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A Procedure for *Agrobacterium tumefaciens* Mediated Genetic Transformation of Sweet Potato

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

Many sweet potato plants have been successfully transformed but the transgenic plants regenerated, however, have been limited to a few genotypes. Reported in this paper is a procedure in which several explants of three sweet potato genotypes from Papua New Guinea (PNG) were used to transform and regenerate transgenic plants. To achieve stable transformation, an efficient shoot regeneration system for different explants was developed. The shoot regeneration protocol developed enabled for a reproducible stable transformation mediated by *Agrobacterium tumefaciens* strain 1065. The plasmid pVDH65 contains the npt II gene for kanamycin (km) resistance, hpt gene for hygromycin resistance and Gus-intron reporter gene (GUS) for β-glucuronidase. Explants inoculated with the bacterial strain were co-cultured for 3, 5 and 7 days (d) in the dark on Murashige and Skoog (MS) medium without growth hormones. After co-cultivation, the explants were washed in liquid MS medium containing 500 mg L^{-1} cefotaxime, rinsed in sterile, deionised water for 10 mins and cultured on km selection medium containing 100 mg L^{-1} km followed by transfer of explants to 125 mg L^{-1} km after 14 d of culture. The km-resistant shoots selected on the former km concentration were transferred to the latter for double selection. Km-resistant shoots obtained at 125 mg L^{-1} were rooted on MS based medium also containing 0.008 mg L^{-1} IAA, 0.03 mg L^{-1} kinetin and 0.001 mg L^{-1} folic acid. This double selection method led to effective elimination of escapes (up to 75%) and successful recovery of transgenic plants from stem explants at more than 25%, leaf discs 10% and petioles 13.3% of each sweet potato cultivar. Polymerase chain reaction (PCR) analysis of the three km-resistant and GUS-positive plants

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revealed the presence of the expected fragment for npt II. This is the first report of successfully transforming sweet potato plants with bacterial strain 1065 and selection of transgenic plants at km concentrations higher than 100 mg L^{-1}.

Keywords: Crop genetic improvement; genetic transformation; sweet potato; tissue culture.

1. INTRODUCTION

Sweet potato [*Ipomoea batatas* L. (Lam.)] is a dicotyledonous plant belonging to the Convolvulaceae family. Early cytological studies have revealed in the genus *Ipomoea* the presence of polyploid plants having 2n=30, 60 and 90. The cultivated sweet potato (*I. batatas*) has the largest number of chromosomes (2n=90) but most of the wild type *Ipomoea* species are, 2n=30 and a few, 2n=60 [1]. Additionally, since the basic chromosome number of the genus *Ipomoea* is 15, *I. batatas* is considered to be a hexaploid. Due to incompatibility, male sterility and the hexaploid genome make sweet potato genetic improvement by conventional breeding difficult [2,3,4].

Moreover, genetically improved sweet potato planting materials that are high yielding, resistant to abiotic and biotic stresses are still limited in the farmers' fields [5]. The progress made in the improvement of sweet potato [6] using conventional breeding methods for the transfer of gene of interest together with the selection process is nevertheless, time-consuming and requires highly trained individuals and improved breeding systems. Biotechnology, as a new frontier in agricultural science has opened up new avenues towards the solution for agricultural problems and genetic transformation using recombinant DNA techniques seems to be very promising in increasing sweet potato production, overcoming incompatibility mechanisms, enhance the nutritive values and introduce genes conferring desirable traits into the genomes of sweet potato.

The limitations presented and the lack of any strong conventional genetic improvement programme, make sweet potato an important crop plant to be modified by novel approaches in biotechnology. Such approaches include plant tissue culture, somaclonal variation, somatic hybridisation and genetic transformation, to overcome such limitations, supplement and complement the conventional two breeding methods in sweet potato genetic improvement [7]. Furthermore, biotechnology is not yet fully utilized and developed for sweet potato improvement because of the difficulties of establishing an efficient and reproducible system for plant regeneration from cultured tissues or cells**.** Presented in this paper is a procedure in which explants of three high yielding and widely cultivated sweet potato genotypes of PNG were used to genetically transform and regenerate transgenic plants for crop genetic improvement.

