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***Agrobacterium* Mediated Transformation of Anti-Stress Genes into Cauliflower (*Brassica oleracea* var. *botrytis* L.). 2. Transformation and Confirmation of Stress Tolerance**

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Abstract: Integration of APX and SOD anti-stress genes into cauliflower (*Brassica oleracea* var. *botrytis* L.) plants was achieved by using *Agrobacterium tumefaciens* – mediated transformation method. Cauliflower explants (hypocotyls and cotyledons) and *Agrobacterium tumefaciens* strains (APX, SA, TA) were used in this experiment. The procedure utilizes polymerase chain reaction (PCR) amplification of insert DNA directly after isolation of individual colonies without the necessity of separate procedures for DNA isolation and purification. Integration of the introduced stress gene (APX and SOD) in the plants was confirmed by using β -glucuronidase gene (GUS) and leaf disc assays as a gene fusion and diagnostic marker, respectively. The stable integration of the APX and SOD gene at 478 bp was detected by using polymerase chain reaction (PCR) of the putative transgenic plants. Analysis of APX and SOD gene expression under salt treatment showed that putative transgenic cauliflower survived the salinity stress comparing with the control plants.

Key words: Cauliflower, *Agrobacterium* salt stress, β -glucuronidase gene, DNA, APX and SOD gene.

INTRODUCTION

Direct gene transfer to plants as a method to genetically improve agricultural crops is now well established (Puddephat *et al.*, 1999, Robledo *et al.*, 2004 and Indrajit *et al.*, 2008) and combined with sexual hybridization and induced mutation provides a wide range of options available to modern plant breeders. The main advantage of gene transfer is the potential of adding a single new character to a proven genotype and thereby save time within the slow process of classical breeding. Although several techniques have been published, the *Agrobacterium*-mediated transformation system has proven to be the most commonly used protocol for the introduction of new genes into dicotyledonous plants (Kuginuki and Tsukazaki 2001; Cardoza and Stewart 2004 and Metwali *et al.*, 2005). Cauliflower (*Brassica oleracea* var. *botrytis* L.) is reported as one of the most responsive species for plant tissue culture but one of the most recalcitrant species for genetic transformation (Passelegue and Kelam, 1996; Puddephat *et al.*, 1999 and Ehab 2006). This is in contrast to Brassicas in general which tend to be very amenable to *Agrobacterium*- mediated transformation and several genes, including herbicide tolerance, have been successfully introduced and expressed in Brassica plants (Cardoz and Stewart 2003; Cao and Earle 2003; Cheng *et al.*, 2003 and Das *et al.*, 2006).

One of the most sought after target gene families for plant breeders is that associated with abiotic stress resistance (salt, drought, low and high temperature). Many of these stresses lead to the overproduction of reactive oxygen species (e.g. peroxide, superoxide), which cause extensive cellular damage and inhibition of photosynthesis by oxidative stress and is one of the major causes of plant damage as a result of environmental stress (Sunkar *et al.* 2003). Plants have evolved systems to combat oxidative stress producing enzymes which aid in reducing the active oxygen species (AOS) in order to protect the plant cell from damage (Yamaguchi-Shinozaki *et al.* 2002) and include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione-synthesizing enzymes (Hoque *et al.*, 2007). Manipulation of the expression of the enzymes involved in the AOS-scavenging systems by gene-transfer technology has provided a powerful tool for increasing the present understanding of the potential of the defense network against oxidative damage caused by environmental stresses (Shkryl *et al.*, 2010). This paper reports the protocol and findings of an *Agrobacterium*-mediated transformation of cauliflower with APX and SOD genes and the response of transformants to stress under *in-vitro* conditions.

MATERIALS AND METHODS

Bacterial Strains And Plasmid:

Agrobacterium tumefaciens strains APX, SOD and TA (U. S. Dep. of Agric.) were used in this experiment. The cytosolic APX from *Pisum sativum* and TA used PCGN1578 as a vector while SA strain using PBin plus ARS as a vector. PCGN1578 has 3 35S promoter elements and constructs have a dual CaMV 35S promoter as well as a TEV leader and CMV terminator.

