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Genetic transformation of Cavendish banana (*Musa* spp. AAA group) cv 'Grand Nain' via microprojectile bombardment

Abstract An effective method has been developed for the stable transformation and regeneration of Cavendish banana (*Musa* spp. AAA group) cv 'Grand Nain' by microprojectile bombardment. Embryogenic cell suspensions were initiated using immature male flowers as the explant. Cells were co-bombarded with the neomycin phosphotransferase (*npt*II) selectable marker gene under the control of a banana bunchy top virus (BBTV) promoter or the CaMV 35S promoter, and either the β-glucuronidase (*uid*A) reporter gene or BBTV genes under the control of the maize polyubiquitin promoter. Plants were regenerated, under selection with kanamycin, that were co-transformed with *npt*II and either the *uid*A or BBTV genes. Molecular characterisation of transformants demonstrated that the transgenes had been stably integrated into the banana genome.

Key words *Musa* · Banana · Transformation · Microprojectile bombardment

Introduction

Bananas and plantains are among the most important crops in tropical and subtropical developing countries. World production is estimated at over 85 million metric tons annually (Anonymous 1997). Approximately 15% of this production is for export trade, and this is comprised largely of a single cultivar, 'Cavendish'. Diseases threaten the current level of banana production and, as well as potentially diminishing an important food source, could have a major impact on the balance of trade of exporting countries.

Genetic transformation, in conjunction with pathogen-derived resistance (Fitchen and Beachy 1993), is one potential strategy for developing virus resistance in bananas, which has proven to be difficult to obtain by conventional breeding (Vuylsteke and Swennen 1992). Further, no genes have been identified within the *Musa* gene pool that confer resistance to banana viruses such as banana bunchy top virus (BBTV), the causal agent of the devastating bunchy top disease.

There have been a number of reports on the genetic transformation of banana (Sagi et al. 1995; May et al. 1995). However, there is only one report on the production of transformed Cavendish plants. May et al. (1995) produced transgenic Cavendish cv 'Grand Nain' by co-cultivating wounded meristems with *Agrobacterium tumefaciens*. Although this technique is appealing because of its relatively short regeneration time, it may be of limited value because of the potential generation of chimeric plants (May et al. 1995). Transformation of totipotent single cells would overcome this limitation. Shii et al. (1992) reported the production of embryogenic cell suspensions of several Cavendish cultivars using immature male flowers as the explant. Escalant et al. (1994) and Cote et al. (1996) further refined this system and applied it to cv 'Grand Nain'. The refined system had a very high regeneration capacity, and it was considered that somatic embryos were most likely unicellular in origin. Such tissue is potentially an ideal target for genetic transformation. In this paper, we describe a system for the generation of transgenic 'Grand Nain' by microprojectile bombardment of embryogenic suspension cells derived from immature male flowers.

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Materials and methods

Plant material

Embryogenic cell suspensions of banana cultivars 'Grand Nain' and 'Williams' (*Musa* spp. AAA group, Cavendish subgroup) were initiated and maintained as described by Cote et al. (1996). Suspensions were initiated and maintained in M2 media (Cote et al. 1996) or suspension maintenance media (BL) (Dhed'a et al. 1991). Suspension cells were collected 4 days after subculturing and passed through a 450-μm mesh. The filtrate was then left to settle for 10 min. Sufficient supernatant was removed to leave a settled cell volume:liquid medium ratio of approximately 1:5. Cells were then resuspended and dispensed in 200-μl aliquots onto 70-mm-diameter Whatman filter paper discs in 90-mm Petri dishes containing BL medium solidified with 7 g/l Sigma washed agar. Cells were bombarded 4 days after plating.

Plasmids

Schematic representations of the transformation vectors used are shown in Fig. 1. The plasmids pDHkan and pUGR73 were gifts from Linda Tabe and David McElroy (Division of Plant Industry, CSIRO, Canberra).

The maize polyubiquitin-1 (*Ubi*-1) promoter and first exon and intron were excised from pUGR73 as a *Pst*I fragment and cloned into the *Pst*I site of pGEM3Zf$^+$ (Promega). This construct was designated pGEM-Ubi. The *nos* 3' untranslated region (3'UTR) from pBI121 (Clontech) was excised as a *Sac*I/*Eco*RI fragment and cloned into the *Sac*I/*Eco*RI site of pGEM-Ubi. This construct was designated pGEM-Ubi-nos.

