ISSN 0975-6299 Vol 3/Issue 1/Jan – Mar 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE

BIOTECHNOLOGY

TRANSFORMATION OF GROUNDNUT - *ARACHIS HYPOGEA L. VAR. GG20* WITH THE *COX* GENE-AN ATTEMPT TO DEVELOP SALINITY TOLERANCE

ASHUTOSH VADAWALE*, RITU MIHANI, ASHA MATHEW AND PUSHPA ROBIN

Department of Biochemistry, Faculty of Science,The Maharaja Sayajirao University of Baroda, Vadodara - 390 002. India.

ASHUTOSH VADAWALE
Department of Biochemistry, Faculty of Science,The Maharaja Sayajirao University of Baroda, Vadodara - 390 002. India.

***Corresponding author**

ABSTRACT

Salinity, is one of the most serious environmental factors limiting the productivity of crop plants and agricultural production world-wide. An attempt was made to develop salinity tolerance in Groundnut-*Arachis hypogea* l. var GG20 through *Agrobacterium* mediated transformation with the *cox* gene for synthesis of Glycine betaine. *Agrobacterium tumefaciens* LBA 4404 with binary vector pHS724 containing the cox gene was co-cultivated with different groundnut explants for infection and transformation. Various factors like explant types and co-cultivation period affecting transformation and concentration of kanamycine in selection medium were studied. The successful transformants were confirmed through phenotypic expression as well as through the expression of GUS gene *(uidA* gene) in the regenerated plants.

KEY WORDS

Salinity, Glycine betaine, Groundnut, Transformation

INTRODUCTION

Several environmental factors adversely affect plant growth and development and final yield performance of a crop. Drought, salinity, nutrient imbalances (including mineral toxicities and deficiencies) and extremes of temperature are among the major environmental constraints to crop productivity worldwide (Hamdia et al. 2010). Salinity, among all these is the most serious environmental factors limiting the productivity of crop plants and agricultural production world-wide (Ashraf, 1999). 40% of world's surface has salinity problems (Jadhav et al., 2010). However, despite advances in the increase of plant productivity and resistance to a number of pests and diseases, improvement in salt tolerance of crop plants remains elusive. In spite of considerable efforts through breeding programmes, progress to enhance salt tolerance has been very slow. Classical genetic studies have demonstrated that the ability of plants to tolerate salt stress is a quantitative trait involving the action of many genes. As a result, it has been difficult to obtain salt tolerance crop plants by traditional methods (Foolad and Lin, 1997).

Among various alternatives for development of saline tolerance in plants through genetic engineering, transfer of gene for synthesis of Osmolytes is most sought after by researchers worldwide. Osmolytes are certain organic metabolites of low molecular weight which are known collectively as compatible solutes (Bohnert et al., 1995). Metabolites that serve as compatible solutes differ among plant species and include polyhydroxylated sugar alcohols, amino acids and their derivatives, tertiary sulphonium compounds and quaternary ammonium compounds (Bohnert and Jensen, 1996). Glycine betaine (GB) among all compatible solutes is one of the most potent compatible solutes which protect the cell machinery in plants against various kinds of stresses like salinity, drought, cold etc. (Hayashi et al 1998). Among the plants which can naturally produce GB, three pathways exist for its synthesis (Hayashi et al 1998). It starts with choline and proceeds through a reaction that involves one or two enzymes for the oxidation of choline to GB. Different enzymes catalyze the reaction in different organisms. The overall reaction is

Choline ----------□ Betaine aldehyde ----------□ GB
CMO / COX /CDH *COX / BADH*

CMO : Choline monooxygenase, COX :Choline oxidase, CDH Choline dehydrogenase, BADH : Betaine aldehyde dehydrogenase.

COX pathway has the advantage of being a single enzyme which can catalyze both steps and also it does not require any cofactor for catalysis (Sakamoto et al, 2001).

