pRI-K47 & Agropine & L. Moore, personal communication \\
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\textit{Agrobacterium tumefaciens} & A6 & pTiA6 & Octopine & Schiaky et al., 1978 \\
 & 3667 & pTi3667 & Nopaline & Watson, 1981 \\
 & C2/74 & pTiC2/74 & Nopaline & Anderson, 1978 \\
 & C58 & pTiC58 & Nopaline & Schiaky et al., 1978 \\
 & ACH5 & pTiAch5 & Octopine & Petit et al., 1983 \\
 & NT1 & None & None & Watson et al., 1975 \\
 & LBA 4404 & \textit{disarmed} & ACH5 & None & Hockema et al., 1983 \\
 & A281 & pTiBo542 & Agropine & Petit et al., 1983 \\
\hline
\end{tabular}
\caption{Strains of \textit{Agrobacterium} tested as vectors.}
\end{table}
described for radish, cut into 4-mm-long stem segments, and cocultivated for 2 to 3 days at 25°C with an aliquot of bacterial culture in Murashige and Skoog (1962) (MS) standard medium containing 3% sucrose. Stem segments were analyzed for GUS presence using X-gluc as described previously.

**Tumor formation on apple and peach.** The stem segments were inoculated, as described above, with wild-type and pBI121-containing Agrobacterium strains. Stem segments were washed with water, treated with carbenicillin and cefotaxime at 250 µg·ml⁻¹ to eliminate the Agrobacterium cells, and selected against 50 µg kanamycin/ml.

**Tumor formation in greenhouse seedlings of apple and peach.** Separate populations of apple and peach seedlings were grown in the greenhouse and treated there with the 10 pBI121-containing strains and 10 wild-type strains of Agrobacterium. A section of stem bark 15 mm long and 4 mm wide was removed from each seedling with a razor cut. Bacteria were removed from the culture plates and smeared onto the cut stem surface. Seedlings were left in the greenhouse for observation at standard conditions (24-26°C; 16 hr supplemental daylight).

**Transformation of apple callus from xylem parenchyma.** Apple (Malus pumila cv. Gala), xylem parenchyma from the third internode from the apex was cultured in MS standard medium (plus BA and 2,4-D) for a continuous callus source (Cresso D.O. Ketchie, Tree Fruit Research Center, Washington State Univ., Wenatchee). Segments of this callus were inoculated for 48 hr at 25°C in MS medium with the Agrobacterium strains containing pBI121. Segments were transferred to MS plates containing 50 µg kanamycin/ml and cefotaxime and carbenicillin each at 250 µg·ml⁻¹. As callus enlarged, transfers were made to similar MS plates containing the same antibiotics.

Apple callus inoculated with A281(pBI121) was examined for resistance to kanamycin. A small amount of callus was placed on duplicate plates, each filled with 25 ml MS medium containing kanamycin at 0, 50, 100, 200, or 400 µg·ml⁻¹. Two controls were also included: nontransformed callus and callus inoculated with NT1(pBI121).

**Histochemical localization of the GUS gene product.** Plant parts, after cocultivation with pBI121-containing Agrobacterium strains, were fixed for 45 min with a solution of 0.3% formaldehyde in 0.3 m mannitol, 10 m Mes at pH 5.6. Plant parts were washed in a 0.2 m sodium phosphate buffer, covered with X-gluc solution, and incubated as described above until the solution turned blue. The plant tissue segment was either dehydrated and embedded in paraffin for photomicrography or photographed immediately.

**Opine analysis.** The method of Petit et al. (1984) was used with some modification. Hot water extracts of apple xylem parenchyma callus containing A281(pBI121) were spotted on Whatman 3MM paper with the opine standards mannopine, mannoopinic acid, agropine, and agropinic acid that were provided by J. Tempé (Institut de France Vegetale, Gif-Sur, Yvette, France). Electrophoretograms were run in formic acid, glacial acetic acid, and water (30, 60, and 910 ml, respectively) in a horizontal electrophoresis unit at 500 V. Dried electrophoretograms were stained with AgNO₃, allowed to air dry, developed with NaOH, and fixed with Kodak X-ray fixer. After electrophoretograms were cleared with Kodak hype, washed in water, and dried, opines were seen as brown spots on a light background.

**Kanamycin phosphotransferase [APH(3’)] assay.** Apple xylem parenchyma callus (100 mg) was extracted in Tris buffer as described by Schreier et al. (1985). The APH(3’)II gene product was separated by electrophoresis and was assayed directly in the gel by layering with a secondary agarose gel containing the substrate kanamycin and $\frac{1}{3}$P]ATP (Reiss et al., 1984). The phosphorylated kanamycin was then blotted onto phosphocellulose and developed with X-ray film.