2. MATERIALS AND METHODS

2.1 Plant Materials

Sterile, virus-free plantlets of three high yielding, drought tolerant and widely cultivated sweet potato cultivars of PNG (SK010, WHCH005 and PRAP496), originally obtained from the PNG National Agriculture Research Institute (NARI) were supplied as axenic shoot cultures by the Unitech Biotechnology Centre, for the present study which was conducted at the School of Biosciences, Plant Science Division, University of Nottingham, UK. Shoot cultures with four plants of each cultivar were maintained in jars containing 40 ml of MS medium supplemented with 3% (w/v) sucrose, solidified with 0.8% gelling agent (w/v) and pH adjusted to 5.8 (MS0.8). Shoot cultures were then grown at 26± 2ºC under a 16 h photoperiod of 30 µmol m^{-2} s^{-2} photosynthetic photon flux in a growth chamber. The stock plants were subcultured monthly in MS0.8 medium by culturing the nodal segments, which were maintained throughout the study period to provide source of explants.

2.2 *Agrobacterium tumefaciens* Strain and Plasmid

Agrobacterium tumefaciens strain 1065 (Fig. 1) used to assess transient *GUS* gene expression in the sweet potato cultivars. The plasmid VDH65, together with the super virulent pToK47 [8], was introduced into *A. tumefaciens* LBA4404 [9]. This LBA4404 carries pVDH65 (with the *gus* and *npt* II genes) and the super virulent pTOK47, which possesses additional *vir* genes (*vir* B, C and G genes).

2.3 Bacterial Inoculation of Sweet Potato Explants

After incubation, freshly prepared leaf discs and nodal segments of the three cultivars from *in vitro* and glasshouse-grown plants were used as source of tissues for inoculation with *Agrobacterium tumefaciens* strain 1065. The glasshouse sourced explants were initially surface sterilized with 8% (v/v) Domestos and rinsed three times in sterile reverse-osmosis water. The leaves were cut into sections, the mid-veins scored and the outer borders removed. The petiole fragments and the nodal segments were cut transversely using a sterile scalpel blade under laminar airflow bench. The explants were incubated in the bacterial suspension on a horizontal shaker (150 rpm) for 30 min at 30ºC in the dark and were then blotted dry on a couple of sterile filter papers under laminar-flow bench, and two co-culture media [MS0.8 and MS.8 (see below)] were tested for transient gene expression. Five jars each containing five explants of each sweet potato cultivar were cultured on the two co-culture media and replicated four times. Co-cultivation was carried out in the dark, for 3 d at 24±2ºC in a growth chamber.

After co-cultivation, the explants were washed on medium containing 500 mgL^{-1} cefotaxime (Cx) for 10 min, rinsed three times in sterile de-ionized water, and then blotted dry on a couple of sterile filter papers. To develop a selection system, medium containing 100 and 125 mg L^{-1} km, previously identified by kill curve as either having an inhibitory effect on regeneration or lethal, leading to the deaths of explants were used with nodal segments. The nodal explants were chosen because of its ease of direct shoot regeneration from the dormant buds. All other explants co-cultured were used for transient gene expression studies. Five jars containing 5 nodal segments each was replicated 4 times. The experiment was repeated twice.

Putatively transformed plants were selected using a double selection method. The initial stage of selection was carried out on containing 100 mg L^{-1} km plus 250 mg L^{-1} cx for 2 wk followed by transfer of km resistant-shoots onto 125 mg L^{-1} km for 14 d (double selection). The entire selection procedure was carried out in the dark at 24±2ºC. Fourteen days old km resistant shoots on medium with 125 mg L^{-1} were isolated and transferred to MS0.8 medium without km for rooting. For histochemical *GUS* assay, 15 leaf discs (1 cm^2), internodes and nodal segments of each cultivar were assessed for GUS activity after 3, 5 and 7 d of co-cultivation. The same numbers of explants not treated with *Agrobacterium* were employed as control. The experiment was repeated twice. Similar experiments were carried out with leave, petiole and stem explants of 14 d old km resistant plants.