Ground nut – *Arachis hypogea* L. is a major cash crop of Gujarat. It is rich source of protein, oil, and fodder and plays an important role in the agricultural economy of the state. An attempt was therefore made to develop salinity tolerance in Ground nut – *Arachis hypogea* L. var GG20.

MATERIALS AND METHODS

Plant Material, culture initiation and maintenance

The pods of peanut cultivar var. GG20 was obtained from the Agriculture University, Junagadh, Gujarat.

Mature dry seeds were washed under running tap water for 25 min. It was then subjected to, treatment with a solution of detergent Extran for 10 min, treatment of antifungal agent Bavistin for 10 min and finally washed thoroughly in sterile double distilled water and

then surface-sterilized in 0.1% aqueous mercuric chloride for 10 min, rinsed 3–4 times with sterile double distilled water. The seeds were left soaked 4-6 hours in sterile double distilled water.

Experiments were conducted on MS (Murashige and Skoog, 1962) medium fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol and 30 g/l sucrose.

The media were solidified with 0.65% agar (colloids). The pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were maintained at 28 ± 1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux.

Explant preparation

Three different kinds of explants i.e. embryo axis, young immature leaves and cotyledon explants were used for transformation .

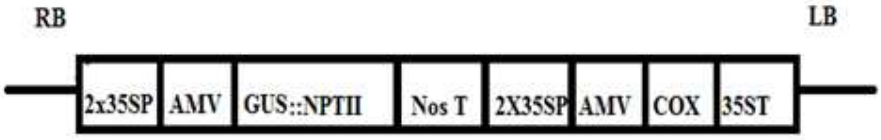
Mature dry seeds were imbibed in sterile double distilled water for 6-h after surface sterilization and kept for germination on autoclaved filter paper bridges in sterile double distilled water in test tubes. In case of embryo axis and cotyledon explants, the explants were collected from 3-day-old seedlings. While in case of young immature leaves the explants were taken from 7 day old seedlings.

In case of embryo axis, the seed coat and the radical were removed and the cotyledonary nodes were excised by cutting both epicotyls and hypocotyls approximately 2-3 mm above and below the nodal region and the embryo axis was bisected along the longitudinal plane. The meristematic region present in the nodal region was macerated by 6-8 diagonal shallow cuts by a sterile surgical blade. From each seed, two explants were obtained.

In case of cotyledon explants the embryos were surgically removed from the cotyledons and two explants were obtained.

Agrobacterium strains and plasmid vectors

The disarmed *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al. 1983) harboring the binary plasmid pHS724, kindly provided by Dr. Gopalan Selvaraj, Plant Biotech Inst., NRCC, Canada, was used as a vector system for transformation. The uidA or GUS (8 glucuronidase) gene driven by the CaMV 35S promoter and terminator sequences served as reporter gene. Plasmid pHS724 contains the gene of interest - *cox* for choline oxidase gene and kanamycin resistance gene for bacterial selection driven by a double 35S promoter, and terminated by 35S poly A signal and the *nptII* and *uidA* genes.



pHS724

The vector contains Right border and Left border sequences which can specifically recombine with the right and left border of the Ti plasmid. The *cox* gene is under the control of viral 35S promoter. GUS represents the eukaryotic reporter gene while in case of primary selection, the transformants can be screened by kanamycin resistance. The size is 15.6 Kb.

Co-cultivation and transformation

A single colony of Agrobacterium strain was incubated in 50 ml of YEP medium and grown overnight on a shaker at 200 rpm at 28^{o}C to an OD at 600 of 1.4–1.6. The bacterial culture was centrifuged at 5,000 rpm and the pellet was resuspended in 50 ml of liquid suspension medium containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968), 100 mg/l

myo-inositol, 30 g/l glucose and 100 ml acetosyringone. The suspension was kept at 28°C for 30 min and used for explant infection.