The major open reading frame (ORF) of BBTV DNA-5 (Burns et al. 1995) and the internal ORF of BBTV DNA-1 (Beetham et al. 1997) were amplified by polymerase chain reaction, (PCR) using primers pairs BTutORF5-1 (5'-TGA GAG TTG GAA TCG TC-3') BTORF5-2 (5'-TTA GAG TTA TGT TAC ATC ATA GTC-3') and BTintORF1-1 (5'-GCG GAT CCA TGA TAA TTT ATT TGA TGT C -3'), BTintORF1-2 (5'-GCG AGC TCT CAT CTC TGC TTG TAC T -3'), respectively. A 50-μl PCR reaction mix contained the primers (0.4 μ*M* final concentration of each), 0.5 U DNA polymerase (Boehringer Mannheim Expand™), 200 μ*M* of each dNTP, 1×reaction buffer (Boehringer Mannheim buffer system 3) and 0.1 ng of BBTV

ssDNA extracted from purified virions. PCR conditions were 1 cycle of 94 °C/5 min followed by 30 cycles of 94 °C/30 s, 45 °C/30 s, 72 °C/1 min, with a 72°C/10-min final extension. The amplified 483-bp (BBTV DNA-5 ORF) and 126-bp (BBTV DNA-1 internal ORF) fragments were individually subcloned into the *Sma*I site of pGEM3Zf$^+$. The BBTV ORFs were subsequently excised from pGEM3Zf$^+$ as *Bam*HI/*Sac*I fragments and each inserted into the *Bam*HI/*Sac*I sites between the maize polyubiquitin promoter and the *nos* 3'UTR, in the plasmid pGEM-Ubi-nos. These constructs were designated pUbi-BTutORF5 and pUbi-BTintORF1, respectively (Fig. 1).
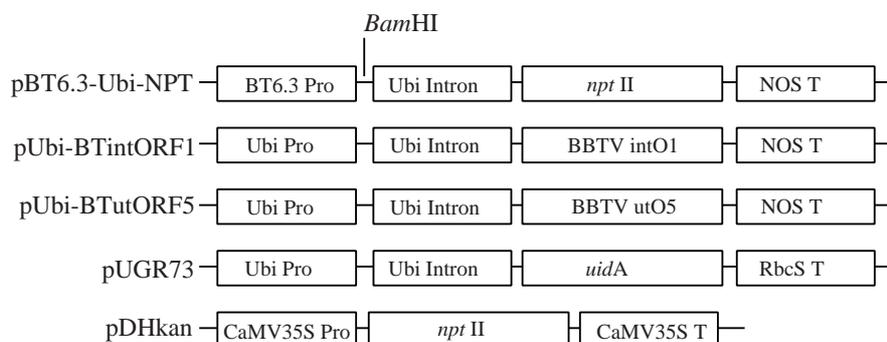
Promoter BT6.3 (239 bp) was isolated from BBTV DNA-6 (Burns et al. 1995) by PCR using primer BT6C1:[5'-GGA TCC GCT TCG TCC TTC CGC T-3' (BBTV DNA-6 sequence complementary to nt 264–280 with *Bam*HI restriction site)] and primer BT6V2 [5'- CTG CAG CAT GAC GTC AGC AAG G-3' (BBTV DNA-6 sequence from nt 42–57 with *Pst*I restriction site)]. The PCR conditions were as previously described. The amplified product was cloned into *Pst*I/*Bam*HI-digested pUC19 as a *Pst*I/*Bam*HI fragment. This construct was designated pUC-BT6.3. An *npt*II -35S 3' UTR cassette was excised from pDHkan with *Kpn*I and inserted into the *Kpn*I site located downstream of the BT6.3 promoter in pUC-BT6.3. This construction was designated pUC-BT6.3-NPT. The 1010-bp*Ubi*-1 intron with the short upstream exon flanking sequence (41 bp) was excised from pGEM-Ubi as a *Bgl*II/*Sma*I fragment and directionally cloned between the BT6.3 promoter and *npt*II gene into the *Bam*HI/*Sma*I sites in pUC-BT6.3-NPT. This construction was designated pBT6.3-Ubi-NPT.

