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Biolistic Transformation of Rose (*Rosa hybrida* L.)

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A reproducible method has been developed for the Biolistic transformation and regeneration of transgenic plants from embryogenic callus of rose (*Rosa hybrida* L.) cv. Glad Tidings. DNA delivery was optimized using the β-glucuronidase (*gus*) gene. The distance between the stopping screen and target explants and supplementation of pre- and post-bombardment culture media with 0·25 M myo-inositol influenced the transformation efficiency. Prior to culture on selection medium containing 250 mg l^{-1} kanamycin sulphate, embryogenic calli were bombarded, using optimized gene delivery parameters, with a plasmid carrying the neomycin phosphotransferase (*npt* II) gene. Somatic embryo-derived kanamycin-resistant plants were regenerated and subsequently transferred to glasshouse conditions. Transformation was confirmed by kanamycin resistance of calli and plants, NPT II ELISA assay and Southern analysis. All transgenic plants were morphologically normal (true-to-type).

Key words: Biolistic, genetic engineering, rose, *Rosa hybrida* L., transformation.

INTRODUCTION

The high commercial value and widespread cultivation of rose makes it the most important of the ornamental crops. Traditionally, rose improvement has depended on sexual crossing and selection, together with the identification of natural mutations (sports). While such techniques will continue to be exploited, they are ultimately restricted by the heterozygosity of the crop and by a limited gene pool to which only a few species have contributed. Genetic engineering strategies are highly desirable for rose as they facilitate the introduction (or modification) of single gene traits without disruption of the pre-existing, commercially valuable phenotypic characteristics of the target variety. A range of transgenes are of potential use in rose, including those for pest and disease resistance, flower colour, morphology and vase life, together with plant architecture.

In 1994, Firoozabady *et al.* reported the successful transformation of *Rosa hybrida* cv. Royalty following co-cultivation of friable embryogenic callus with *Agrobacterium tumefaciens* strain LBA4404. However, this transformation procedure is strongly cultivar dependent (Burchi *et al.*, 1996) and employs an extended callus phase, increasing the risk of somaclonal variation in the regenerated plants. More recently, van der Salm *et al.* (1997) regenerated transgenic plants from roots derived from stem slices of rootstock *R. hybrida* cv. Moneyway following co-cultivation with *A. tumefaciens* strain GV3101 containing the *npt* II gene and individual *rol* genes from *A. rhizogenes*. For molecular breeding to be widely adopted as a method of rose improvement, there is a requirement to develop and to optimize species/cultivar independent methods of transformation.

Microprojectile-mediated gene delivery, which lacks species, cultivar or tissue-type specificity, was first conceived by Sanford *et al.* (1987) and has been successfully employed with a range of target tissues and species, including woody plants such as poplar (McCown *et al.* 1991), spruce (Robertson *et al.*, 1992; Ellis *et al.*, 1993) and ornamentals, including chrysanthemum (Yepes *et al.*, 1995) and carnation (Zuker *et al.*, 1996). Several factors have been shown to influence the genetic engineering of plants by the Biolistic technique (Southgate *et al.*, 1995). This paper reports methods to optimize Biolistic gene delivery in rose and the first successful recovery of transgenic rose plants using this technique.

MATERIALS AND METHODS

Plant material and tissue culture

Somatic embryogenic callus of *Rosa hybrida* L. cv. Glad Tidings (Tantide, Tantau, Germany) was initiated following an established procedure (Marchant *et al.*, 1996). Briefly, petioles from *in vitro*-grown shoots were maintained in the dark at 28 ± 2 °C on preculture (P) medium consisting of Schenk and Hildebrandt's (1972) basal salts and vitamins with 5·0 mg l^{-1} 2,4-D(2,4-dichlorophenoxyacetic acid), 30·0 g l^{-1} sucrose, 300·0 mg l^{-1} L-proline and 4·0 g l^{-1} agarose (pH 5·8). After 14 d culture, explants were transferred to embryo proliferation (EP) medium, of the same composition as P medium, but containing 3·0 mg l^{-1} 2,4-D, and were maintained under identical conditions. Proliferating calli were subcultured by division every 28 d onto EP medium. Somatic embryogenesis occurred after 42–56 d repeated subculture.