**Results and Discussion**

**Bacteria transformation.** All of the pBI121-containing bacterial strains grew on kanamycin-containing type 523 plates, while nontransformed, wild-type strains failed to grow. The pBI121-containing strains of bacteria, except A4(pBI121), hydrolyzed X-gluc to form a blue pigment, whereas the wild-type strains did not hydrolyze X-gluc (Table 2). The time for color formation varied from ≈2 to 20 hr. Expression of the GUS gene product by the bacteria led to problems that are discussed below.

The presence of pBI121 in the bacteria was confirmed by re-isolating the pBI121 plasmid DNA from each strain and digesting it with restriction enzymes for comparison of restriction fragments from purified pBI121 (data not shown). These positive results showed that the tumorigenic wild-type strains of bacteria were transformed with pBI121, as determined by resistance to kanamycin, plasmid re-isolation, and formation of the positive blue pigment when incubated with X-gluc.

The strong bacterial expression of GUS presented a problem for identifying tissue transformation (Table 2). Unless bacteria are removed from plant samples after inoculation, one cannot be sure that the blue pigment formed with X-gluc is from the bacteria or the transformed tissue. Agrobacterium characteristically adhere to various surfaces; this is an important feature of the infection process (Matthysse et al., 1981). Thus, multiple water washes and soaking treatments with carbencillin and cefotaxime will not completely rid plant sections of bacteria.

**Transformation of radish, peach, and apple.** Nine of 10 radish slices or peach stem segments inoculated with pBI121-containing strains gave positive blue pigment when incubated with X-gluc (Table 2). Controls not treated with pBI121 strains failed to become blue. Photomicrographs of radish sections showed blue pigment presence for NT1(pBI121). Since the NT1(pBI121) strain was cured of Ti plasmid and thus was incapable of mediating transformation of plant cells, the positive X-gluc reaction could only be due to the presence of viable bacteria in the

| Strain | Radish | Positive X-gluc reaction* | Peach | Apple* | Bacteria* |
|--------|--------|---------------------------|-------|--------|----------|
| A4(pBI121) | +       | –                         | + (slow) | + (slow) | –        |
| A6(pBI121) | +       | + (slow)                  | + (slow) | + (slow) | + (slow) |
| CS8(pBI121) | +       | +                         | +      | +      | +        |
| K47(pBI121) | +       | +                         | +      | +      | +        |
| ACH5(pBI121) | +       | + (slow)                  | +      | +      | + (slow) |
| NT1(pBI121) | +       | +                         | +      | +      | + (slow) |
| A281(pBI121) | +       | + (slow)                  | +      | +      | + (slow) |
| LBA4404(pBI121) | + | +                         | +      | +      | + (slow) |
| 3667(pBI121) | +       | +                         | +      | +      | + (slow) |
| C2/74(pBI121) | –       | + (slow)                  | +      | +      | + (slow) |

* * Indicates a positive blue pigment; – indicates no color formation; slow = color formed after 20 hr of incubation.
*Seven of 10 apple stems were infected with fungus and no X-gluc reactions were conducted.
No blue pigment formed in bacterial strains unless transformed with pBI121.
Table 3. Presence of GUS activity in apple xylem parenchyma callus following two transfers from original inoculation with A. tumefaciens strain.

| Strain            | Color | Growth | Positive X-gluc reaction* after time indicated (hr) |
|-------------------|-------|--------|---------------------------------------------------|
|                   |       |        | 2       | 6   | 24  |
| ACH5(pBI121)      | tan   | +      | ++      | +   | +   |
| LBA4404(pBI121)   | tan   | +      | ++      | +   | +   |
| A281(pBI121)      | white | +++    | ++++    | +++ | +++ |
| C58(pBI121)       | white | +      | +       | +   | +   |
| NT1(pBI121)       | brown | -      | -       | -   | -   |
| K47(pBI121)       | tan   | -      | -       | +   | +   |
| Parenchyma callus | (untransformed) | tan | +      | -   | -   |

*Growth of callus after 3 weeks of transfer on MS plates with kanamycin at 50 µg·ml⁻¹: – is no growth, + is poor growth, ++ is fair growth, +++ is good growth.

A281(pBI121), NT1(pBI121), or no inoculation after 7 weeks of exposure to kanamycin.