Different explants as explained above were infected with *A. tumefaciens* suspension culture for 20 min at room temperature under continuous shaking and transferred onto co-cultivation medium and incubated for 5 days at 21°C in the dark. Experiments were conducted on MS (Murashige and Skoog, 1962) medium fortified with B5 vitamins (Gamborg et al., 1968), 100 mg/l myo-inositol, 30 g/l sucrose, BAP (4.44-17.76 µM) and NAA (0.53-1.06 µM).

Selection of transformants, multiplication and growth conditions

After co-cultivation for 5 days, the explants were rinsed 5–6 times with sterile water separately aseptically. All explants were blotted on sterile paper to remove excess bacterial suspension and further placed on shoot induction medium which is the same as co-cultivation medium but additionally containing Cefotaxime (Alkem, India) (200 mg/l) to eliminate overgrowth of *A. tumefaciens*. All the cultures were maintained at 28±1°C under a continuous 16/8 h (light/dark) photoperiod with light supplied by cool white fluorescent lamps at an intensity of about 1600 lux.

The bud forming region of the culture were transferred to shoot multiplication and selection medium consisting of MS salts, B5 vitamins, 100 mg/l myo-inositol, 30 g/l sucrose, 6.5 g/l agar BAP (2.22-8.88µM), 200 mg/l cefotaxime and different concentrations of kanamycin (Macleods, India).

Two subcultures of 4 week intervals were done and multiple shoot buds were subcultured onto respective fresh medium. After two cycles of continuous kanamycin selection the shoots that attained > 2 cm length were excised and transferred onto MS medium supplemented with 4.30 µM α-naphthalene acetic acid to induce roots

GUS assay

The ß -glucuronidase (GUS) histochemical assay was used as a rapid way to detect the presence of the *uidA* gene (GUS) in the putative transformants as described by Jefferson *et al*. using leaf segments in regenerated shoots from explants. GUS assay was carried out by incubating the whole transformed plant tissues in GUS assay solution overnight at 37°C.

RESULTS AND DISCUSSION

Explants were co-cultured with Agrobacterium strain LBA4404 carrying the binary vector pHS724 and then transferred to respective shoot induction medium to select for transformed shoots. After 5 days of incubation the Agrobacterium infection was conspicuously seen in ring fashion on cotyledons (Fig-1).

Co-cultivated explants swelled and developed shoot buds with little callus after 3 weeks on selective shoot regeneration medium. Shoot clumps which survived this selection step were sub-cultured to shoot multiplication medium. The green shoot buds along with yellowish-white shoots were transferred to fresh medium with 60 mg/L kanamycin sulfate along with respective controls. The yellowish white coloration of shoot and leaves was observed which might be due to extra genetic load of the gene of interest along with *uid*A gene and *npt*II gene present and indicate the positively transformed shoots.

There was significant reduction in qualitative growth performance of control plants compared to transgenic plants on Kanamycin rich MS medium. The kanamycin resistant transformed elongated shoots were rooted in respective medium. Root induction was observed within 3 weeks of culture. The transformation frequency in terms of number of explants producing kanamycin resistant shoots was better using co-cultivated embryo axis explants (32%) than for cotyledons explants (21%). Different factors which

affected the transformation frequency were studied.

Effect of Explant type on transformation

Co-cultivated explants showed different responses upon inoculation with Agrobacterium. The efficiency of shoot bud formation from embryo axis or cotyledon explants was not affected by Agrobacterium infection when compared with uninfected explants. On respective shoot induction medium, 50% embryo axis explants showed multiple shoot bud induction while 30% explants showed shoot bud formation in case of cotyledon explants at the proximal end. In case of young leaves explants shoot bud formation could not be achieved even after four weeks of incubation on the medium. Subsequent transfer of leaves explants also did not yield any bud formation. This might be because the leaves were too tender and young when co-cultivated with agrobacterium.