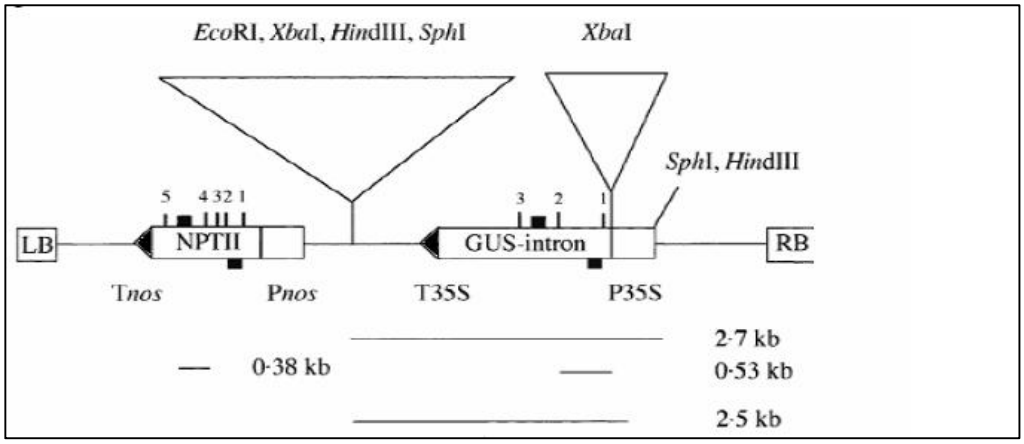

Fig. 1. T-DNA restriction map of pVDH65. The *nptII* gene driven by the nopaline synthase promoter (Pnos) inserted near to the left border (LB) of the T-DNA. The *gus*-intron gene under the CaMV35S promoter (P35S) with a CaMV35S terminator sequence (T35S) sited near to the TDNA right border (RB). The horizontal lines of 2.5 kb (*XbaI*) and 2.7 kb (*SphI, HindIII*) represent the fragments released when the genomic DNA of transgenic plants cut with the restriction enzymes. Numbers 1-5 represent *Sau 3a*, *NdeII* restriction sites, where the fragments of size 0.38 kb and 0.53 kb are released from the *nptII* and *gue* genes of transgenic plant DNA

2.4 Evaluation of Explants Sensitivity to Antibiotics

To determine the optimum antibiotic concentration for use in the selection of potentially transformed plant tissues, kanamycin (km) concentrations ranging from 0, 25, 50, 75, 100 and 125 mg L^{-1} were evaluated. Leaf discs, petiole fragments and nodal segments were prepared as described earlier and cultured in jars containing 40 ml of MS0.8 medium supplemented with the different km concentrations (MSK.8) above. The same numbers of explants were cultured on the same medium-lacking km as the control. The cultures were placed under growth chamber conditions suitable for direct plant regeneration.

2.5 Histochemical Glucuronidase Assay (GUS)

The detection of *GUS* gene activity was performed using *GUS* assay after 3, 5, 7 and 8 d of co-culture for inoculated and non-inoculated explants. *GUS* histochemical assay buffer solution was prepared by dissolving 10 mg X-Gluc in 1 ml of EGME and then adding 20 ml of 0.2 M sodium phosphate (12.2 ml NaH_2PO_4 plus 7.8 ml $Na_2H_2PO_4$) and 0.02% (w/v) sodium azide, pH 7.4 [10]. Prior to fixation, the surfaces of 30 (10 of each cultivar) explants (leaf, petioles and nodal) of km resistant plants and 45 (15 of each cultivar) explants of non-transformed parent stocks were wounded with a blade to enable more staining solution to enter the cells.