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Preparation of tissues for Biolistic gene delivery

Embryogenic calli with globular, white pre-embryos, were subjected to Biolistic gene delivery 14 d after subculture. Pieces of embryogenic callus (approx. 5 mm diameter) were placed in the central 3 cm radius of a 9 cm Petri dish (30 explants per dish), the latter containing 20 ml of EP medium (Marchant *et al*., 1996). In order to determine the effect of osmotica on the efficiency of gene transfer, the medium was also supplemented, where appropriate, with 0·25 M myo-inositol, 0·125 M sorbitol and 0·125 M mannitol, or 0·25 M sucrose.

Plasmid DNA

Plasmid pMJD67 was used to optimize the bombardment conditions. This plasmid, described by Rech, Vainstein and Davey (1991), is based on the vector pTZ18 with the CaMV35S promoter upstream of the *gus* (*uid* A) coding region. The DNA used for stable transformation was pBI101 (Jefferson, Kavanagh and Bevan, 1987; Clonetech Laboratories Inc., Palo Alto, USA) from which the *gus* gene and its associated terminator sequence had been deleted, leaving the plasmid containing only the *nos* promoter upstream of the *npt* II (neomycin phosphotransferase II) gene and *nos* terminator. Both plasmids were maintained and amplified in *Escherichia coli* strain HB101 and isolated and purified using the Wizard Megaprep Plasmid Isolation Kit (Promega, Southampton, UK). Purified plasmid was resuspended in TE buffer [1 mM Tris HCl (pH 7·8), 0·1 mM Na_2EDTA] and was stored at −20 °C until required.

Coating of microprojectiles

Microprojectiles were coated with DNA using a $CaCl_2$/spermidine precipitation technique. Under continuous vortexing, 5 μl of plasmid DNA (1 $\mu g\ \mu l^{-1}$ in TE buffer), 50 μl of $CaCl_2$ (2·5 M) and 20 μl of spermidine (0·1 M, free base) was added to a 50 μl sterile preparation of gold particles (60 mg ml^{-1}; 0·4–1·2 μm diameter; Heraeus GmbH, Karlsruhe, Germany) in a 1·5 ml Treff Eppendorf Tube. Vortexing of this mixture was continued for 3 min, followed by centrifugation (10 s pulse). The supernatant was removed and the pellet resuspended in 250 μl of absolute ethanol. Centrifugation was repeated and the supernatant removed. Coated particles were resuspended in 75 μl of absolute ethanol. Following vortexing, 10 μl of the mixture was pipetted immediately into the centre of each macroprojectile (which was retained within the steel macrocarrier holder), and was left at room temperature for 10–20 min to dry.

Bombardment conditions

The helium driven Biolistics PDS-1000/He device (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) was used for all bombardments. Parameters which were maintained constant throughout the experiments were a 30 mm distance between rupture disc and macrocarrier, 10 mm between macrocarrier and stopping screen and a vacuum of 73·6 cm Hg. Target tissue was placed 70, 100 or 130 mm from the stopping screen; rupture disc pressures of 7500, 9000 or 10500 kPa were employed. Target tissues were subjected to a single bombardment.

Post-bombardment handling of tissues

One h post-bombardment, target tissues were returned to the incubation conditions described previously for embryo proliferation. After 24 h incubation, tissues that had been maintained on EP medium containing additional osmotica were subcultured onto medium of the same composition, but lacking these supplements. After a further 24 h, those tissues used for stable transformation were subcultured onto EP medium supplemented with Km (kanamycin sulphate) at 250 mg l^{-1}. Embryogenic callus was maintained on Km-supplemented EP medium with subculture every 14 d. In order to recover transformed plants, calli surviving four cycles of subculture on EP medium containing the selection agent were subjected to the embryo maturation, germination and rooting procedures described previously (Marchant *et al*., 1996). During these stages, all media used were supplemented with Km (250 mg l^{-1}). Following embryo germination and rooting, regenerated plants were transferred to glasshouse conditions (Marchant *et al*., 1996). Each Km-resistant plant arose from an individual and independent bombardment event.

GUS (β-*glucuronidase*) *histochemical analysis*

In order to assay for transient *gus* gene expression when optimizing the bombardment parameters, calli were removed from the culture medium 48 h post-bombardment and placed into the wells of a 5 × 5 (25) well dish (Bibby-Sterilin Ltd, Stone, UK). Approximately 200 μl of GUS reaction buffer, modified from Jefferson *et al*. (1987) and containing 1·5 mM 5-bromo-3-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Gold BioTechnology, St. Louis, USA), 20 mM $Na_2HPO_4 + NaH_2PO_4$(pH 7·0), 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6 . 3H_2O$, 10 mM Na_2EDTA and 0·1% (v/v) Triton X-100 (Sigma), was added to each well. Tissues were incubated in the dark at 37 °C for 16 h. The number of indigo blue GUS expression units per callus was counted using a stereo microscope. Each blue spot observed was scored as a single GUS expression unit, regardless of its size or intensity.

