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Expression of *GUS* and *GFP* reporter genes in transgenic hairy roots of tomato and potato plants via *Agrobacterium rhizogenes* mediated transformation.

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ABSTRACT

In response to the threats caused by phytopathogens and plant disease, this investigation looks at the potential of genetic modification technology as a strategy to protect plant crops globally. Specifically, it will explore the usefulness of *GUS* (β-Glucuronidase) and *GFP* (Green fluorescent protein) as reporter genes in *Agrobacterium*-mediated transformation of tomato (*Lycopersicum esculentum* L.) and potato (*Solanum tuberosum* L.) hairy roots. Transformation of *Agrobacterium* with *GUS* and *GFP* will be analysed using PCR to evaluate the efficacy of this vector. Compiling knowledge of genetic markers and transformation techniques will allow for high yield and efficient crop plant transformations.

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INTRODUCTION

Plants have many defence systems to endure and ward off pests and diseases, but these still manage to cause devastating effects in many plant species. For example, in Mediterranean countries vine plants are increasingly being infected with fungal trunk disease pathogens (Aroca *et al.,* 2006). Generally these infections have also been shown to reduce the aesthetic value and storage life of some crops (Punja and Utkhede, 2003). Therefore combating fungal disease by enhancing plant defence and resistance strategies are vital to maintain crops (Rajam, 2012). It is essential to find other methods that can help crops fight fungal pathogens, and one way to achieve this is by creating transgenic plants. Such as one which carries disease resistant genes. It is important to have efficient gene delivery systems in order to produce large populations of transformants (Bornhoff *et al.,* 2005; Li *et al.,* 2006; Gago *et al.,* 2011).

GUS and *GFP* are competing DNA markers used in many studies, these reporter genes can also be used to detect multiple integration and unstable transgenes within a transformant (Filipecki and Malepszy, 2006). They work by attaching to a fragment of DNA of interest, e.g. in the case of increasing a plant defences, a pathogen resistant gene, and can then identifying successfully transformed genes within an organism.

A. rhizogenes is an important experimental tool in producing transgenic material that can transfer foreign DNA into plant cells based on the autonomous root-inducing (Ri) plasmid and cause hairy root formation (Deng *et al.,* 2011; Al-Mallah and Mohammed, 2012).

This investigation aimed to study the efficiency of 35S *GUS* and 35S *GFP* uptake by *A.rhizogenes* R1000 and the reliability of using these separate markers within this construct. These transgenic bacteria harbouring 35S *GUS* and *GFP* used to create transgenic hairy roots in both tomato (*lycopersicum esculentum* L.) and potato (*Solanum tuberosum* L.) plants.

MATERIALS AND METHODS

Agrobacterium rhizogenes R1000 transformation:

Two eppendrof tubes containing 100µl A. *rhizogenes* R1000 competent cells were kept on ice, one labelled with pBI121 35S *GFP* and the other labelled with pBI121 35S *GUS*. 2.0µl of each vector was added to each tube and immediately placed in water bath at 37^0C, 5 min. with agitating to ensure the DNA is mixed. One ml of LB broth medium was added to each tube and the cells were transferred into separate falcon tubes. *Agrobacterium* suspension was then incubated in an orbital shaker (GallenKamp, UK) at180rpm, 28^0C, 2h. The culture was then plated out onto the surface of agar-solidified LB plates containing 50 µg/ml kanamycin for

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transformed bacterial selection. Plates were placed on a static incubator (GallenKamp, UK) at 28^0C (Sambrook and Russel, 2001).

After colonies had grown 48h later, plates were removed. One *GFP* and one *GUS*, of *Agrobacterium* culture were used for the rest of the experiment.

PCR of Agrobacterium rhizogenes constructs:

For each construct of *A. rhizogenes*, a yellow tip was used to scoop up some bacteria from the plate and transferred into a 0.2ml PCR tube containing 10µl sterile water. The *GUS* construct tubes were labelled 1 & 5 and the *GFP* construct tubes were labelled 2 & 6 for ease of allocating a place in the PCR wells (Table1).

Table 1: Tubes set up for use in both *A. rhizogenes* 35S *GUS* PCR and 35S *GFP* PCR.