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Culture of Bacteria and Bacterial Colony Template Preparation:

Agrobacterium tumefaciens strains were grown in solid or liquid Lauria Broth (LB) medium supplemented with different combinations of antibiotic selection 50 mg/l kanamycin + 10 mg/l gentamycin, 50 mg/l kanamycin + 12 mg/l tetracyclin and 10 mg/l gentamycin + 12 mg/l tetracycline respectively. All cultures were incubated at $28 \pm 1^\circ\text{C}$ in the dark for 24 to 48 hours. After colonies had grown sufficiently to be visible on the agar medium a sample from a single cell derived colony was obtained using a sterile wooden toothpick and re-inoculated onto fresh medium or used to determine the presence of the transgenes. In order to test for the presence of the transgene the sample was re-suspended in sterile double deionized water (20 μl) until no clumps were visible and then heated at 95°C for 20 minutes then centrifuged briefly to pellet debris. The majority of the sample was then placed directly into 50 μl of PCR mixture contained in a microfuge tube.

PCR Protocol:

Primers 5'-TTTCGGAACAATTAAGCACCAA-3', 5'-AAGAGGGCGGAATACAGAGTCAGT-3' were used for PCR reaction of APX strain and 5'-CAACATGGGAAAGGCTGTGGTTG-3', 5'-GTTGGAAAGCACAACCTTAACC-3' for SA and TA strains. The total amount of each PCR reaction mixture was 50 μl including: 25 μl PCR mixture, 0.2 μl of each primer, 5.6 μl template and 19 μl deionized autoclaved water. Before starting the first PCR cycle, the thermocycler (Gene Amp PCR system 9700 DNA thermal cycler) was heated to 95°C for 30 min, and then the sample was heated to 94°C for 2 min. This was followed by 30 cycles of 55°C for 1 min, 75°C for 1 min. In the final cycle, the reaction period at 75°C was extended to 10 min. DNA gels were prepared using 0.8 g agarose and 100 ml of 1x TBE buffer thoroughly mixed and boiled in