Analysis of kanamycin-resistant plants

NPT II ELISA assay. NPT II protein was detected using a commercial enzyme-linked immunosorbent assay (ELISA) kit (5 prime-3 prime, Boulder, USA). Soluble proteins were extracted by grinding leaf discs (3 × 0·01 g f. wt from the penultimate, fully expanded leaves of each plant) in 0·1 M Tris HCl (pH 8·0) buffer containing 1·0 mM PMSF (phenylmethylsulfonyl fluoride) and 1·0% (w/v) PVP-10 (polyvinyl pyrrolidone), in a 1·5 ml Eppendorf tube using a Kontes pestle (Scotlab, Coatbridge, UK). Protein content was determined following the method of Bradford (1976), and 10–200 mg of protein was applied to each ELISA sample well. NPT II protein content was determined according to

the manufacturer's instructions. All assays were standardized against authentic NPT II enzyme. NPT II analysis was undertaken on all putatively transformed plants and on non-transformed control plants.

Southern analysis. Genomic DNA was extracted, using the Nucleon Phytopure Plant DNA extraction kit (Scotlab, Coatbridge, UK), from 1 g f.wt of leaf tissue from the penultimate fully expanded leaves on young shoots of each of the putatively transformed and non-transformed (control, somatic embryo-derived) plants all maintained under glasshouse conditions. The procedure used for Southern blot analysis was essentially as described by McCabe *et al*. (1997). In order to estimate transgene copy number, isolated genomic DNA (10 μg) was digested with 40 units of *Bgl* I restriction endonuclease (Boehringer-Mannheim UK Ltd., Lewes, UK), which does not cleave with the plasmid used for transformation, for 16 h at 37 °C and subsequently electrophoresed on 0·8% (w/v) agarose gel (molecular biology grade agarose; NBS Biologicals, Huntingdon, UK) at 25 V for 16 h. DNA was immobilized onto a positively charged nylon membrane (Hybond N+; Amersham International plc, Amersham, UK) by alkaline transfer. Following transfer, the membrane was probed with a polymerase chain reaction-digoxigenin (DIG)-labelled fragment of the *npt* II gene (labelled according to Lion and Haas, 1990). Hybridization (37 °C for 16 h) and washing were according to standard techniques (McCabe *et al*., 1997). Chemiluminescent detection was undertaken using CDP-Star substrate (Tropix, Bedford, USA). The membrane was subsequently exposed to Hyperfilm MP X-ray film (Amersham International plc) for 1–4 h at 22 °C.

RESULTS AND DISCUSSION

Optimization of bombardment parameters

Transient *gus* gene expression 48 h after bombardment was used as an initial indicator of the efficiency of gene transfer (Fig. 1A). These data were exploited to optimize the parameters for gene delivery which were subsequently employed to generate stably transformed rose plants.

The effect of firing distance (distance between the stopping screen and explant) and rupture disc pressure are shown in Fig. 2. Irrespective of the rupture disc pressure employed, the level of GUS expression was consistently greater when a firing distance of 70 mm was used compared to a distance of 100 mm. GUS expression was not detected when target material was bombarded at a distance greater than 100 mm. Maximal GUS expression was observed with a rupture disc pressure of 9000 kPa. Effective penetration of the target plant tissue by the microprojectiles carrying the DNA is essential for successful gene delivery (Southgate *et al*., 1995) and the effects observed in relation to firing distance and rupture disc pressure are likely to reflect the relative velocity of the microprojectiles on reaching the surface of the explants.

In some plant transformation systems, successful microprojectile-mediated gene delivery has also been attributed to the osmoticum of the culture medium on which the explants are bombarded or maintained post-bombardment (Vain, McMullen and Finer, 1993; Ye *et al*., 1994; Southgate *et al*., 1995). It has been suggested that the osmoticum induces cell plasmolysis and that the reduced turgor of plasmolysed cells prevents leakage of their protoplasm when cell walls are perforated by the microprojectiles. A comparison was made of the effect of the three osmotica, myo-inositol, sorbitol with mannitol, and sucrose, in the EP medium on which explants were maintained for either 24 h post-bombardment, or for 4 h prior to bombardment and for 24 h post-bombardment. Supplementing EP medium by the addition of 0·25 M myo-inositol more than doubled GUS expression 48 h post-bombardment (Fig. 3). Preconditioning target explants on medium containing myo-inositol for 4 h before bombardment and for 24 h post-bombardment produced a greater increase in gene expression than when the osmoticum was applied for only 24 h post-bombardment (Fig. 3).