Tube Label	Contents
1	*A. rhizogenes* 35S *GUS* sample
2	*A. rhizogenes* 35S *GFP* sample
3	10µl of a 1.0 in 1000 dilution of 35S GUS DNA (positive control)
4	10µl of sterile water (negative control)
5	*A. rhizogenes* 35S *GUS* sample
6	*A. rhizogenes* 35S *GFP* sample
7	10µl of a 1.0 in 1000 dilution of 35S GFP DNA (positive control)
8	10µl of sterile water (negative control)

Each tube was then boiled for 10 min. in the PCR block, boiled samples were placed in ice to cool for a few min., pulsed briefly in a centrifuge and placed back in ice.

Mastermix solution was prepared for use in the PCR reaction for 35S *GUS*. To a pre-made tube of mastermix (Table 2) 5µl of *GUS* F (forward primer) and 5µl of *GUS* R (reverse primer) was added which will amplify a section of the *GUS* reporter gene. This mastermix was briefly vortexed to ensure thorough mixing.

Table 2: Mastermix for PCR reactions.

Contents	µl per 190µl
$MgCl_2$	7.5
Bioline buffer	25
2mM dNTPs	25
Biotaq red	7.5
Sterile water	125

Four 0.2ml tubes were set up for use in the PCR reaction for 35S *GUS* labelled 1-4, and 40µl of the prepared mastermix (Table 2) was added to each tubes 1-4 and mixed using a pipette, fresh tip was used with each tube to avoid cross-contamination, and were kept on ice.

Similarly PCR test for 35S *GFP*, four 0.2ml tubes were set up labelled 5-8 and 40µl of the prepared mastermix was added to each of these tube, mixed and placed on ice. Polymerase Chain Reaction took place in a PCR machine (Eppendrof Thermo Mixer Comfort, Germany). Programme was at 94°C, 3min., [94°C, 20 sec., 55°C, 1.0 min., 72°C, 40 sec.] for 30 cycles, 72°C, 10 min. Agarose gel (Sigma, UK) of 1% prepared by using 0.5g of agarose and 50ml of TBE buffer, placed in a 250ml conical flask and microwaved for 2 min. Once the flask had cooled to hand hot temperature, 5µl GelRed™ was added. This molten agarose was then poured in a gel tray, firmly sealed with red ends with two sets of five wells comb added, and left to solidified. The combs were carefully removed from the gel, and 10µl of each PCR sample in tubes 1-4 were loaded in the first 4 wells of the gel. 10µl of DNA marker,(Bioline Easyladder1) was loaded into the next well, and 10µl of each PCR sample in tubes 5-8 was loaded in the first 4 wells of the second row of the gel. Again, 10µl of the DNA marker was added in the next well. The reaction was ran at 80v, 30 min. (Sambrook and Russel, 2001).

Tomato Transformation:

Production of aseptic seedlings:

Twenty seeds of tomato were placed in a tube, mixed with 5.0 ml of 10% bleach for 5 min, and were thoroughly washed with sterile D.W.(six times). The surface sterilized seeds were distributed onto four petri dishes each containing 25 ml of 1% water agar, 5 seeds/plate. They kept in dark condition at 24^0C in culture room for a week (Christy and Braun, 2005).

Direct injection of tomato seedlings with 35S GUS and 35S GFP labelled A. Rhizogenes:

One week old tomato seedlings were removed from the water agar plates. Each root decapitated seedlings was inoculated with 35S *GUS*-labelled *A.rhizogenes*. *Agrobacterium* was scraped along the cell surface with a hypodermic needle. Where then used to stab each individual hypocotyls, in 4 or 5 positions. Each of the

inoculated seedlings were placed on a 9.0 cm diam. plastic petri dish containing 25 ml of 1/2 MS (Murashige and Skoog, 1962) medium.

The above procedure was repeated with other tomato seedlings using 35S *GUS* construct and was then carried out for other two plates with the 35S *GFP*-labelled *A. rhizogenes*. All plates were kept at 24^{0}C in dark for 3 days to permit growth of transgenic roots (Rajesh *et al.,* 2007). The hypocotyls were transferred to 1/2 MS plates containing kanamycin 50 µg ml^{-1} and cefotaxime 100 µg ml^{-1} to eliminate bacteria from plant material. Plates were sealed with micropore tape, wrapped in foil and returned to 24^{0}C.

