Environment & Ecology 35 (4) : 2609—2615, October—December 2017
Website: environmentandecology.com ISSN 0970-0420

Transgenic Tobacco (*Nicotiana tabacum* cv FCV) Plants Over Expressing A Bacterial *cod A* Gene Show Reduced Oxidative Damage under High Light Stress

Poornima R., Lokesha A.N., Noor Nawaz A. S., Shankar A.G.

Received 24 November 2016 ; Accepted 26 December 2016 ; Published online 13 January 2017

Abstract Transgenic tobacco (*Nicotiana tabacum* cv FCV) special plants expressing a choline oxidase (*cod A*) gene from *Arthrobacter globiformis* were obtained through *Agrobacterium tumefaciens* mediated transformation. The *cod A* gene expression was targeted to chloroplast. Stress tolerance was evaluated in transgenic plants by exposing the leaf discs to methyl viologen (MV) induced oxidative stress and measured by TTC reduction test. At 2 µM of methyl viologen the percent reduction in cell viability was as high as 25.5% in wild type, whereas it was only 11.8% and 6.3% in line 1 and line 2 of transgenic plants respectively. At 5 µM methyl viologen, percent reduction in cell viability for wild type was 30.9% and for *cod A* transgenics is 13.9 and 11.1% in line 1 and line 2 respectively. The chlorophyll retained in transgenic plants was higher especially in those expressing *cod A* gene and similar trend was also seen regarding the accumulation of pheophytin levels especially at 5 µM MV. In wild type plants, the pheophytin accumulated was high and the least was seen in transgenic plants expressing *cod A*. Our results indicate that the introduction of *cod A* gene from *Arthrobacter globiformis* into tobacco could be a potential strategy for improving the plant tolerance to oxidative stress and by protecting the chloroplast from reactive oxygen species.

Keywords Choline oxidase, Oxidative Stress, Glycine betaine, *Arthrobacter globiformis*, *Nicotiana tabacum*.

Poornima R.*
PhD Scholar, Department of Crop Physiology, UAS, Dharwad 05, India

Lokesha A. N.
PhD Scholar, Department of Crop Physiology, UAS, GKVK, Bangalore 65, India

Noor Nawaz A. S.
PhD Scholar, Department of Agricultural Microbiology, UAS, Dharwad 05, India

Shankar A. G.
Professor, Department of Crop Physiology, UAS, GKVK, Bangalore 65, India
e-mail: poornimarjoshi@gmail.com
*Correspondence

Introduction

Glycine betaine (GB), a quaternary ammonium compound is amongst the most effective compatible solutes which accumulate in a wide variety of organism viz., bacteria, cyanobacteria, algae, animals and higher plants (Chenopodiaceae and Poaceae) as an adaptive means to tolerate abiotic stresses. It can stabilize the structure of proteins and maintain the integrity of cell membrane, thus enhancing tolerance against salinity, higher light, high temperature and cold stress [1, 2]. Unlike in higher plants where two enzymes are involved in oxidation of choline to glycine betaine,

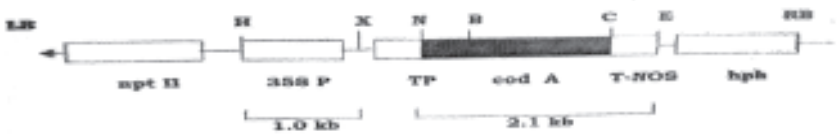

Fig. 1. Structure of T-DNA region of the binary vector plasmid *pGAH/cod A* that carry the genes for choline oxidase. RB : right border ; *Kmr* : kanamycin resistance gene ; 35S pro : the 35S promoter of cauliflower mosaic virus ; *rbcStr* : the sequence that encodes transit peptide of subunit of Rubisco from tobacco ; *cod A* : the gene encoding choline oxidase (COD) ; NOS ter : NOS terminator ; P_1 and P_2 are upstream and downstream primers, respectively ; *hph* : hygromycinphosphotransferase.

the biosynthesis of betaine is catalyzed by a single enzyme choline oxidase (COD) in certain microorganisms such as the soil bacterium *Arthrobacter globiformis* [3].

$$\text{Choline} \xrightarrow[(\textit{Choline Oxidase})]{\textit{Cod A}} \text{Glycine betaine}$$

To achieve this goal the *Agrobacterium tumefaciens* mediated transformation procedure was adopted to transfer *cod A* gene into the model plant tobacco. In this study, we evaluated the transgenic plants for their tolerance to high light stress by examining their membrane stability and chlorophyll content. We report here the enhanced tolerance observed in these transgenic plants against wild type plants under stress.

