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TRANSFORMATION OF ALFALFA (*MEDICAGO SATIVA* L.) PLANTS WITH GUS MARKER CONTAINING INTRON

SAIRA SHABNAM, YUSUF ZAFAR AND KAUSER A. MALIK

National Institute for Biotechnology & Genetic Engineering (NIBGE), P.O. Box 577, Faisalabad, Pakistan.

Abstract

Alfalfa (*Medicago sativa L.* cv Regen SY) plants were regenerated from roots induced by inoculation of leaf discs with *Agrobacterium rhizogenes* containing the vector p35S GUS INT. The DNA transformed to the plant genome with T-DNA based vector contained genes which encoded neomycin phosphotransferase (NPT II), that confers resistance to kanamycin and intron containing ß-D glucuronidase (GUS) gene. Histochemical staining for all the tested kanamycin resistant plants exhibited GUS activity and some differences in the phenotypic characters were also noted in the transformed and untransformed plants.

Introduction

Agrobacterium rhizogenes is a gram negative soil borne plant pathogen that causes proliferation of secondary roots at a wound site (Elliot, 1951). Rhizogenic ability is conferred to plant cells by a fragment of DNA (T-DNA) which is transferred from large root inducing (Ri) plasmid of the bacterium to plant genome where it is stably integrated and expressed (Taylor *et al.*, 1985). The mechanism of transfer of T-DNA from Ri plasmid to plant cell appears to be the same as that employed by the Ti plasmid (Nester *et al.*, 1984).

For selection of transformed cells, reporter gene such as ß-D glucuronidase (GUS) from *E.coli* is now widely being used. Being bacterial in origin, it often provides false positive results. Vancanneyt *et al.*, (1990) were the first to construct an intron-containing GUS marker gene. Intron splicing system exists in eukaryotes but not in prokaryotes. Therefore, intron-GUS gene is useful to monitor the *Agrobacterium* mediated transfer and expression of foreign genes in plant cells avoiding false positive expression mediated by bacterial cells. There are only a few reports where intron-containing marker genes have been used to produce transgenic plants like apple (Norelli & Aldwinckle, 1993), flax (Dong & Mchughen, 1993), spring wheat (Chibbar *et al.*, 1991), cowpea (Penza *et al.*, 1991), lentil (Warkentin & Mchughen, 1992) and tobacco (Ohta *et al.*, 1990). *Medicago sativa* L., is one of the wide spread forage legumes found in temperate region and a major protein source for livestock feeding. The present report describes transformation of *Medicago sativa* cv. Regen SY with *A. rhizogenes* strain K599 containing intron in the coding sequence. The establishment of such a system is an essential pre-requisite for our long term project of conferring salt tolerance to the salt sensitive leguminous crops. In this regard a 1.9 kb DNA fragment for Glycine-betaine uptake has already been identified and cloned for transformation studies.

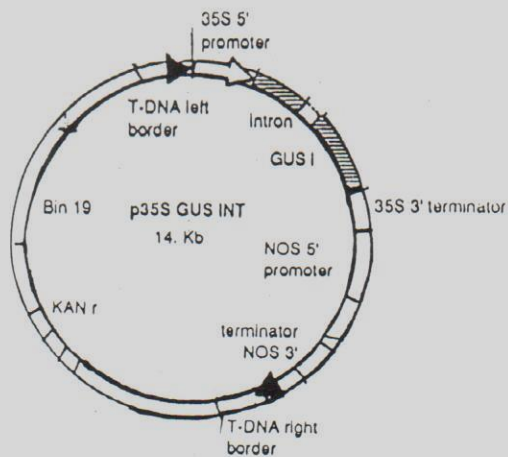

Fig.1. Restriction map of GUS vector for *A. rhizogenes* strain K599.

Materials and Methods

Plant material: *Medicago sativa* L. cv. Regen SY (courtesy of Prof. Ann Hirsch, UCLA. USA) was used in this study. Five to six months old green house grown fully expanded trifoliate leaveswere surface sterilized by rinsing for 15 second in soapy water. Leaves were immersed for 3 minutes in 20% commercial bleach (sodium hypochlorite) alongwith 2 drops (0.1 ml) of tween 80. Finally the leaves were repeatedly washed in sterile distilled water.

Bacterial strain and plasmid: *Agrobacterium rhizogenes* strain K599 (a gift from University of Tennessee, USA) was provided with the binary plasmid p35S GUS intron. It harbors two genes, the neomycin phospho-transferase gene (NPTII) which confers resistance to kanamycin regulated by NOS promoter, and intron containing-glucuronidase (GUS) gene controlled by CaMV 35S promoter (Fig 1).