GUS Staining Of Transformed Hairy Roots:

A *GUS* stain was consisted of 0.75ml of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) at concentration of 20mgml^{-1} ,in DMF (Dimethylformamide) solvent. 14.25ml of X-Gluc buffer was added to this to make a 1.0 in 20 X-Gluc stain solution. Three cm explants of young hairy roots were excised from the plates. They were thoroughly washed with sterile D. W. to remove any residual agar. Hairy roots were placed in a vial and to this 2.0ml of X-Gluc stain was added. The vial was kept at 37^{0}C overnight to allow imitation of stain, after 24h the stain was replaced by water. Stained root specimens were placed in a Petri dish with little water, and observations were made of the expression patterns seen.

Tomato seedlings transformed with 35S *GFP* were observed microscopically (Olympus SZX12 microscope) for visualising *GFP* fluorescence and expression. Microscope was attached to a UV light source, which will cause the *GFP* to fluoresce if successfully transformed into roots. Also has suitable filters for allowing *GFP* excitation at 395nm and emission at 509nm.

Potato transformation:

Co-cultivation of potato leaves with 35S GUS and 35S GFP- labelled A. Rhizogenes:

One week potato detached leaves, taken from fully grown plants, were placed in a sterile Petri dish. The leaves were given wounding sites by cutting slits horizontally across the blade width, for penetration of *Agrobacterium* whilst keeping the edges intact. Two sterile Petri dishes were half filled up with a liquid culture of 1/2 MS. To one of these dishes 100µl of 35S *GUS*-labelled *A. rhizogenes* suspension was added and to the other 100µl of 35S *GFP*-labelled *A. rhizogenes* suspension was made. Five wounded potato leaves were immersed into agrobacterial suspension in each dish for 30 min., then they removed from suspension and blotted dry using separate blotting equipment for each *Agrobacterium* to avoid cross-contamination. The leaves were transferred to 1/2 MS plates with the lower epidermis in contact with the medium. Specimens were incubated at 20^{0}C, 2-3 days to allow growth of hairy roots.

Similarly, potato plants were transferred to 1/2 MS plates containing selection antibiotics, kanamycin 50 µgml^{-1} and cefotaxime 100 µgml^{-1} to clear bacteria from plant material. Plates were sealed with micropore tape, wrapped in foil and incubated at 20^{0}C.

GUS expressing potato plates were visually analysed after sufficient hairy root growth had occurred using X-Gluc stain. Also potato plants transformed with 35S *GFP* were analysed under an appropriate microscope mentioned previously with tomato hairy roots.

Results:

PCR of A.rhizogenes R1000 transformed with 35S GUS and 35S GFP:

Gel electrophoresis with an intercalating stain which help to assess the presence and length of different DNA fragments generated from the PCR samples. Bioline Easyladder1 was used to create standard bands to which the DNA samples could be compared. This developed 5 bands for easy identification of the fragments and these bands experimentally should be 2000bp, 1000bp, 500bp, 250bp and 100bp, which can be seen in the last lanes of both PCRs. If the 35S *GFP* gene was present in the DNA sample a band was expected to be seen around the 714bp mark. If the 35S *GUS* gene was present in the DNA sample a band was expected to be seen at the 401bp mark (Fig. 1).

The results of *GUS*-PCR was expected to be present in lanes 1 and 3, therefore a band appeared at 401bp. These expected bands were shown in the PCR output. In lane 1 there was also a band of smaller bp which indicates DNA fragments of a smaller size were also present in this sample.

Identically *GFP*-PCR in the second set of wells, the *GFP* gene was expected to be seen in lanes 6 and 7, therefore a band was produced at 714bp. The expected band was shown in lane 7 of the PCR output but very faint in lane 6.

For both PCRs no bands were expected to be seen in lane 4, as the sample was sterile water and this was clear in the PCR image.