Effect of co-cultivation periods on transformation

The transformation frequency of different explants was highly influenced by the co-cultivation period. Transformation frequencies of different explants co-cultivated on respective shoot regeneration medium for different periods are shown in Table 1. After two cycles of sub culture, the maximum transformation frequency occurred in embryo axis explants (32.20%) followed by cotyledon explants (21.10%) after 5 days of co-cultivation than after 1, 2, and 3 days of co-cultivation. Thus five days of co-cultivation resulted in the highest percentage of shoot regeneration on selection medium. These differences might be because of the fact that the concentration of *Agrobacterium* was higher after 5 days of cultivation which increases the possibility of gene transfer in groundnut. Similar observations were made in alfalfa by Chabaud *et al* (1988). Similar results were also observed in cowpea (Muthukumar *et al.* 1996) and in pea (DeKathen and Jacobsen 1990, Lulsdorf *eta.* 1991).

Table 1.

Effect of co-cultivation period on shoot bud regeneration on three explants of groundnut on shoot induction medium with 60 mg/l kanamycin sulfate

Co-cultivation period Without kanamycin (No of days)	Shoot bud regeneration frequency* (mean + SD)		
	Embryo axis	Cotyledon explants	Young leaves
1	5.50 ± 1.08	5.20 ± 1.03	3.10 ± 1.37
2	13.50 ± 2.37	10.20 ± 1.23	-
3	18.40 ± 1.07	12.60 ± 1.84	-
4	26.10 ± 3.07	18.60 ± 1.51	-
5	32.20 ± 2.94	21.10 ± 2.69	-

.* 30-35 explants were cultured per experiment and replicated three times.

However, extended co-cultivation period for more than 2 days for young leaf led to explant abortion caused by bacterial contamination. Thus young leaf explants could not yield any transformed shoots. These results do not agree with that of Eapen et al., (1994) who obtained an average of 6.7% of shoot regeneration on selection medium containing 50 mg/l kanamycin. Cheng et al. (1996) reported that the frequency of transformed fertile plants was 0.2% to 0.3% of the leaf explants inoculated. The difference in results could be explained by the fact that they used mature leaf segments while here we used tender young immature leaves which are fragile comparatively.

Effect of kanamycin concentration in selection:

To find out optimum concentration of Kanamycin and its effect on different explants, various concentration of kanamycin were added to respective medium as shown in table 2 and their effect were observed prior to Agrobacterium transformation. At 80 mg/L, kanamycin caused necrosis in all explants. Concentrations of 80 mg/L and 100

a b

Figure - 1.

a: Comparison of transformed plant with the control plant growing on MS medium without stress

b: Comparison of performance of transformed plants with control plants.

L: control plants in MS medium without NaCl, M: control plants in MS medium with 100 mM NaCl, R: Transformed plant in MS medium with 100 mM NaCl.

mg/L kanamycin completely inhibited shoot bud formation. Kanamycin concentration of 100 mg/L caused all explants to become necrotic within two weeks of incubation of culture and killed almost all the type of explants. At 60 mg/L, kanamycin level produced highest transformation efficiency (45% and 40 % for embryo axis and cotyledon explants respectively) with normal growth of explants without any deleterious effect on both embryo axis as well as cotyledon explants while in case of young leaves even 40-60 mg/ml kanamycin also caused chlorosis and death of the explants. Thus Transformation efficiency was decreased when there was an increase in the kanamycin sulfate level beyond 60 mg/l. Thus higher concentration of kanamycin caused necrosis of explants. Accordingly, in all the experiments with transformation kanamycin concentration of 60 mg/L was used in multiplication and selection medium for the initial selection of transformants. A concentration of 40 mg/l kanamycin sulfate was used for selecting transformants in subsequent subculture to prevent possible escapes. Similar findings were also reported by Kar et al., (1996) in chickpea.

Table 2
Transformation frequency of groundnut var. GG20 as influenced by explant type and level of kanamycin sulfate.