In order to maximize transformation, all subsequent bombardments were undertaken using 9000 kPa rupture discs with a firing distance of 70 mm; explants were maintained on EP medium supplemented with 0·25 M myo-inositol for 4 h prior to bombardment and for 24 h post-bombardment.

Selection of kanamycin-resistant tissues and regeneration of transgenic plants

Calli bombarded using the optimized parameters became dark brown/black within 3 d of transfer onto EP medium containing 250 mg l^{-1} Km. However, by the time of their first subculture (14 d post-bombardment), small areas of white/cream proliferating callus/embryos were observed (Fig. 1B). On subculture, such white/cream sectors of tissue were excised from the brown/black callus mass and were placed onto fresh EP medium containing Km. Some further tissue necrosis was observed, but this was again accompanied by the development of healthy proliferating tissue sectors which were subcultured to fresh Km-supplemented EP medium. This process was repeated every 14 d over a 42 d period until only white/cream embryogenic calli, which proliferated on Km-supplemented medium, were retained. Embryos enlarged and proliferated from the surface of the calli when placed on embryo maturation medium containing Km (Fig. 1C). Fifty to 70% of the rose somatic embryos developed normally. The remainder either lacked distinct cotyledons or possessed fused cotyledons; such embryos failed to germinate when transferred to embryo germination medium. Somatic embryos that germinated developed normally, producing elongated shoots (6–12 mm stem) with three to six leaves. Twenty putatively transformed rose plants were recovered from a total of 90 bombardments. All of the recovered plants rooted when transferred to the appropriate medium (Marchant *et al*., 1996) containing 250 mg l^{-1} Km (Fig. 1D). Subsequently, these regenerated plants commenced flowering within 12 weeks of transfer to glasshouse conditions and appeared morphologically true-to-type (Fig. 1E).

Biochemical and molecular analysis of kanamycin-resistant plants

NPT II ELISA assays revealed that all putatively transgenic plants contained 2–9 times more NPT II protein