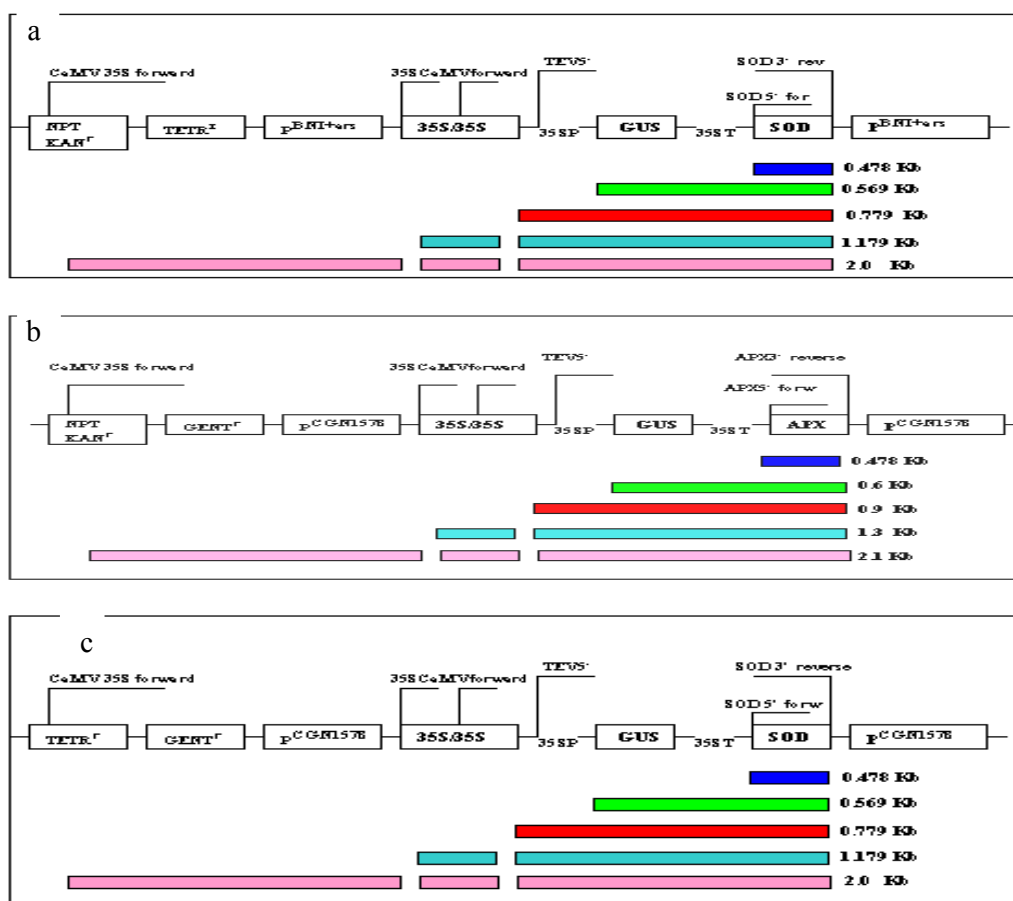


Fig. 1: Schematic presentation of the construct maps for 3 strains of *Agrobacterium tumefaciens* a) APX, b) SOD and c) TA (courtesy of USDA).

a microwave oven for 3-4 minutes, then 2.5 μl ethidium bromide was added and the mixture poured in a gel former (gel tank volume of 50 ml). After the PCR process, 5 μl from each microfuge tube was transferred to a new microfuge tube, 1 μl of loading buffer added and the mixture mixed. Then 6 μl of each sample was loaded into the sample wells of the gel together with DNA ladders. The gel was run at 100 Volts for approximately 45

minutes. After running the gel was viewed by illumination with UV light using a transilluminator/gel documentation system.

Transformation Protocol:

Plant Materials and Culture Conditions:

Seeds of cauliflower variety Medallion were surfaced sterilized as described in (Ehab, 2006). Cotyledon explants with 1-2 mm petioles and hypocotyl sections of 3 mm in length were cut from 8 day old germinated seedlings and pre-cultured for two days on pre-culture medium (S23 medium) and explants placed in a *A.tumefaciens* suspension for 30 m with gentle shaking to ensure the infection of the wounded area of the explants. *A. tumefaciens* suspension cultures were prepared from fresh colonies grown on Luria Broth plates and kept at 4 °C. Single colonies were transferred to 20 ml LB liquid medium and grown overnight at 28 °C to an O.D of 0.5 (at 600 nm). Explants were blotted dry then cultured with for 2 days on S23 medium at 22 °C. The explants were then washed for 30 second with 250 mg l⁻¹ cefotaxime, followed by three washes with sterilized distilled water and cultured for a further 3 days of growth on S23c medium (S23b medium with 250 mg l⁻¹ cefotaxime). Transformation was assessed using the Beta-Glucuronidase Assay. Explants were then transferred to S23f medium (S23 supplemented with 3 mg/l BAP, 0.2 mg/l NAA and different concentrations of antibiotic dependent on *Agrobacterium* strains viz: APX strain (25 mg l⁻¹ kanamycin + 6 mg l⁻¹ gentamycin), SA strain (15 mg l⁻¹ kanamycin + 6 mg l⁻¹ tetracycline) and TA strain (6 mg l⁻¹ gentamycin + 9 mg l⁻¹ tetracycline for strain TA). A Leaf disc assay was applied to detect the transformation during this step.

1- Beta-Glucuronidase Assay:

The detection of gene activity was performed using the histochemical and fluorogenic GUS assay according to Jefferson *et al.*, 1987 after 3 days of co-cultivation.

2- Leaf Disc Assay:

Leaves were removed from *in-vitro* green shoots growing on transformation selection medium. The leaves were cut into small sections and the leaf pieces were carefully pressed into petri dishes containing S23f medium (containing antibiotics). Leaf discs from wild type tissue culture regenerated plant were used as controls. The plates were sealed and incubated at 25 °C for 7-14 d. Putative transgenic plants was assessed using 0 or 1 scoring system based on the change of the leaf colour and callus formation

3- Plant DNA Analysis (PCR):

DNA from putatively transformed (positive GUS assay) and randomly selected non transformed plants were extracted using a hexadecyltrimethylammonium bromide (CTAB) method according to Maniatis *et al.*, 1982 and detection of the APX and SOD genes in the sample was conducted using PCR with the specific primers as described earlier. The PCR reaction was performed in a 25 µl microfuge tube using a preparation of Promega Master Mix and DNA was amplified using the following programme in the thermal cycler: 1 cycle of 94 °C for 4 min; 42 cycle of 94 °C for 30 sec; 36 °C for 30 sec; 72 °C for 2 min; and 1 cycle of 72 °C for 2 min. Samples were separated by agarose gel electrophoresis and visualised as described earlier.