Plasmid DNA was prepared using a Qiagen Plasmid Maxi Kit according to the manufacturer's instructions. The plasmid pDHkan was co-transformed with pUGR73, whereas the plasmid pBT6.3-Ubi-NPT was co-transformed with either pUbi-BTutORF5 or pUbi-BTintORF1. The plasmids were mixed at equimolar concentrations before preparation of the gold particles.

Bombardment conditions

Suspension cells (cv 'Grand Nain') were bombarded using a particle inflow gun (Finer et al. 1992). Gold particles (Bio-Rad), 1.0 μm in diameter, were used as microprojectiles. To prepare gold particles for bombardment, we washed 120 mg of gold particles three times in ethanol and three times in sterile distilled water before suspending them in 1 ml of sterile 50% (v/v) glycerol. Microprojectiles were prepared for bombardment by first sonicating 25 μl of gold particles for 30 s, then mixing them with 2 μg of plasmid DNA (equimolar concentration of co-transforming plasmids), 25 μl of 2.5 *M* CaCl$_2$ and 5 μl of 0.1 *M* spermidine-free base. All solutions were kept on ice. The gold was kept in suspension for 5 min by occasional vortexing, and then allowed to precipitate for 10 min on ice; 22 μl of supernatant was subsequently removed. The remaining suspension was vortexed, and 5-μl aliquots were used for each bombardment. Target tissue was placed 7.5 cm from the point of particle discharge and covered by a 210-μm stainless steel mesh baffle. Helium pressure was 550 KPa and chamber vacuum was –84 KPa.

Fig. 1 Schematic representation of gene constructs used for banana transformation. *CaMV 35S Pro* Cauliflower mosaic virus 35S promoter, *uid*A β-glucuronidase (GUS) reporter gene, *NOS T* nopaline synthase gene 3'UTR, *npt*II neomycin phosphotransferase gene, *CaMV 35S T* cauliflower mosaic virus 35S gene 3'UTR, Ubi Pro promoter from maize polyubiquitin gene, Ubi intron first exon and intron from maize polyubiquitin-1 gene, *rbcS T* tobacco rubisco gene 3'UTR, *BT6.3 Pro* promoter from DNA 6 of banana bunchy top virus, *BBTV intORF1* the internal open reading frame of BBTV DNA 1, *BBTV utORF5* the open reading frame of BBTV DNA 5 in an untranslatable form

*Bam*HI

pBT6.3-Ubi-NPT	BT6.3 Pro	Ubi Intron	*npt* II	NOS T
pUbi-BTintORF1	Ubi Pro	Ubi Intron	BBTV intO1	NOS T
pUbi-BTutORF5	Ubi Pro	Ubi Intron	BBTV utO5	NOS T
pUGR73	Ubi Pro	Ubi Intron	*uid*A	RbcS T
pDHkan	CaMV35S Pro	*npt* II	CaMV35S T	

Selection and regeneration of plants

Ten days after bombardment, embryogenic cells, including non-bombarded controls, were subcultured on BL medium containing 100 mg/l kanamycin and solidified with 7 g/l agar. The cells were subcultured by transferring the supporting filter paper to fresh medium. After 3 months (monthly subcultures), plants were regenerated as described by Cote et al. (1996) by culturing for 4 months on M3 medium containing 100 mg/l kanamycin to allow for embryo formation and maturation. After this, putatively transgenic embryos were removed from the filter paper and placed in direct contact with M4 medium (embryo germination) containing no antibiotic. Embryos were cultured on this medium until they germinated, which took up to 3 months. Germinated embryos were then rooted on M5 medium containing 100 mg/l kanamycin. Rooted plantlets were maintained *in vitro* and analysed to verify stable transformation. Non-transformed Cavendish plants (cv 'Williams') were also regenerated from embryogenic cell suspensions as described by Cote et al. (1996), acclimatized in the glasshouse and then transferred to the field. After 14 months (after they had produced fruit), 33 non-transformed plants were assessed for the level of somaclonal variation.

Histochemical β-glucuronidase (GUS) assay

For β-glucuronidase GUS assays, tissue was incubated in buffer containing 100 mM sodium phosphate (pH 7.0), 50 mM ascorbate and 1 mM 5-bromo-4-chloro-3-indolyl β-δ-glucuronide (x-gluc) according to Jefferson (1987). Samples were incubated overnight at 37°C and cleared with acetic acid:ethanol (1:3).