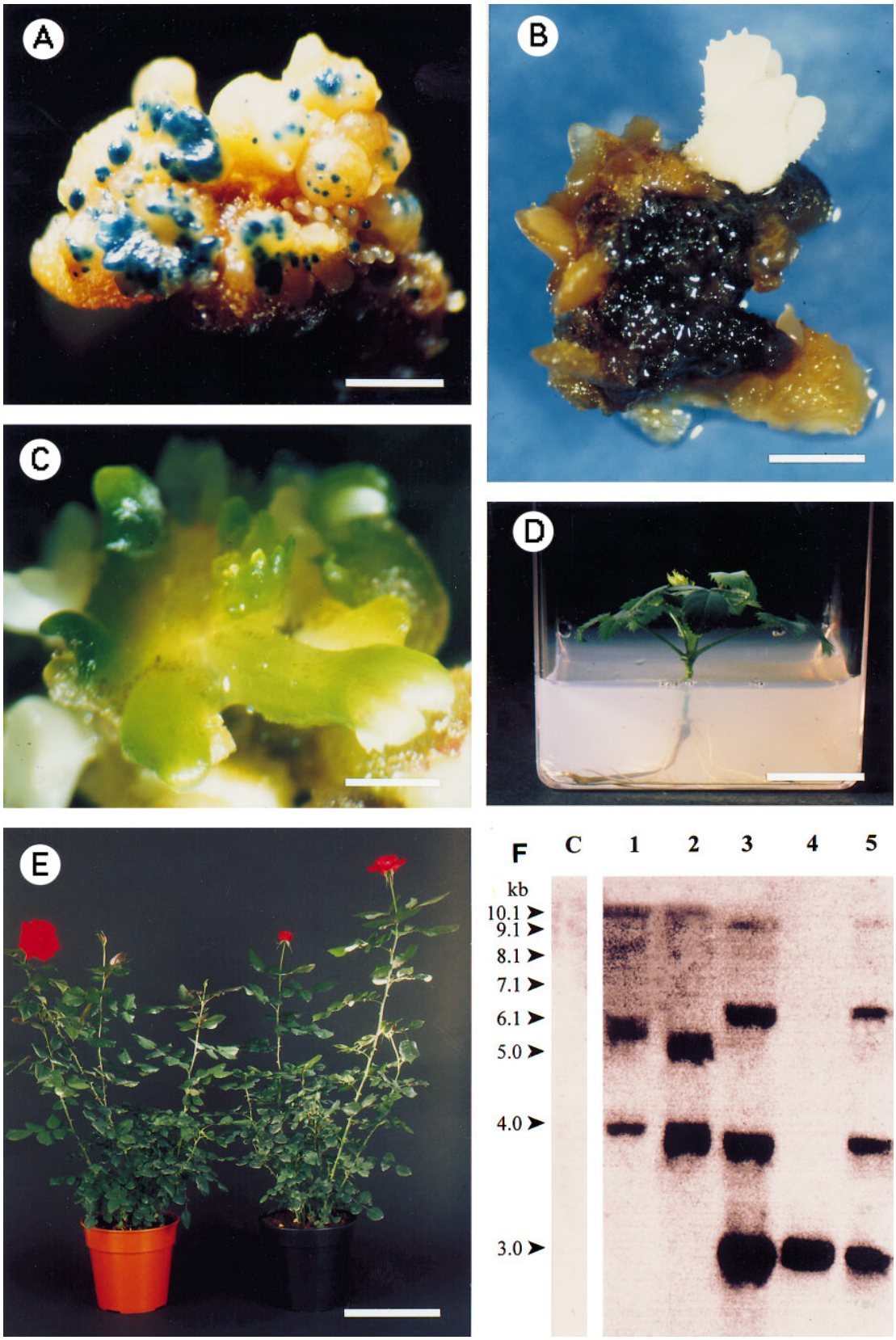

FIG. 1. For legend see facing page.

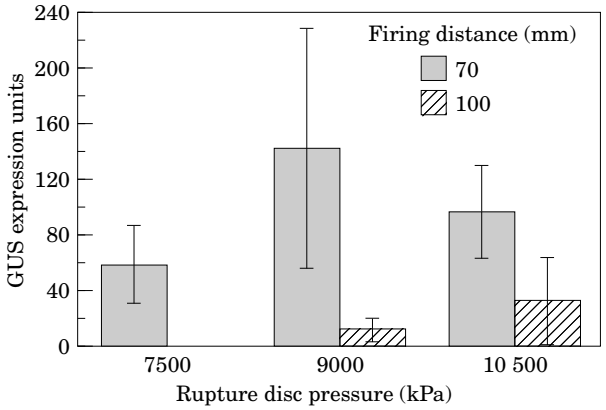

FIG. 2. Effect of rupture disc pressure and firing distance on GUS expression 48 h postbombardment. Data are mean number of GUS expression units per callus ± s.e. from five bombardments (30 explants per bombardment). All bombardments used a distance of 30 mm between rupture disc and macrocarrier and 10 mm between macrocarrier and stopping screen; vacuum was 73·6 cm Hg.

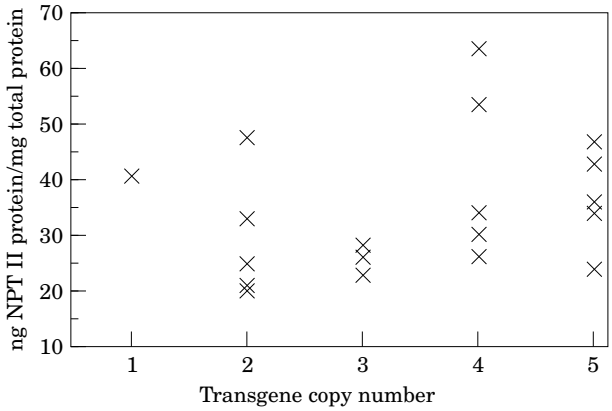

FIG. 4. Relationship between the level of NPT II protein and transgene copy number in transformed rose plants. Each data point represents an individual transformed plant. Transgene copy number was estimated following Southern analysis and NPT II protein levels were determined by ELISA assay; see Materials and Methods for full details.