Expression of Tolerant Genes in the Putative Transgenic Plant Under Salt Stress:

This experiment was conducted to determine the expression of the APX and SOD genes after integration in the plant genome. Stress was applied in this experiment to test the effect of salt stress in the putative transgenic plants compared with non-transformed plants as a control. Shoot tips of transformed and control plants *in-vitro* culture were aseptically subcultured onto solid medium supplemented with 300 mM NaCl and the jars were incubated in the growth chamber. After 4 weeks incubation on salt and control media the following parameters were measured

1. Shoot Fresh Weight:

After four weeks shoot fresh weight was measured (mg)

2. Sodium, Potassium, Calcium and Magnesium Content:

Dried shoots of each treatment (control and 300 mM NaCl) were freeze dried for three days to obtain dried samples. The concentration of Na and K were determined by flame emission spectrophotometry according to Jackson 1958, while Ca and Mg concentration were determined by flame absorption spectrophotometer using a GBC Model 902 AAS-AES according to Cheng and Bray 1951.

3. Estimation of Free Proline Content:

The first fully expanded leaf was then removed for determination of leaf proline content. Leaves were immediately frozen in a -80 °C freezer and then dried in a freeze drier to constant weight. HPLC was used to

determine proline levels using a Dionex AAA-Direct Amino Acid Analyser System according to *Bates et al., 1973*. *PeaknetTM* software gave output curves from which the concentration of proline was integrated.

Results:

Molecular Analysis of Agrobacterium Plasmid DNA Using PCR:

Bands on the agarose gel at 478 bp confirmed that all three of the *A. tumefaciens* strains used contained the appropriate T-DNA constructs (Figure 1).

Putative Confirmation of Transformation:

GUS activity was confirmed in hypocotyl and cotyledon explants used in the experiments (Figure 2). Samples taken from plants derived from both explant sources showed GUS activity in the leaves which indicated the presence of functional enzymes in the regenerated plantlets (Figure 3).

Leaf Disc Assay:

Untransformed leaf pieces from control plants did not swell or produce callus on this medium and turned white within 5-6 days. In contrast leaf pieces from putatively transformed plants expanded in size and remained green and produced callus after 14 days (Figure 4).