Genomic DNA isolation, PCR and Southern blot hybridization analysis

Genomic DNA was isolated from leaves according to a modified CTAB protocol (Stewart and Via 1993). Full length transgenes were amplified by PCR using the following primer pairs: GUS gene, GUS-1 (5′-ATG TTA CGT CCT GTA GAA ACC C-3′) and GUS-2 (5′-TCA TTG TTT GCC TCC CTG CTG C-3′);*npt*II gene, NPT-1 (5′-ATG ATT GAA CAA GAT GGA TTG C-3′) and NPT-2 (5′-TCA GAA GAA CTC GTC AAG AAG G-3′); BBTV DNA1 internal gene, BTintORF1-1 and BTintORF1-2; untranslatable BBTV DNA5 gene, BTutORF5-1 and BTORF5-2. PCR conditions were as previously described except that 1 μg of isolated genomic DNA was used as the template, and the primer annealing temperature was 50°C. For Southern blot hybridisation analysis, 10 μg of each genomic DNA sample was digested with *Bam*HI and electrophoresed through a 1% agarose gel in 1×TAE buffer. DNA was transferred to a positively charged nylon membrane (Boehringer Mannheim) by the method of Southern (1975). DIG-labelled probes, specific for the *npt*II gene, were prepared using a PCR DIG probe synthesis kit (Boehringer Mannheim) and primers NPT-1 and NPT-2. Membranes were probed using a DIG luminescence detection kit (Boehringer Mannheim) as per the manufacturer's instructions. The hybridisation signal was detected by autoradiography.

Results and discussion

Initiation of suspension cultures and regeneration of non-transformed plants

Embryogenic callus from immature male flowers of 'Grand Nain' and 'Williams' were placed in either M2 or BL medium to compare the quality of suspension cultures. In both media, cell suspensions formed within 2 months and, after 4 months, were sufficiently homogeneous for use in transformation experiments. Suspensions cultured in both media consisted of individual, elongated, highly vacuolated cells and clusters of embryogenic cells, as previously described by Dhed'a et al. (1991). However, more highly vacuolated cells were observed in suspensions cultured in M2 medium. When plated on M3 embryo formation medium (Cote et al. 1996), the large vacuolated cells became necrotic and died, whereas the embryogenic cell clusters produced somatic embryos. Suspensions cultured in both media were capable of producing somatic embryos; however, cells from the BL medium produced more embryos and had less necrosis than cells from the M2 medium.

The longer the embryos were allowed to mature on M3 medium, the higher the frequency of germination, as also reported by Grapin et al. (1996). For instance, embryos matured on M3 medium for four months had a germination frequency of 50–60% compared with 10–20% after a 3 month maturation.

Somaclonal variation was assessed in 33 regenerated non-transgenic 'Williams' plants. After 14 months growth in the field, all of the 33 plants appeared phenotypically normal. However, two suckers from 1 plant were 'mosaic' somaclonal variants (narrow, thickened, chlorotic leaves), which is a common 'off-type' produced during tissue culture of Cavendish (Israeli et al. 1991). Other suckers from the same plant were normal, indicating that the plant was chimeric. If banana somatic embryos are unicellular in origin, as proposed by Cote et al. (1996), then the somaclonal variation probably did not occur in suspension culture but rather after formation of the multicellular embryo. The occurrence of only 1 plant with 'off-type' suckers indicated that the level of somaclonal variation in Cavendish plants derived from embryogenic cell suspensions was low. However, larger numbers of plants need to be assessed to more accurately determine the frequency of somaclonal variation.

Regeneration of transgenic plants

Embryogenic suspension cells of 'Grand Nain' were bombarded with the *uid*A reporter gene and potential virus resistance genes 6 months after the suspension was initiated in BL medium. In previous experiments, suspension cells were bombarded 4 and 5 months after initiation; however, very few transgenic plants were produced (results not shown). This may have been due to the different transformation constructs used and/or to the age of the culture because suspensions reached their highest regenerative capacity at 6 months.