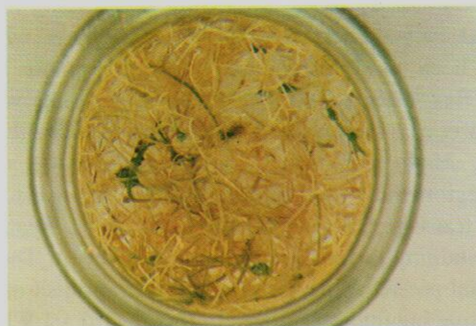

Fig.2. Hairy roots multiplication of transformed explant on B_5H_0 selection medium. After screening, the sample was regenerated via callus phase.

Agrobacterium mediated transformation: *A. rhizogenes* was grown in BMM medium (Bergersen, 1961). Over night grown bacterial culture (1 ml) was added to 10 ml of liquid B_5 basal medium (Gamborg *et al.*, 1968). The leaf discs were immersed in bacterial suspension for 15 minutes. Infected leaf discs were cocultured for 4 days on B_5 solid medium under 16/8 h photoperiod at $26 \pm 2°C$ with light intensity of 3000 Lux and then transferred to selection medium (B_5 medium with 250 mg/L carbenicillin and 25 mg/L kanamycin). Uninfected leaf discs were also placed on selection medium as a negative control.

Hairy root induction, callus culture and plant regeneration: Newly developed adventitious hairy roots were used as explants for callus induction on B_5H medium (B_5 with 1 mg/L 2,4-D, 0.1 mg/L kinetin and 25 mg/L kanamycin) and tips (3-5cm) of these actively growing roots were also placed on B_5 medium with kanamycin (25 mg/L) for further multiplication. One month old transformed calli were regenerated on hormone free B_5 medium containing kanamycin (25 mg/L).

Establishment of plants in soil: Transformed plantlets (6-8 cm in height) with well-developed root system were removed from the culture jars. Roots were washed with warm water, immersed in 0.2% Dithane M45 for few seconds and then potted into moist vermiculite in Magenta jars (Sigma) which were covered individually with other jars. Plants were placed under high humid conditions and acclimatized to less humid conditions by progressive slitting of the corners of the upper jar. Overlying jars were removed after 28 days and plants were transferred to soil.

Results and Discussion

Hairy root induction and GUS assay: Age of the plant material was found to be critical for infection. Fresh trifoliate light green, thin (2-3 weeks old) leaves responded better than dark green, dentate and thick leaves (6-8 weeks old). Similar response was earlier observed by Spano *et al.*, (1987). To determine the optimum level of kanamycin for selection of transgenic tissues, non-cocultivated leaf discs were cultured on B_5h_o medium with 25,50,75 and 100 mg/L kanamycin. Neither callus induction nor rooting was observed even at the lowest (25 mg/L) level of kanamycin. Preliminary experiments with co-cultivated tissues indicated that a minimal level of carbenicillin (250 mg/L) was required for reliable control of *Agrobacterium* during the selection phase. Adventitious hairy roots appeared 6 weeks after incubation on B_5 selection medium. Rooting response at wound site was 55%, quite higher than reported by Golds *et al.*, (1991) using stem sections and cotyledons. Abundant root proliferation was initially observed from small calli after two weeks of infection. Callusing response was less evident when older leaves were used. These observations are in agreement with the results reported by Spano *et al.*, (1987). Hairy roots when used as explant grew vigorously on hormone free solid B_5 medium supplemented with kanamycin (Fig 2). They showed macroscopic differences compared to control root culture, the former being larger in size and faster in growth. Histochemical GUS assay (Jefferson, 1987) of these transformed and control roots showed that 96% of transformed roots turned blue (Fig.3a) which suggest that the GUS gene has been introduced and correctly spliced. Staining was more dense in root tips (Fig.3a) which is highly meristematic region and thus provides good prospects for achieving stable integration (Wilmink *et al.*, 1992).