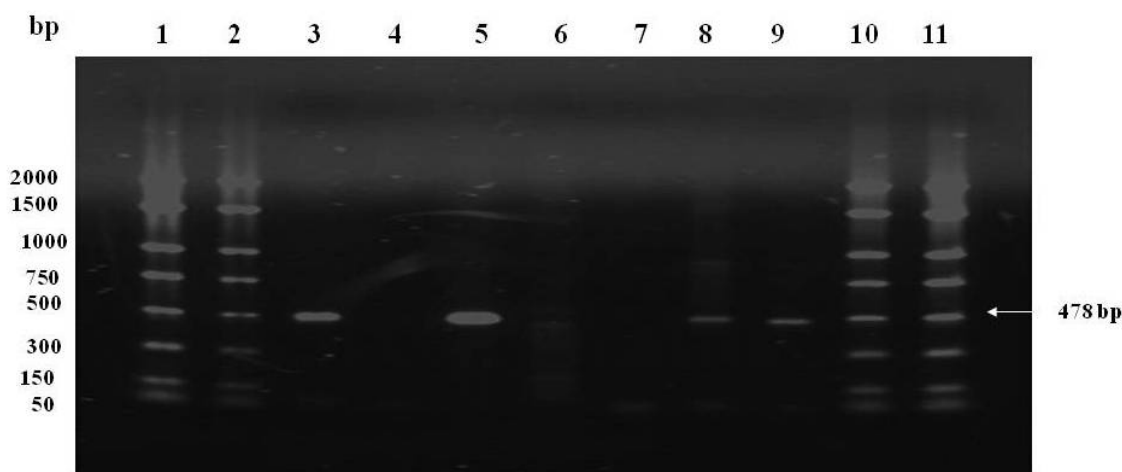


Fig. 1: PCR detection of insert DNA in recombinant plasmids harboured in *Agrobacterium*. A. PCR product from *Agrobacterium* colonies contained recombinant plasmid with APX or SOD insert. Lanes 1, 2, 10 and 11 are DNA markers; lanes 3 and 5 are from colonies harbouring PBIN + ARS (SOD insert) and PCGN1578 (SOD insert); lanes 8 and 9 are from colonies harbouring PCGN1578 (APX insert), consequently; lanes 4 and 7 are the result of PCR without bacterial colony; lane 6 is a colony harbouring vector without insert.

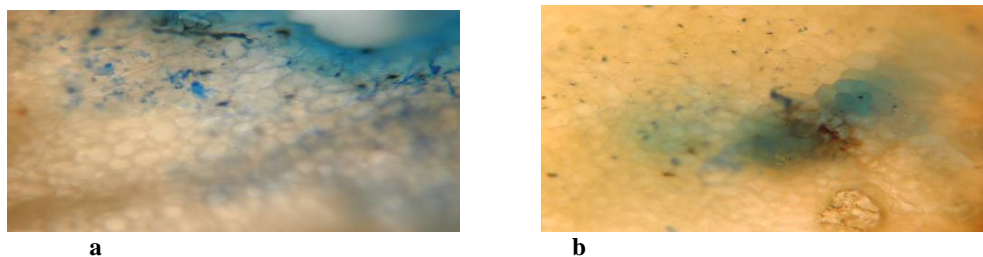


Fig. 2: Transformation evidence in the peripheral region of an (a) cotyledon and (b) hypocotyls after co-cultivated with *A. tumefaciens* for 3 days and assayed for GUS activity.

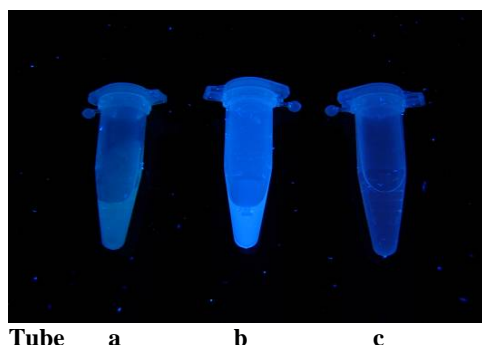


Fig. 3: GUS assay showing strong fluorescence a) from *A. tumefaciens* culture, b) from transformed leaf tissues of cauliflower Plantlets, c) Non transformed a was detected from leaves of non-transformed plantlets.

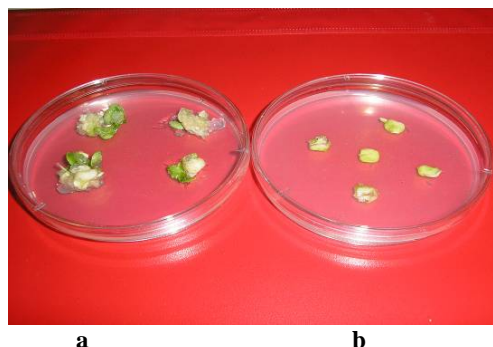


Fig. 4: Leaf disc assay. a) leaf pieces from transformed plants remained green and produce callus. b) Leaf pieces from untransformed control turned white without callus formation.

DNA Analysis (PCR):

DNA from leaf tissues of putatively transformed plants was analysed and a DNA extract from the APX and SA strains was used as a positive control and DNA from non-transformed cauliflower leaves was used as a negative control. PCR results confirmed that a fragment of about 478 bp appeared in transgenic plants and the positive controls whilst it was not present in non-transformed plants (Figure 5).