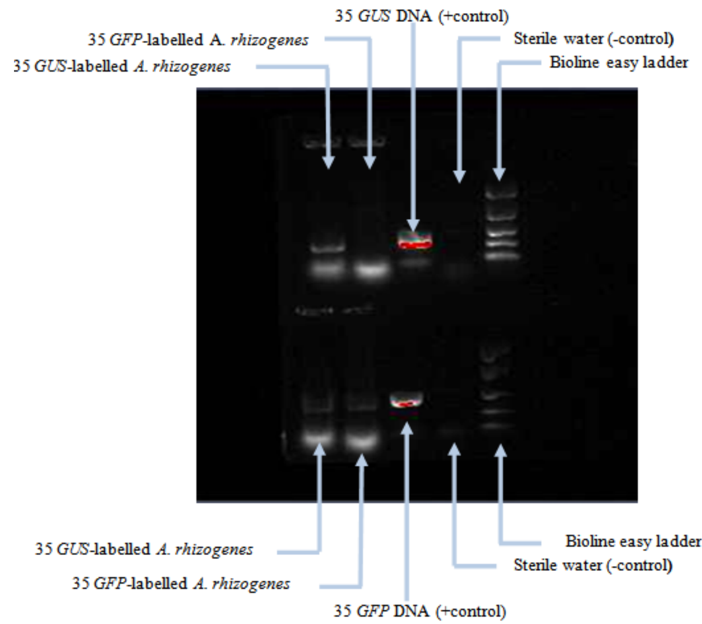

Fig. 1: PCR pattern of *A. rhizogenes* R1000 samples harbouring *GUS* and *GFP* genes.

Transgenic Tomato:

The data indicate the transformation of tomato seedlings by each *GUS*-labelled *A.rhizogenes* R1000, and *GFP*-labelled *A. rhizogenes* R1000. Hairy roots were induced on these seedlings after a week of inoculation (Fig. 2A). Staining of these transgenic tissue with X-Gluc exhibit a distinct blue colour visualised with the naked eye (Fig. 2B). Light microscope examination of blue-coloured transgenic hairy roots suggested that they expressing the blue stain constitutively confirming their transformation to express 35S *GUS* gene (Fig. 2C).

Microscopic examination of *GFP* stained tomato hairy roots were visualised under a UV where by *GFP* expressing cells will fluoresce. These transgenic hairy roots showed constitutive green fluorescence proved their successful transformation (Fig. 2D). These results demonstrated the incidence of transformation with 35S *GFP*.

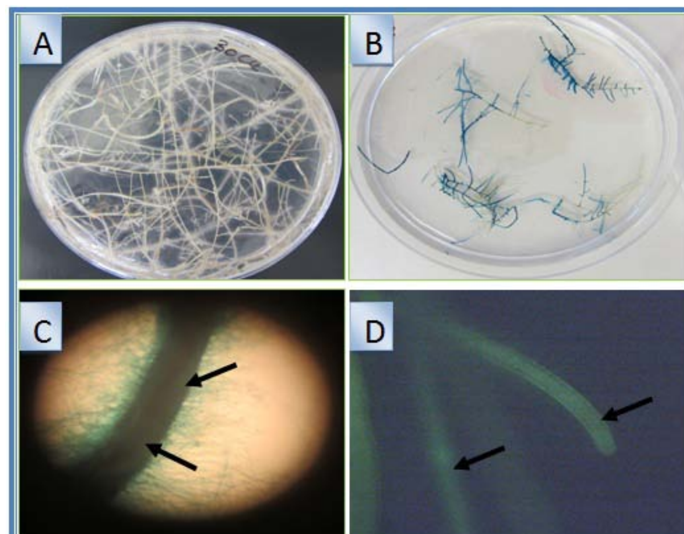

Fig. 2: Transgenic hairy roots induced on tomato (*Lycopersicum esculentum* L.) seedlings by each of *GUS* and *GFP*-labelled *A. rhizogenes* R1000.

A) Culture of hairy roots grown on agar-solidified1/2 MS supplemented with kanamycin 50 µg $ml^{-1}$and cefotaxime 100 µg ml^{-1} .

B) *GUS* stained hairy roots in (A).

C) *GUS* stained hairy roots (arrowed) under light microscope.

D) *GFP* stained hairy roots (arrowed) under UV microscope.

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Transgenic Potato:

The results exhibit the induction of white colour hairy roots on potato leaves after their co-cultivation with each of pBI121*GUS* and pBI121 *GFP* labelled *A. rhizogenes* R1000 (Fig. 3A). *GUS* staining of hairy roots with X-Gluc showed a distinct blue colour visualised with naked eye (Fig. 3B).

Light microscope examinations showed that most roots were stained and acquired blue colour, some showed no *GUS* expression (Fig. 3C). New root tips did not express the stain and showed up a brown colour.

GFP stained potato roots were visualised under UV microscope whereby cause green fluorescent protein expressing cells to fluoresce (Fig.3D). *GFP* transgenic potato roots did not show as much green fluorescence as tomato roots, and some regions of roots showed up red colour indicating the absence of transformation in those cells. However, microscopic observations indicated that most of potato roots had successfully transformed.