Explant type	Kanamycin sulfate (rng/l)	No. of explants Co-cultured	No. of explants producing shoots	Transformation frequency (%)
Embryo axis	40	35	19	54.29
	60	35	16	45.71
	80	35	14	40.00
	100	35	8	22.86
Cotyledon	40	35	18	51.43
	60	35	14	40.00
	80	35	13	37.14
	100	35	3	8.57
Young leaves	40	35	11	31.43
	60	35	6	17.14
	80	35	4	11.43
	100	35	0	0.00

Confirmation of transformants
Phenotypic Expression:

Shoot growth was determined to assess the stress tolerance of the transgenic plants. There was a significant reduction in the growth of transformed plants even under non-stress conditions compared to control plants but this might be due to kanamycin-containing agar medium to select for only transgenic plants. Transformed plants which survived the selection pressure for two subcultures were inoculated in MS medium containing 100mM NaCl along with the control plants and incubated under standard growth condition. All transgenics and their corresponding untransformed controls suffered a growth reduction under conditions of salinity (100mM NaCl). This correlates with the results obtained in case of *Brassica napus* and *Arabidopsis* by Huang et al., (2000). Growth reduction due to salinity was less severe in the transformed plants than in the untransformed control (Fig-1) in presence of 100 mM NaCl in medium.

After 12 days of incubation it was observed that the control plants which were healthy and were green in color started showing sign of leaf burning and curling from the leaf margins while the transformed plants did not show such sign of burning of leaf edges

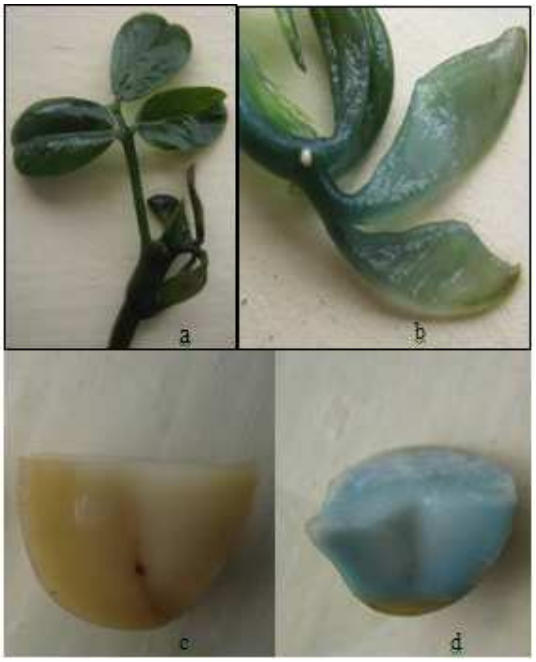


Figure -2.
X – gluc staining in the control and transformed tissues
a: control leaves with no blue staining, b: Transformed leaves with blue staining.c: control cotyledon with no blue staining, d: Transformed cotyledon with blue staining.

and remained healthy and continued to grow. This indicates the accumulation of glycine betain as a consequence of expression of *cox* gene in the transformed plants increased saline tolerance up to 100 mM level. Hayashi et al., 1998 also reported development of different level of saline tolerance in transgenic *Arabidopsis* transformed with the cholin oxidase gene from Arthrobacter globiformis (*cod* A).

GUS Expression

The whole regenerated plants along with cotyledons were subjected to in situ GUS assay. The expression of uidA gene was verified by histochemical staining of the leaf of the transgenic plants. The GUS positive regenerants showed the typical indigo blue colouration of X-Gluc treatment, while the control did not. Young leaves were more densely stained than other tissue of the plant and showed various GUS spots and GUS positive sectors on different leaf, shoot and cotyledon parts (Fig. 2). Both, leaves and the cotyledon parts of the transformants showed dense Gus positive sectors and not only GUS spots which indicate uniform transgene integration.

In conclusion, transgenic groundnut plants expressing cox gene were successfully generated through *Agrobacterium tumefaciens* mediated transformation conferring salinity tolerance.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Gopalan Selvraj, Plant Biotechnology institute, NRCC, Canada for providing the cDNA clone of *cox* (choline oxidase) gene and to the GSFC Science Foundation for funding the project.