After fixation, all samples were incubated overnight at 37ºC in 20 ml assay buffer containing 1 µM X-Gluc. After incubation, the *GUS* assay buffer was removed and replaced with 70% (v/v) ethanol for 4 d in order to remove chlorophyll from the explants and to improve visualisation of the blue colour from the *GUS* activity in the explants. The explants were removed from ethanol and transferred to a Petri dish and the intensity and localisation of the blue staining was quantified using a stereomicroscope. The explants on co-cultivation media for 3, 5 and 7 d were treated separately. Untransformed explants of the three sweet potato cultivars on co-culture media for the specified number of d of co-cultivation were treated separately as the controls for *GUS* assay.

2.6 DNA Extraction from Leaf Tissues

In order to confirm the presence of transgenes (*nptII*) in the genomes of putatively transgenic plants and confirm the absence in non-transgenic plants, polymerase chain reaction amplification was used. Additionally, the *nptII* forward and reverse primers [20 base pair (bp) in length] (Table 1) which amplify a 280 bp region of the *nptII* gene, were used. The primers were diluted to a stock solution of 100 pmol $µl^{-1}$ with sterile purified water (356 µl to the forward primer and 429 µl to the reverse primer), mixed well and pulsed for 3 sec and stored at -20ºC prior to use. The working solutions were prepared by addition of 20 µl (20 pmol $µl^{-1}$) to the reaction mixture (stock solution) plus 80 µl of purified water.

Table 1. NptII primers and their sequences

Primers	Sequences
nptII forward	5' ACA AGA TGG ATT GCA CGC AGG 3'
nptII reverse	5' AAC TCG TCA AGA AGG CGA TAG 3'

Source: MWG Biotech, Ebersberg, Germany

The total genomic DNA for the template was isolated from the leaves of *in vitro*-cultured plants following a modified method [11]. DNA samples were extracted from putative transgenics including two untransformed plants as controls by removing a leaf disc of each cultivar using the lid of a 1.5 ml microfuge tube and flash frozen in liquid nitrogen (-196ºC). The frozen leaf tissues were then quickly grounded to a fine powder using a micropestle followed by addition of 1-2 µL of RNase from stock solution (10 mg ml^{-1}) to 1ml of extraction buffer. Before thawing the samples, 600 µL aliquot of the extraction buffer was added to each of the tissue samples. The samples were vortexed on a vortex mixer for three seconds and incubated in a water bath at 45ºC for 15 min.

Samples were then centrifuged for 2 min (13000 rpm). 500 µl of the supernatant was drawn using a micropipette (1 ml) and transferred to a new microfuge tube and 500 µl of ice cold isopropanol was added to the supernatant. The original microfuge tube containing the leaf tissue was discarded. The supernatant was then incubated on ice for 5 min followed by centrifugation for 5 min. After centrifuging, the supernatants were drawn by a micropipette and discarded and 600 µl of ethanol (70% v/v) and centrifuged again for 3 min. The supernatants were discarded as described previously and the pellets air-dried in a laminar airflow bench and the pellets re-

suspended for 15-20 min later in DNase free water (50 µl).

2.7 PCR Amplification

For PCR amplification, a total volume of 65 µl (5 x 13) of master mix was used (3 genomic DNA, 1 positive control (*nptII*) and 1 negative control (without DNA) [12]. A total volume of 13 µl of the reaction mixture (2 µl of purified water, 7µl of RED Tag DNA polymerase; 2 µl each of *nptII* forward and reverse primers) (Table 1) were used. Thirteen µl of the master mix was added to 5 PCR tubes followed by addition of 2 µl of the appropriate DNA in each tube or 2 µl de-ionized water for the negative control. The reaction mixture was pooled by vortex and pulse centrifugation (10 sec) to collect all the components to the bottom of the tubes. PCR amplification was performed in a DNA Thermal Cycler 480 under the following amplification conditions; 1 cycle at 94ºC for 5 min (initial denature), 35 cycles at 94ºC each for 1 min (annealing), 35 cycles at 57ºC for 1 min (extension), and 1 cycle at 72ºC for 10 min (final extension) and 1 cycle at 4ºC for 5 min (termination).