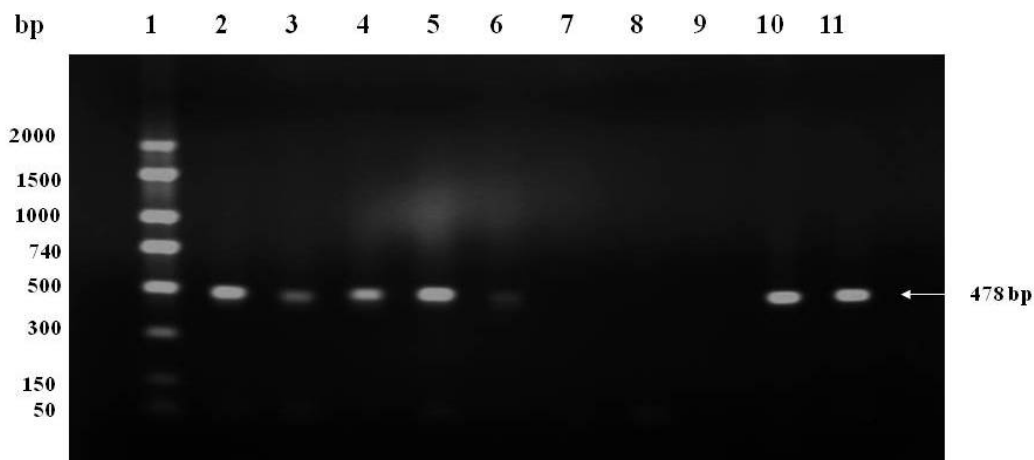


Fig. 5: PCR analysis of the presence of the APX and SOD gene in putative transgenic plant. DNA molecular size marker (lane 1), negative control (non-transformed cauliflower leaves) (lane 7 & 9), transformed plants carrying SOD gene (lane 2, 4 & 5), positive control (*Agrobacterium* DNA of SA or TA Strain) (lane 3 & 6), transformed plant carrying APX gene (lane 10), positive control (*Agrobacterium* DNA of APX strains) (lane 11) and water (lane 8).

Effect of Salt Tolerance on Shoot Fresh Weight of Transgenic Plants:

In control plants, shoot fresh weight was adversely affected by the salinity treatment, whilst in transgenic plants carrying APX and SOD this depression in growth was significantly less (Figure 6).

Effect of Salt Treatment on Proline Content of Transgenic Plants:

For the control plants the level of proline was low and stayed low when challenged with NaCl (Figure 7). The transgenic plants were also low in proline under non-stressing conditions but showed a significant rise in proline when challenged with NaCl of the order of 5 to 6 times control levels (Figure 2).

Effect of Salt Tolerance on Na, K, Ca and Mg Content of Transgenic Plants:

Under non-stressing conditions, transgenic plants were not significantly different to controls for Na, K, Ca and Mg concentrations of leaf material (Figure 3). When stressed with 300 mM NaCl transgenic plants showed

significant differences for Mg, K and Na whilst Ca was the same as for the controls. In all plants (controls and transgenic) NaCl stress reduced both Mg, Ca and K whilst levels of Na increased. The transgenic plants appeared to tolerate higher levels of Na and also maintained higher levels of Mg and K than the controls when stressed (Figure 8).

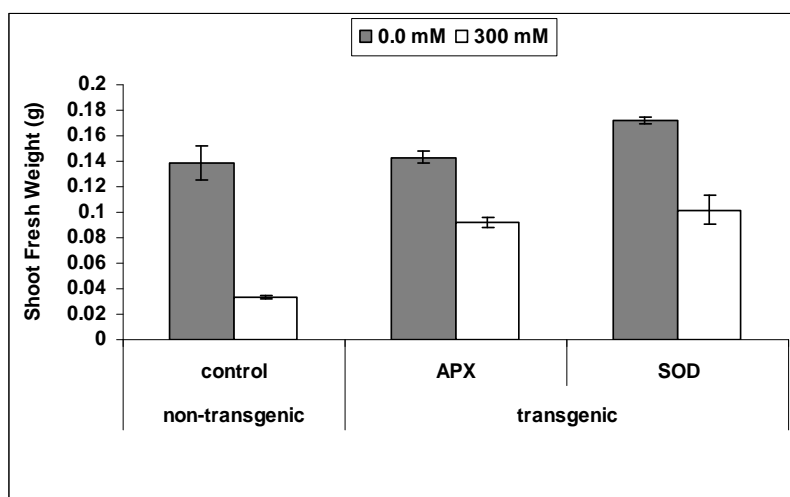


Fig. 6: Effect of salt treatment on fresh weight in transgenic and non-transgenic plants. (bar= \pm 1 SE, n=15).