Bombarded cells were selected for 3 months on BL medium followed by 4 months of selection on M3 medium (both media containing 100 mg/l kanamycin). Embryos were then transferred to M4 germination medium without antibiotic. Studies on transformation of 'Bluggoe' (*Musa* spp. ABB group) embryogenic
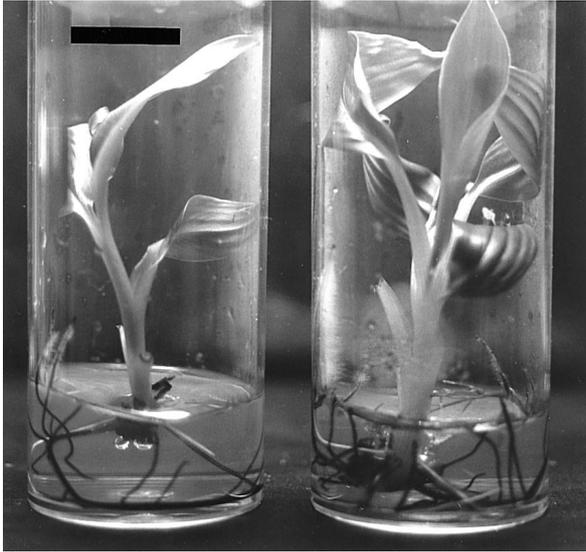
Fig. 2 Transgenic 'Grand Nain' plants on rooting medium containing 100 mg/l kanamycin. Plants have produced roots, healthy pseudostems and leaves. No plants were regenerated from unshot cells placed on selection medium. *Bar*: 20 mm

Fig. 3A, B Histochemical GUS stain of transgenic and non-transformed 'Grand Nain' tissue. The *uid*A gene driven by the maize polyubiquitin-1 promoter resulted in strong GUS activity in leaf and root tissue (*B*) whereas no GUS activity was observed in tissue from untransformed plants (*A*) *Bar*: 5 mm

suspension cells have shown that geneticin allows the formation of transgenic embryos, but interfers with embryo germination (Becker, Harding and Dale, unpublished results). The inhibitory effect of kanamycin in the regeneration phase of plant transformation has also been previously reported in other crops (Yao et al. 1995; Bretagnesagnard and Chupeau 1996). Thus, kanamycin was omitted from embryo germination media and re-introduced at the rooting stage. As bombarded cells had already been on selection for 7 months and no embryos had formed from nonbombarded cells on selection, it was considered unlikely that the embryos formed were 'escapes'. On rooting medium containing kanamycin, all germinated embryos formed roots and continued to actively grow (Fig. 2). When assayed for GUS expression, all 4 plants transformed with the plasmid pUGR73 showed a high expression in leaf and root tissue, whereas no activity was observed in untransformed plants (Fig. 3).

Two constructs with BBTV genes were also prepared and used to bombard banana embryogenic suspension cells. The BBTV DNA-5 gene and the BBTV DNA-1 internal gene both encode proteins that may be involved in virus replication (Wanitchakorn, Hafner, Harding and Dale, unpublished results), and these may be candidate genes for virus-derived resistance.

Analysis of transgenic plants

Southern blot hybridisation analysis of 8 plants transformed with pBT6.3-Ubi-NPT confirmed the stable integration of *npt*II int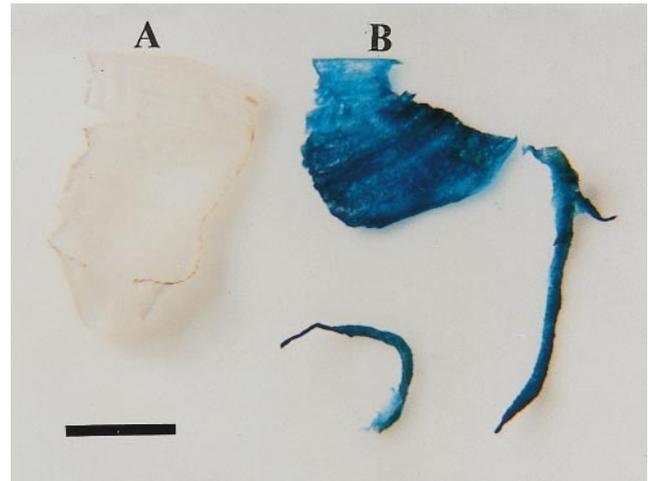o the genome. The hybridisation signals in lanes containing genomic DNA of transgenic plants were of different molecular weights to the positive control plasmid (5.5 kb), whereas no signal was detected in the non-transformed control lane (Fig. 4). The copy number ranged from 1 to 14 based on the number of bands present in each lane. The transfer of banana genomic DNA to the membrane appeared to block non-specific binding of the probe, resulting in
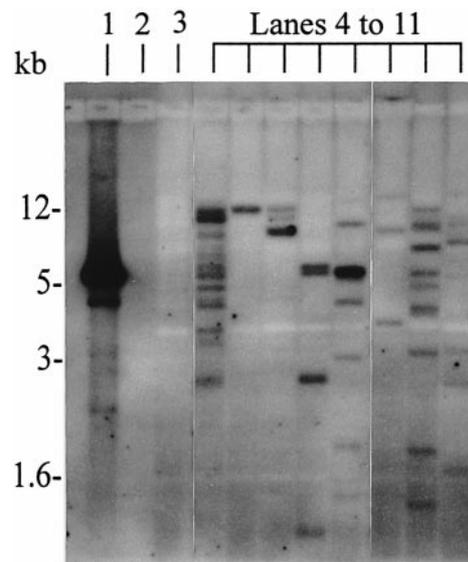