The amplification products (10 µl from each sample) were analysed by gel electrophoresis in 1.5% (w/v) agarose at 120 V, 250 mA for 2 h in 0.5 TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0). The gel was stained by incubation in 5 µl ethidium bromide stock solution (10 mg ml^{-1}) dissolved in 100 ml reverse-osmosis water, for 15 min. The separated DNA bands were visualized on a UV transiluminator. For each lane, sample-primer combination, amplification products were identified and their molecular weight calculated by comparison with molecular weight standard.

3. RESULTS AND DISCUSSION

3.1 Sensitivity of Non-transformed Explants to Antibiotic

The leaf discs, petiole fragments and nodal segments of the three cultivars turned brown and died after 2 wk of culture on MSK.8 medium containing 125 mg L^{-1} km (Fig. 2F). At 100 mg L^{-1}, over 90% of leaf discs, 85% of petiole fragments and 76% of nodal segments were inhibited from rooting and shoot proliferation (Fig. 2E). At 25, 50 and 75 mg L^{-1} km, more than 30-80% of the explants survived and the nodal segments regenerated normal plants with well-developed roots and shoots compared to the other explants cultured on medium lacking km [Fig. 2 (A)].

3.2 Establishing Optimal Parameters for Transformation

Of the two co-cultivation media evaluated, MS.8 medium was found to be optimal for transient *gene* expression in leaf discs and petiole fragments. The frequency of transient *GUS* gene expression, measured as the percentage of explants with at least one blue spot, was over 25% (3/10) after 3 d of co-cultivation in the dark. Based on the km kill-curve evaluated previously, all nodal segments cultured on lower km concentration survived except at 100 and 125 mg L^{-1}.

When km at 100 mg L^{-1} was tested; all non-inoculated nodal segments turned yellow and explant proliferation was inhibited while those cultured on medium with 125 mg L^{-1} turned brown within 14 d and died thereafter. Similar results were obtained from leaf disc explants when tested separately on the same medium.

3.3 GUS Activity in Regenerated Plants after Bacterial Inoculation

Histochemical analysis failed to show high levels of *GUS* gene activity on explants of km-resistant plants, selected using the double selection method. In all the cultivars, *GUS* activity was observed at low levels in leaf discs and petiole fragments for the bacterial dilutions [1:2 or 1:3 (v/v)] used for explant inoculation when assayed. No *GUS* activity was detected on nodes. Similar results were obtained from explants on the co-cultivation medium (7 d after co-cultivation). The levels of *GUS* activity (% of explants showing blue staining at low levels) were; SK010 (leaf discs was 40% (4/10), petioles 20% (2/10) and nodal segments 10% (1/10), WHCH005 (leaf discs was 50% (5/10), petioles 30% (3/10) and nodal segments 20% (2/10) and for PRAP496 was 30% (3/10) on leaf discs but no blue staining was detected on petiole or nodal explants.

Additionally, samples of explants co-cultivated for 3, 5 and 7 d failed to show *GUS* activity among the sweet potato cultivars. Non-inoculated nodal segments, leaf discs and petiole fragments of all the sweet potato cultivars and inoculated explants of PRAP496 failed to exhibit *GUS* gene activity at 3, 5 or 7 d of co-cultivation. The results were similar when co-cultivation was extended

for 10 d. The *GUS* activity obtained from cultivar WHCH005 was 4% (6/15) from petiole fragments and 2% (2/15) from leaf discs respectively. No *GUS* activity was detected on nodal explants. No *GUS* activity was detected on the other two cultivars (SK010 and PRAP496). In an earlier study, genotypic differences in the susceptibility among the cultivars of sweet potato to infection with *A. rhizogenes* A13 was reported [13]. Similar findings have been reported in kale [*Brassica oleracea var. acephala* [14,15] and faba bean [*Vicia faba* [16].