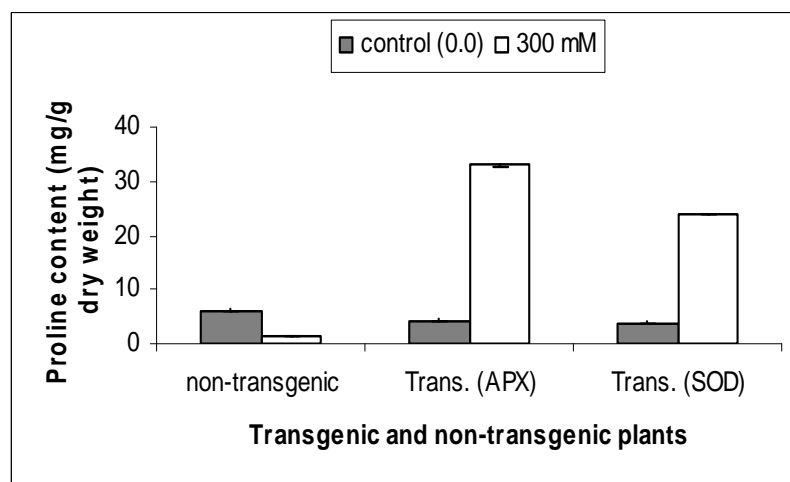
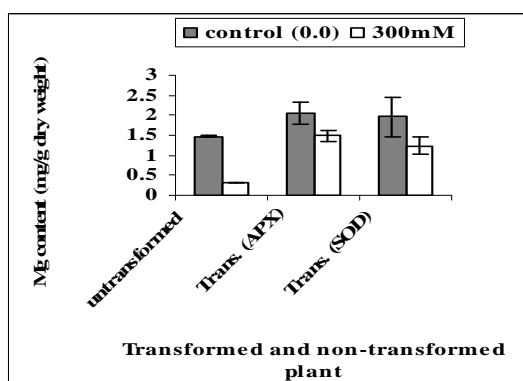
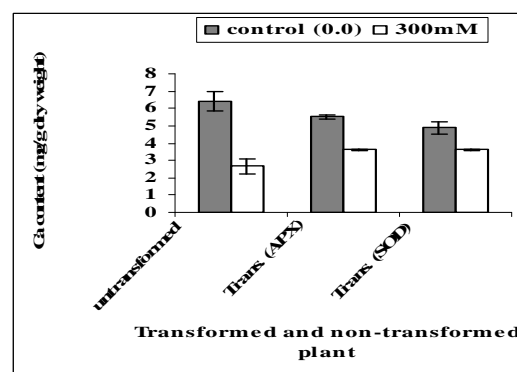


Fig. 7: Effect of NaCl on proline content in transgenic and non-transgenic plants. (bar = \pm 1 SE, n=3).



a)



b)

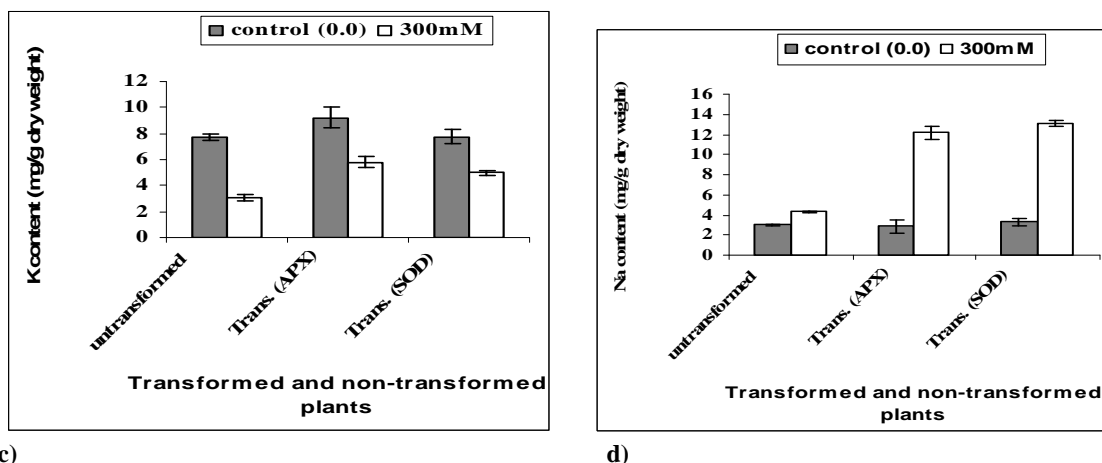


Fig. 8: Effect of salt treatment on a) Magnesium b) Calcium c) Potassium d) Sodium content in transgenic and non-transgenic plants. (Bar= \pm 1 SE, n=3).