Table 4. Growth of apple xylem parenchyma callus inoculated with A281(pBI121), NT1(pBI121), or no inoculation after 7 weeks of exposure to kanamycin.

| Treatment          | Kanamycin conc (µg·ml⁻¹) |
|--------------------|-------------------------|
|                    | 0           | 50          | 100         | 200         | 400         |
| A281(pBI121)       | ++++++      | ++++        | ++++        | ++++        | ++++        |
| No Agrobacterium   | ++++++      | ++++        | +           | 0           | 0           |
| NT1(pBI121)        | ++++++      | +           | 0           | 0           | 0           |

None of the 10 pBI121 or wild-type strains tested in the greenhouse produced tumors on treated peach or apple seedlings. Anderson and Moore (1979) found apple and peach to be resistant to tumor formation following Agrobacterium treatment in the greenhouse. In one of their tests, apple showed pathogenic reaction to 22 of the 89 strains tested. These results led to our search for tissue of similar type that could be manipulated following inoculation to avoid bacterial presence. We tested apple callus maintained in culture and amenable to transfer.

Apple xylem parenchyma callus. Apple callus first initiated from xylem parenchyma was inoculated with A281(pBI121). The treated callus survived on kanamycin plates and gave the positive X-gluc reaction (Table 3). Neither NT1(pBI121)-treated callus nor the nonbacterial-treated callus showed the positive X-gluc reaction. This experiment indicated putative transformation of the A281(pBI121)-inoculated apple callus, as the subculture technique should have been sufficient to escape continued bacterial presence from the original bacterial treatment. Further, A281(pBI121)-inoculated callus survived on media containing concentrations of kanamycin ranging from 50 to 400 µg·ml⁻¹ for 7 weeks, whereas NT1(pBI121) and the nontransformed callus were killed with as low as 50 µg kanamycin/ml (Table 4). As expected, both the nontransformed callus and NT1(pBI121)-treated callus did not have resistance to kanamycin. Subsequently, samples of A281(pBI121)-inoculated apple callus were placed on agar medium devoid of antibiotics to determine if viable bacteria were still present in the tissue. There were no visible signs of bacterial growth on the media after 2 weeks.

If transformed, the apple xylem parenchyma callus should synthesize the opines mannopine, mannopinic acid, agropine, and agropinic acid (Petit et al., 1984). These opines tested positive in A281(pBI121)-treated apple callus, but not in the nontransformed callus (Fig. 1).

The last evidence sought for putative transformation of the apple xylem parenchyma callus was by analysis for the enzyme APH(3')I, providing resistance to kanamycin. The enzyme was present in pBI121-treated apple callus, but was absent from nontransformed callus (Fig. 2).

R.A. Jefferson (personal communication) recognized that Agrobacterium containing pBI121 would result in high background activity for the X-gluc reaction. He attributed this activity to the strong promoter CaMV35S and read-through transcription from the lacZ transcript of pBIN19 into the GUS coding region and subsequent translation. At least three solu-
tions to this problem exist. R.A. Jefferson (personal communication) has found the background decreases some 20 times when the GUS cassette is inverted with respect to the lac promoter. A second solution to this problem will be through the introduction of an intron into the coding region of GUS. Recently, a third alternative has been explored, and GUS recombinants displaying diminished expression in bacteria have been constructed by Janssen and Gardner (1989). These chimeric constructs contain either modifications in the promoter sequence (Janssen and Gardner, 1989). These chimeric constructs contain either modifications in the promoter sequence or deletion in the bacterial ribosome-binding site (Janssen and Gardner, 1989).

Our results for putative transformation show that apple xylem parenchyma callus inoculated with Agrobacterium strains carrying pBI121 were kanamycin resistant, synthesized opines, and expressed GUS and APH(3')II enzymatic activity. Taken together, these data strongly argue for plant transformation. Southern analysis remains a future goal to conclusively prove genetic transformation. The goals of this investigation were met. Ten strains of Agrobacterium containing the GUS markers were constructed. A. tumefaciens, strain A281, was identified as the best vector of those tested. The GUS marker system is useful for early selection but cannot serve as a sole evaluation criterion, as bacterial expression of the marker severely compromises its effectiveness. Callus tissue, such as the xylem parenchyma used in this investigation, has a special value. Because of the ease in handling callus, its routine use for initial screening of bacterial strains for transformation is encouraged. The best candidate strains can be subsequently tested on leaves, stems, or other appropriate organs from the genus of interest.

Fig. 1. Analysis for presence of opines in nontransformed and transformed apple xylem parenchyma callus. For callus and callus + GUS lanes: contain 1, 2, 3, or 4 mg fresh weight of callus.

Fig. 2. Analysis for presence of kanamycin phosphotransferase, providing kanamycin resistance in transformed callus. Longer exposure was necessary for the callus and callus + GUS lanes.

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