It was observed too that leaf explants inoculated with *Agrobacterium* exhibited blue *GUS* histochemical staining at wound sites throughout the explants, indicating expression of the *GUS* gene. On the other hand, blue *GUS* staining was observed on petiole fragments only at the cut edges, from the older portion of the petioles. No *GUS* gene activity was detected on nodal segments of each of the cultivar. Additionally; all the *GUS* positive explants were of glasshouse origin. No *GUS* gene activity was detected in explants sourced from *in vitro* regenerated plants that were selected on kanamycin selection. One of the possible reasons drawn from this observation was that glasshouse grown plants were anticipated to produce considerable amount of secondary metabolites that could help more plant tissues to be infected by *Agrobacterium*. No differences however were found on transformation frequency between stem explants from *in vitro* stock cultures and field-grown plants inoculated with *Agrobacterium* strain EHA 1065 [4].

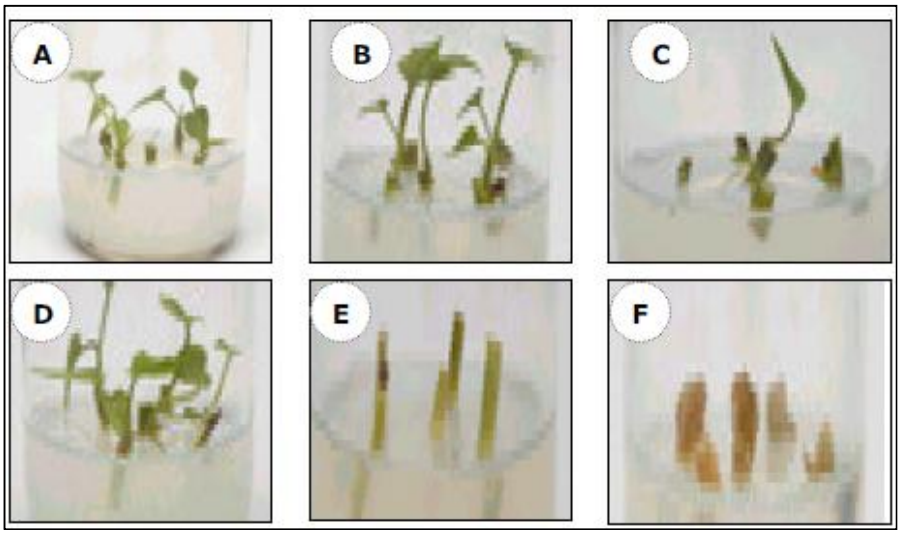

Fig. 2. Sensitivity of nodal explants of sweet potato on MS.8 medium containing different concentrations of km. Regeneration of shoot A (0), B (25), C (50), D (75), E (100) and F, (125) mg L^{-1} of km

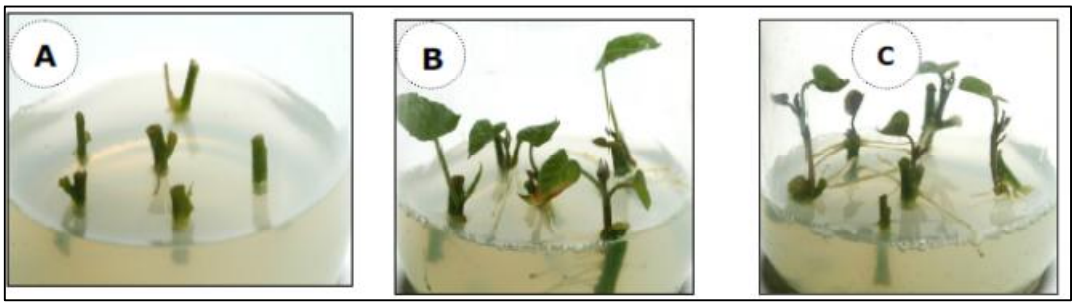

Fig. 3. Double selection of transgenic tissues after inoculation with *A. tumefaciens* strain 1065 on MSK.8 medium. A, Non-inoculated nodal fragments at 100 mg L^{-1} km after 14 d of culture. B, Inoculated nodal segments at 100 mg L^{-1} km after 14 d; and C, km resistant explants from B rooting on medium containing 125 mg L^{-1} km 7 d after transfer (double selection)