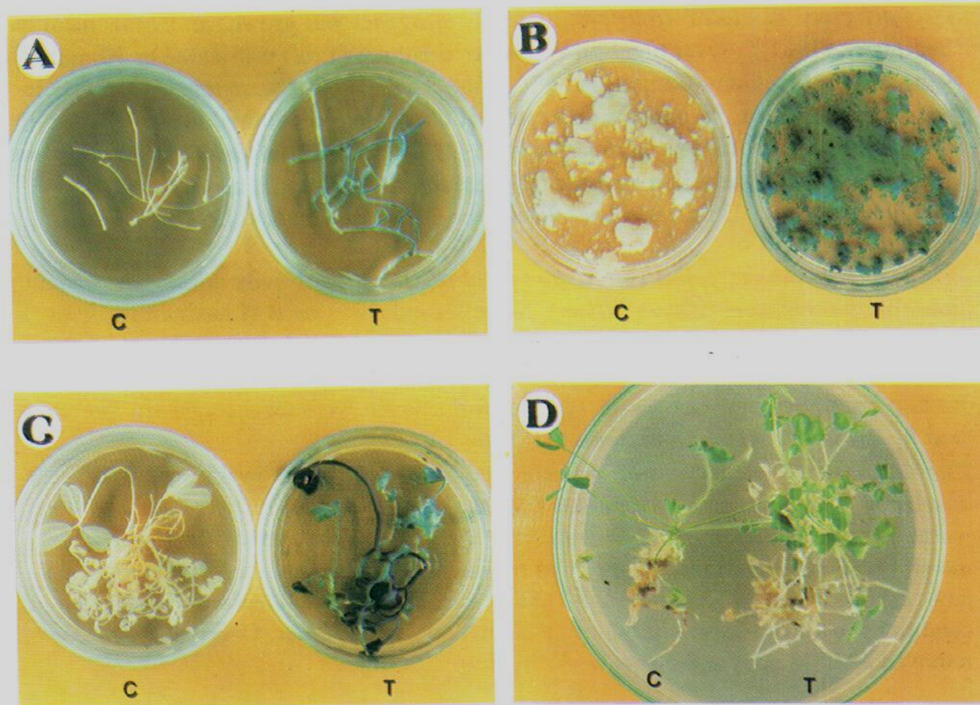

Fig.3. Regeneration from transformed hairy root (explant) on B_5H_0 medium with kanamycin (25 mg/l). C: untransformed control plant; T: transformed sample. GUS staining was performed at each stage
A) Cultured hairy roots grown on selection medium. B) Subsequent development of callus. C) Transgenic plants of *M. sativa.*, D) Completely transformed shoot just before transfer to green house. Profuse rooting and multiple branching are clearly visible. Control plants (C) at the same stage.

Callus induction from transformed roots and GUS assay: The addition of auxin, 2,4-D (1 mg/L) and kinetin (0.1 mg/L) in B_5 basal medium inhibited root multiplication while promoted vigorous callus formation. Friable and creamy embryogenic calli were developed after three weeks of incubation on B_5 H medium in light (3000 Lux). The callus developed from hairy roots grew faster compared to control (untransformed roots). The average number of embryos (11) on B_5 H medium per transformed callus was 30 % less than the number of embryos (16) obtained from untransformed root derived callus. GUS staining was dense in embryogenic transformed calli while no colour developed in non-transformed calli indicating lack of indigenous GUS activity (Fig.3b).

Regeneration of transformed calli: Embryos were developed when creamy calli (2-4 weeks old) were placed on hormone free B_5 medium with (25 mg/L) and without kanamycin. These somatic embryos were sub-cultured on the same medium for further development which produced small shoots and leaflets with prolific root system.

Number of plantlets developed on kanamycin free medium were double as compared to medium with kanamycin. The reason for the suppression of plantlet number on kanamycin supplemented medium was morphologically abnormal embryos which failed to produce plants. Histochemical *GUS* assays showed that 93% and 80% of the plants regenerated on medium with and without kanamycin, respectively exhibited GUS activity. From this data it is clear that it is better to regenerate transformants on selection medium which inhibit further development of untransformed tissues.

Increased number of shoots with a reduced internodal distance resulted in dwarf and compact plants (Fig.3d). Similar morphology of transformed plants of *Onobrychis viciifolia* was observed by Golds *et al.*, (1991).

Shoots appeared 6 weeks after the transfer of calli to the respective medium while roots developed later. Histochemical GUS assay showed blue coloration in leaves, stem and roots (Fig.3c). Hairy roots have earlier been shown to regenerate into whole plants (Spano *et al.*, 1987). The same has been confirmed in our experiments.

Spontaneous shoot production was not observed in root cultures as described by Spano *et al.*, (1987) and Golds *et al.*, (1991). Golds *et al.*, (1991) have used the same variety of *M. sativa* and got 25% transformation with stem and cotyledons tissues. In the present study leaf discs were used with a higher transformation efficiency (55%).

The present study resulted in obtaining transgenic plants which exhibited stable transformation after 3 months of their establishment in soil. The inheritance of the foreign gene is being published separately. Development of reliable transformation technology paved the way of transformation of *M. sativa* plants with other available genes with useful agronomic traits. One such gene under study is 1.9 kb DNA fragment, harboring glycine betaine uptake/transport mechanism for conferring salt tolerance to this sensitive plant.

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