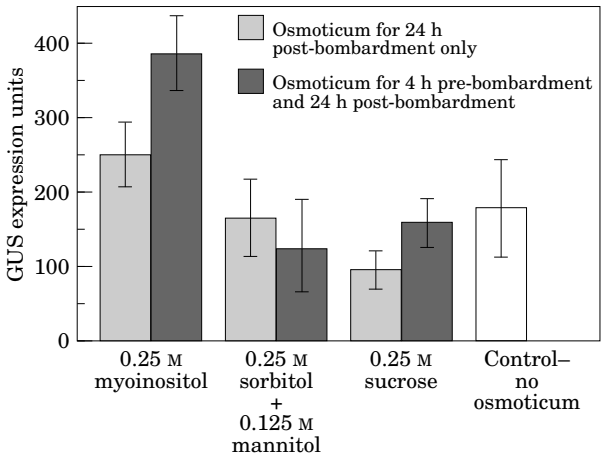

FIG. 3. Effect of osmotica and timing of application on GUS expression 48 h post-bombardment. Data are mean number of GUS expression units per callus ± s.e. from five bombardments (30 explants per bombardment). All bombardments used 9000 kPa rupture discs, a distance of 30 mm between rupture disc and macrocarrier, 10 mm between macrocarrier and stopping screen and 70 mm between stopping screen and explant; vacuum was 73·6 cm Hg.

(20–64 ng NPT II protein mg^{-1} total protein; mean 35·6 ng NPT II protein mg^{-1} total protein) than the non-transformed control (7 ng NPT II protein mg^{-1} total protein).

Putatively transformed plants and non-transformed control plants were subjected to Southern analysis. Genomic DNA was digested with *Bgl* I restriction enzyme which does not cleave with the plasmid used for transformation. However, the restriction enzyme will cleave genomic DNA at naturally occurring restriction sites throughout the genome, releasing random sized DNA fragments some of which will contain the transgene of interest. Fragments of genomic DNA are resolved following gel electrophoresis and, after Southern blotting and probing of the membrane, any fragments containing the gene of interest will result in a positive signal being obtained. The number of such positive signals provides an indication of the quantity of transgenes incorporated within the genome. All of the samples from the Km-resistant plants showed strong positive signals of high molecular weight, confirming incorporation of one to five copies of the *npt* II gene into the rose genome (Fig. 1F). The probe failed to hybridize to DNA from the non-transgenic control plants (Fig. 1F). Southern analysis of two plants produced similar integration patterns (Fig. 1F, lanes 3 and 5) even though these plants arose from individual bombardments of separate masses of callus.

The inactivation of recombinant genes that have been introduced into transgenic plants has become a widely recognized phenomenon in recent years (Meyer, 1996). After microprojectile-mediated gene delivery, the number of detected transgene copies varies widely. Commonly reported numbers range from one to more than 20 copies (Pawlowski and Somers, 1966) accompanied by a wide spectrum of gene expression. Variation in transgene expression levels and transgene silencing can result from several factors. Expression levels can be influenced by the number of copies of the integrated transgene (co-suppression) or by the position of the transgene(s) within the recipient genome (positional effects). Figure 4 shows the relationship between the level of

FIG. 1. Stages in the production of transgenic plants of *Rosa hybrida* cv. Glad Tidings. A, GUS expression in rose embryogenic callus 48 post-bombardment and after 16 h incubation in X-Gluc solution. Bar = 1·5 mm. B, Embryogenic callus proliferating from non-transformed callus mass 14 d after transfer to embryo proliferation medium containing 250 mg l^{-1} Km. Bar = 1·0 mm. C, Enlarged transformed embryos proliferating on the surface of the callus after 14 d maintenance on embryo maturation medium containing 250 mg l^{-1} Km. Bar = 2 mm. D, Rooting of a transformed plant on medium containing Km. Bar = 20 mm. E, Transformed plant (right) and somatic embryogenesis-derived, non-transformed plant (left) flowering after transfer to glasshouse conditions. Bar = 125 mm. F, Representative Southern analysis of transformed rose plants. *Bgl* I-digested rose genomic DNA (10 μg) from a non-transformed plant (Lane C) and five Km-resistant plants (Lanes 1–5), hybridized with a DIG-labelled fragment of the *npt* II gene. The position and lengths, in kilobase pairs, of the molecular size makers are indicated.

NPT II protein and *npt* II transgene copy number estimated by Southern analysis in the transformed rose plants in the current study. The level of NPT II protein did not positively correlate with transgene copy number. It is therefore concluded that, in the rose system, positional effects due to differences in the integration site of the introduced DNA were more critical than transgene copy number in terms of gene expression.

Biolistic gene delivery into embryogenic callus is clearly an effective method for the production of transformed rose plants of rose cv. Glad Tidings and this procedure should be applicable to other rose cultivars. In turn, this DNA delivery option will be evaluated for the introduction of genes relevant to rose improvement, ultimately leading to commercially improved cultivars.

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