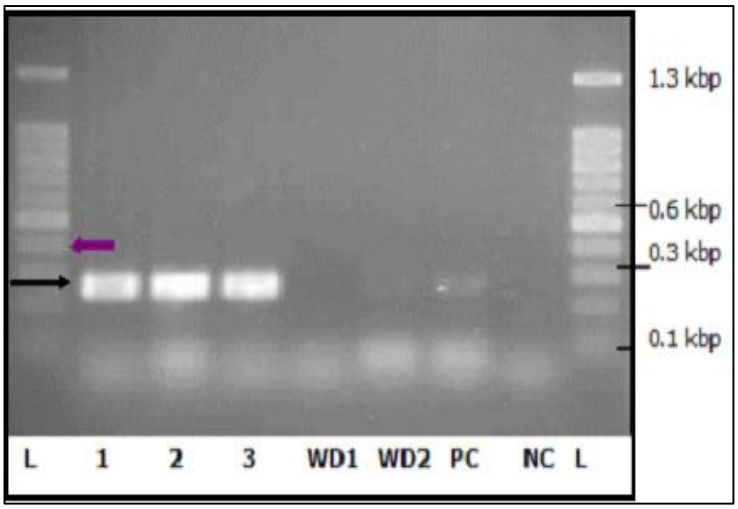

Fig. 4. Gel electrophoresis results of PCR products of putative transgenic plants. L (DNA ladder as marker (1kb), Putatively transformed cultivars 1 (SK010), 2 (WHCH005), 3 (PRAP496), untransformed parents (WD1 & WD2), PC (Positive control) and NC (Negative control). The positive control contained 1 µl of primer (*npt II*) and the negative control 1 µl of water respectively. The arrows show the expected band size of 0.28 kbp from *npt II* gene on the DNA ladder (top) and 0.27 kbp in the putative transgenic sweet potato plants (bottom), respectively

Among the independent km-resistant plants recovered from different nodal explants using the double km selection method previously described, one km-resistant plant of each cultivar was selected for PCR. The expected band size of 0.28 kbp fragments released from the *nptII* gene of transgenic plant DNA was at least found in all the putatively transformed plants (Fig. 4). When each lane of the amplification product for transgenic plants and sample-primer combinations were compared using a molecular weight standard (DNA Ladder), the band size amplified in the transgenic plants were estimated to be 0.27 kbp. All of the three cultivars had a single band with a single pattern. Comparatively, no band was amplified in the genomic DNA of the two wild types included for comparison as control, together with the positive (*nptII*) and negative (without DNA) controls respectively (Fig. 4).

4. CONCLUSION

This is the first report of successfully transforming sweet potato plants with *A. tumefaciens* strain 1065 and selection of transgenic plants at kanamycin concentration higher than 100 mg L^{-1} compared to previous reports of sweet potato genetic transformation and selection of transgenic plants on different kanamycin concentration to date. Consequently, the study has clearly established an important procedure to be used for sweet potato genetic transformation work for genetic improvement of the crop in the light of climate change related natural extremes such as drought, frost and ever increasing invasive pests and diseases of the crop that are currently affecting and will affect the production of this important crop in the future.

DISCLAIMER

Some part of this manuscript was previously presented and uploaded in the following conference: Proceedings of the TWAS Regional Young Scientist Conference. In 'Food, Health and Fuel: Plants for the Future in November 2009, At Selangor, Malaysia. Web Link of the proceeding:https://www.researchgate.net/publication/274139366_Tissue_culture_and_genetic_transformation_of_sweet_potato_for_crop_genetic_improvement.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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