Discussion:

In this study, the most reliable results were obtained if small quantities of cells and fresh bacterial colonies (one to two weeks after streaking) were analyzed using PCR method. Bacterial cells were deposited into the microfuge tube by scraping the toothpick along the tube wall facilitated efficient cell recovery. It may be possible to improve the sensitivity of the PCR by heating the *Agrobacterium*, increasing the annealing temperature or by reducing PCR cycle if non-specific amplification is a problem. This method can be used routinely to evaluate insert sequence constructed into vectors harboured in individual bacterial colonies, substantially reduces time and effort required to evaluate the authenticity of inserts in *Agrobacterium* binary vectors, Ping *et al.*, (1997). SOD and APX genes were detected after PCR amplification from appropriate colonies at 478 bp. This method can be used routinely to evaluate insert sequence constructed into vectors harboured in individual *Agrobacterium* colonies, substantially reduces time and effort required to evaluate the authenticity of inserts in *Agrobacterium* binary vectors (Ping *et al.*, 1997; Jones *et al.*, 1987). Although Prem and Nicole (1998) indicated that PCR is a fast and sensitive method, it is expensive and susceptible to cross-contamination. On the other hand, the leaf disc assay has an advantage as it is rapid, simple, requires minimal use of chemicals and plant tissues, and causes no permanent damage to the plant.

In order to identify transformed cells or plants that have been growing on a selective medium, it is necessary to have an easily assayable reporter gene. The most useful reporter genes encode an enzyme activity is the *E. Coli* β glucuronidase, Bronwyn *et al.*, 2002 and Parasharami *et al.*, 2006. In this study, *Agrobacterium* containing some of the GUS plasmids showed significant GUS activity. The substrate which used in this study was (X-Gluc), the substrate works very well but the qualities of the histochemical localization are effect by numerous variables such as tissue preparation and fixation. The results of transformation with Bt-gene construct indicated that the transient GUS assay approach is a easy reliable method of establishing optimal conditions of transformation, Chakrabarty *et al.*, (2002).

Also the result showed that the reduction in shoot fresh weight in transgenic plants was less than non-transgenic plant, it is clear that transgenic plant (carrying APX and SOD genes) have a high level of superoxidase dismutase and ascorbate peroxidase isozymes which are essential effectively to maintain the antioxidant system that protect plants from oxidative damage due to abiotic stresses, this explanation agrees with that of Chen *et al.*, 2005. This confirmed that APX and SOD successfully integrated into the plant and it is expressed under salinity treatment as superoxidase dismutase and ascorbate peroxidase isozymes and transgenic plant become more tolerant to salinity treatment than non-transgenic plants. Transformed plants with both the APX and the SOD transgenes demonstrated improved resistance to NaCl stress in-vitro compared to control plants. When analysed it was shown that the transformants could tolerate significantly higher tissue levels of Na than controls and maintain higher levels of Ca, Mg and K, this results agree with Cramer and Jones 1996; Eric 2005 and Ding *et al.*, 2010. Furthermore, transformants upregulated their proline content 5 – 6 fold in response to NaCl stress whilst control plants were incapable of achieving this. Similar results was obtained by Hasegawa *et al.*, 2000; Silveira *et al.*, 2003; Qasim *et al.*, 2003 and Hoque *et al.*, 2008, while Fuller *et al.*, 2006 concluded that elevated proline is not essential for improved resistance to abiotic stress in cauliflower, but where it does occur it does improve resistance.

Due to restrictions applying to the laboratory it was not possible to take the in-vitro transformants plants to in-vivo and therefore not possible to fully assess the stability of the inserted transgenes.

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