Fig. 4 Southern blot hybridisation analysis of eight putatively transformed 'Grand Nain' plants. Positive control plasmid and total genomic DNA extracted from a non-transformed control plant and selected transgenic plants were all digested with *Bam*HI. The positive control plasmid (pBT6.3-Ubi-NPT) contained a single *Bam*HI site and was 5.5 kb in size. *Lane 1* pBT6.3-Ubi-NPT (1 ng), *lane 3* non-transformed control, *lanes 4–11*: plants putatively transformed with pBT6.3-Ubi-NPT

Table 1 Summary of banana transformation results and molecular analysis of transgenic plants (*nd* not determined)

	pUbi-BtintORF1[a] pBT6.3-Ubi-NPT[b]	pUbi-BTutORF5[a] pBT6.3-Ubi-NPT[b]	pUGR73[a] pDHkan[b]	Total
Plates bombarded[c]	42	42	6	90
Plates forming embryo clusters[d]	3	6	1	10
Plants regenerated[e]	16	55	4	75
PCR analysis[f]	16/16	55/55	4/4	75/75
Southern blot hybridisation analysis[g]	3/3	5/5	nd	8/8

[a,b]Plants were co-transformed with either a pathogen derived construct or *uid*A reporter gene[a], and a selectable marker construct[b]
[c]Number of plates co-bombarded with each plasmid combination
[d]Number of plates that formed transgenic embryo clusters. Plates only formed one transgenic embryo cluster each; i.e. one plate gave rise to one transformation event
[e]Total number of plants regenerated. More than 1 plant was regenerated from each embryo cluster
[f]Number of plants testing positive for the presence of either the BBTV transgene or *uid*A per total number of plants screened by PCR with gene-specific primers
[g]Number of plants testing positive for the presence of the *npt*II gene per total number screened by Southern blot hybridisation analysis with an *npt*II-specific probe

white bands in lanes containing DNA of both transgenic and non-transgenic plants (Fig. 4).

To confirm that plants were derived from a single transformed cell and were not chimeric, we selected 3 plants that were derived from different bombarded plates, and therefore certainly derived from three different transformation events, for analysis. The 3 plants, which were co-transformed with pUbi-BTutORF5 and pBT6.3-Ubi-NPT, were placed on multiplication media (M5 containing 5 mg/l 6-benzy-laminopurine) with no antibiotic to allow possible non-transformed cells to form suckers. Three suckers from each of the 3 plants were assessed for the presence of pUbi-BTutORF5 using PCR, and all tested positive.

Table 1 shows the number of transgenic plants produced containing *uid*A and the different BBTV constructs. Putatively transformed embryos formed in clusters with only one cluster forming per plate; however, there were as many as 23 plants regenerated from a single embryo cluster. Although not all plants derived from individual embryo clusters were analysed by Southern blot hybridisation, it is most likely that plants from a single cluster were all derived from the same transformation event. The transformation efficiency was ten individual transformation events from 90 bombardments or approximately 11%. All plants were co-transformed, indicating that the potential for co-transformation is very high.

This appears to be the first report of the transformation of 'Grand Nain' by microprojectile bombardment using male flower-derived embryogenic suspension cells as the target tissue. Individual transformed plants appeared to be unicellular in origin and composed of cells derived from a single transformation event. We are currently assessing plants in the glasshouse for virus resistance and also optimising the transformation protocol to enable a more efficient production of large numbers of transgenic lines.

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