REFERENCES

1. Ashraf M., Breeding for salinity tolerance proteins in plants. Crit.Rev Plant Sci, 13: 17 – 42, (1999).
2. Bohnert H, Nelson D & Jensen R., Adaptations to environmental stresses. Plant Cell 7: 1099 – 1111, (1995)
3. Chabaud, M., Passiatore, J.E., Cannon, F. and Buchanan Wollaston, V., Parameters affecting the frequency of kanamycin resistant alfalfa obtained by *Agrobacterium tumefaciens* mediated transformation. Plant Cell Rep. 7:512 516, 1988
4. Cheng, M., His, DCH, Phillips, GC, In vitro regeneration of valencia-type peanut (Arachis hypogaea L.) from cultured petiolules, epicotyl sections and other seedling explants. Peanut Sci. 19, 82-87, (1992)
5. DeKathen, A. and Jacobsen, HJ, *Agrobacteriumtumefaciens* mediated transformation of *Pisum sativam* L. using binary vector and cointegrated vectors. Plant Cell Rep. 9: 276-279, (1990)
6. Eapen S, George L, *Agrobacterium tumefaciens*-mediated gene transfer in peanut (*Arachis hypogaea* L.). *Plant Cell Rep*. 13: 582–586, (1994)
7. Foolad MR, Lin GY, Genetic potential for salt tolerance during germination in Lycopersicon species. HortScience 32: 296- 300, (1997)
8. Gamborg OL, Miller RA, Ojima K, Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res*. 50(1): 151–158, (1968)
9. Hamdia M A and Shaddad M AK, Salt tolerance of crop plants, J. of stress physio. & biochem. 6(3)- *64-90*, (2010)
10. Hayashi H, Alia, Sakamoto A, Nonaka H, Chen THH, Murata N, Enhanced germination under high-salt conditions of seeds of transgenic Arabidopsis with a bacterial gene (codA) for choline oxidase. J Plant Res 111: 357–362, (1998)
11. Hoekema A, Hirsch P, Hooykass P, A binary plant vector strategy based on separation of the vir and T-DNA regions of the *Agrobacterium tumefaciens* Ti plasmid, Nature 303: 179-180, (1983)
12. Huang j, Rozina H, Luc Adam, Kevin LR, Joe KH, Wilf AK, Gopalan S., Genetic engineering of Glycine betaine production toward enhancing stress tolerance in plants. Metabolic limitations.122: 747-756, (2000)
13. Jadhav GG, Salunkhe DS, Nerkar DP, Bhadekar RK, Isolation and characterization of salt-tolerant nitrogen-fixing microorganisms from food. J. Eur. Asia. Bio. Sci., 4: 33-40, (2010)
14. Kar, S., Johnson, T.M., Nayak, P. and Sen, S.K, Efficient transgenic plant regeneration through *Agrobacterium* mediated transformation of chickpea *(Cicer arietinum* L.). Plant Cell Rep. 16: 32-37, (1996)
15. Lulsdorf, M.M., Rempel, H., Jackson, J.A., Baliski, D.S. and Hobbs, SLA, Optimizing the production of transformed pea *(Pisum sativum* L.) callus using disar*med Agrobacterium tumefaciens* strains. Plant Cell Rep. 9: 479-483, (1991)
16. Murashige T, Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol. 15: 473-497, (1962)
17. Muthukumar, B., Mariamma, M., Veluthambi, K. and Gnanam, A, Genetic transformation of cotyledon explants of cowpea *(Vigna unguiculata* L. Walp) using *Agrobacterium tumefaciens.* Plant Cell Rep. 15: 980-985, (1996)
18. Sakamoto A, Alia, Murata N, The Use of Bacterial Choline Oxidase, a Glycinebetaine- Synthesizing Enzyme, to Create Stress-Resistant Transgenic Plants1 *Plant Physio*, , 125: 180–188, (2001)