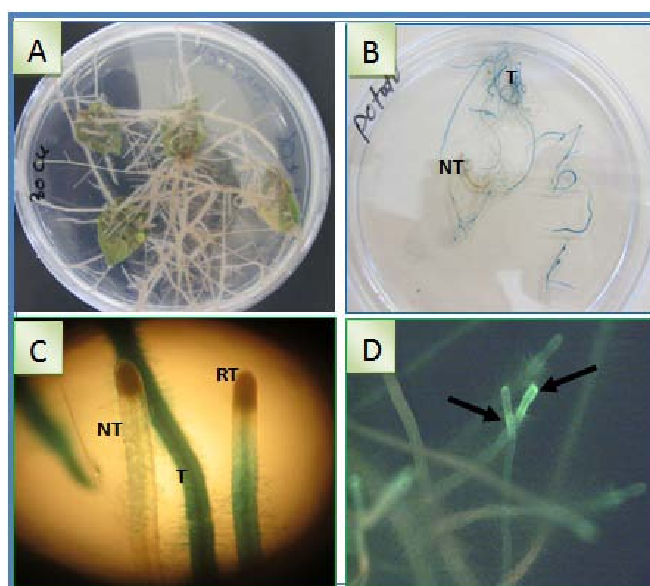

Fig. 3: Production of transgenic hairy roots on leaves of potato (*Solanum tuberosum* L.)by each of *GUS* and *GFP*-labelled *A. rhizogenes* R1000.

A) Culture of hairy roots from leaves grown on 1/2 MS supplemented with kanamycin 50 µg ml^{-1}and cefotaxime 100 µg ml^{-1} .

B) *GUS* stained hairy roots.

C) *GUS* stained hairy roots under light microscope.

D) *GFP* stained hairy roots (arrowed) under UV microscope.

RT: Root Tip.; NT: Non Transformed.; T: Transformed

Discussion:

Production of transgenic material with *GUS*, involved *cauliflower mosaic virus* (CaMV) 35S promoter fused with *GUS* reporter gene in pBI121vector. This vector express the ability to swap *GUS* for *GFP*, which was used as the second transforming agent (Mazarei *et al.,*2008). *Agrobacterium*-mediated transformation produced transformed plant tissues carrying various numbers of integrated copies of the foreign DNA. This is led to production of plant potentially expressing different amounts of gene product. This suggests that the method would have to be improved for use in high yields of transgenic crops of minimal variations between plants. It seems likely that the CaMV 35S was preferentially active in cells during the S phase of the cell cycle (Jefferson *et al.,* 1987). If this is true in future experiments, the pattern observed in *GUS* staining may be representative of cell division activity in these cells. However, many studies reported that CaMV 35S is strong and promoter has been shown to be reliable and consistent. Therefore, one can assume that the used protocol is robust, replicable and to be viable method of protecting crop plants from pathogenic damage through integration of a disease resistant gene.

Generation of transgenic hairy roots is a quick way to introduce new genetic elements into plant material and can be used with many plant species. This technology has been used in various applications of plant genetics, such as gene functions, promoter functions, root and lateral root development, defence and a biotic stress responses as well as other responses (Deng *et al.,* 2011; Al-Mallah and Ibrahim, 2012). Transformation of plants with each of *GUS* and *GFP* labelled *A. rhizogenes* did not interference with cell growth and activity. The proposed explanation that transformations will evade the risks of unintended genetic variations. However, it has been likely that constitutive gene expression within a transformants may affect growth and development causing

adverse outcomes in the host plant (Gago *et al.,* 2011). This can led to sterility, abnormal morphology, yield losses, altered grain composition and transgene silencing (Cai *et al.,* 2007). The finding that the use of tissue specific promoters may be superior to constitutive promoters, which would restrict gene expression in a tissue of interest at given developmental stages (Gago *et al.,* 2011). Interestingly, *Agrobacterium* mediated transformation could be enhanced by using SAAT (sonication assisted agrobacterium transformation) which had been reported as an easy and low cost method to enhance efficiency of transformation in low or non-susceptible plant species (Liu *et al.,* 2005). The protocol is quiet similar to *Agrobacterium*–mediated transformation with one added step.

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