Materials and Methods

The binary vector designed *pGAH/cod A* in *Agrobacterium tumefaciens* (EHA 101) was used for transformation of wild type tobacco (*Nicotiana tabacum* cv FCV) special according to leaf disc method.

pGAH/cod A construct

The gene construct was obtained from Dr. Pardha Saradhi, Plant Physiology and Biotechnology Laboratory, Department of Biosciences, Jamia Millia Islamia, New Delhi. The *cod A* gene from *Arthrobacter globiformis* was ligated with the transit peptide region of the small subunit of *rbcs* from tobacco, the 35S constitutive promoter from cauliflower mosaic

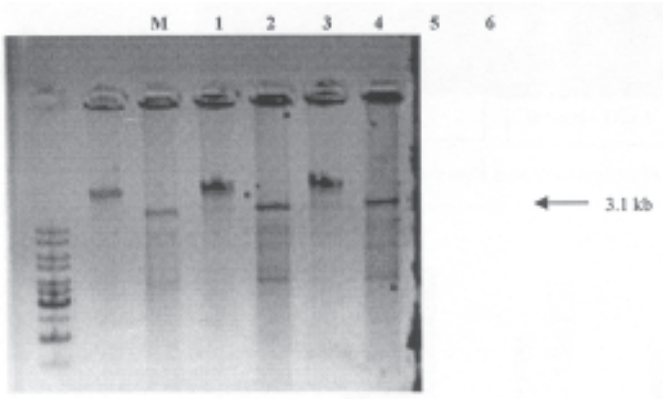

Fig. 2. Agarose gel electrophoresis depicting the release of *pGAH/cod A* insert by restriction digestion analysis. M : 1 kb gene ruler ; Lane 1, 3 and 5 : Undigested DNA of *pGAH/cod A* construct ; Lane 2, 4 and 6 : Digested DNA of *pGAH/ cod A* construct.

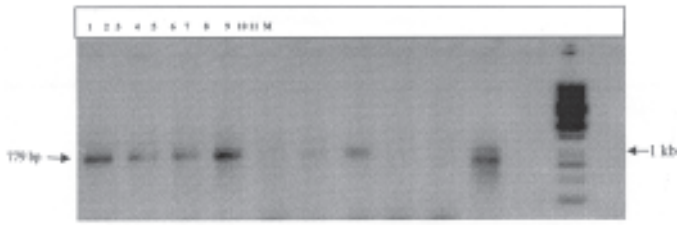

Fig. 3. PCR analysis for genomic DNA from primary transformants carrying *cod A* gene using *cod A* primers. M : 1 kb gene ruler ; Lane 1,2, 3, 4, 6 and 7 : DNA from the putative *cod A* overexpressing plants ; Lane 5, 8 and 9 : DNA from the putative transformants selected on Hygromycin media ; Lane 11 : DNA from wild type plants ; Lane 10 : Plasmid DNA fron *pGAH/cod A* construct.

virus and the nopaline synthase terminator. This construct was inserted into the binary vector *pGAH*, which contained genes for kanamycin and hygromycin resistance (Fig. 1).

Transformation of FCV special tobacco leaf discs with EHA 101 (*pGAH/cod A*)

The leaf discs were inoculated in plates with MS (Murashige and Skoog) morphogenesis media for 48 h. *Agrobacterium* culture was grown overnight and is monitored by measuring the OD at 595 nm. Leaf discs were co-cultivated with the culture and leaf discs were transferred to the respective plates and incubated for 48 h. The discs were removed after the incubation and washed thoroughly in sterile water and blotted on filter paper and transferred to fresh plates with selective media (MS media : 2 mg/L BA + 0.2 mg/L NAA + 500 mg/L cefotaxime and 50 mg/L kanamycin + 30 µg/ml of Hygromycin). After 20 days the regenerated plantlets were later sub cultured and subsequently rooted on MS rooting media containing 0.5 ppm indole butyric acid (IBA). Continuous sub culturing of the shoot lets was carried out and shoot lets showing varied morphology were selected and then tried for rooting. *In vitro* shoot lets with roots were transferred to the pots containing soilrite and were placed in polythene bags and small holes were made to avoid build up of humidity. After two weeks the plants were shifted to the mist chamber

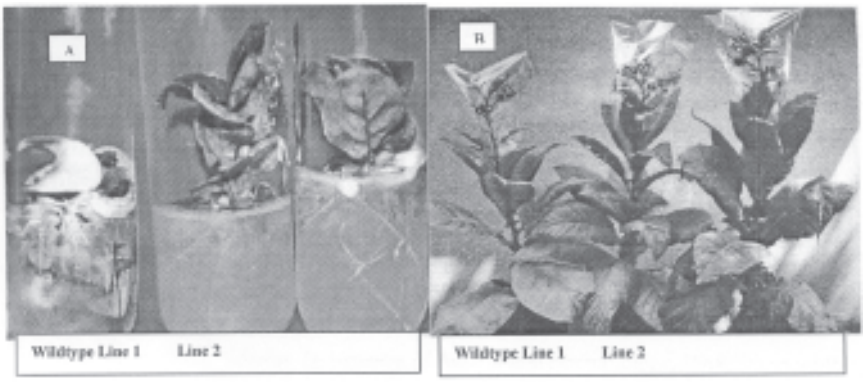

Fig. 4. Wild type and putative transformants carrying *cod A* gene in laboratory (A) and green house (B) conditions.

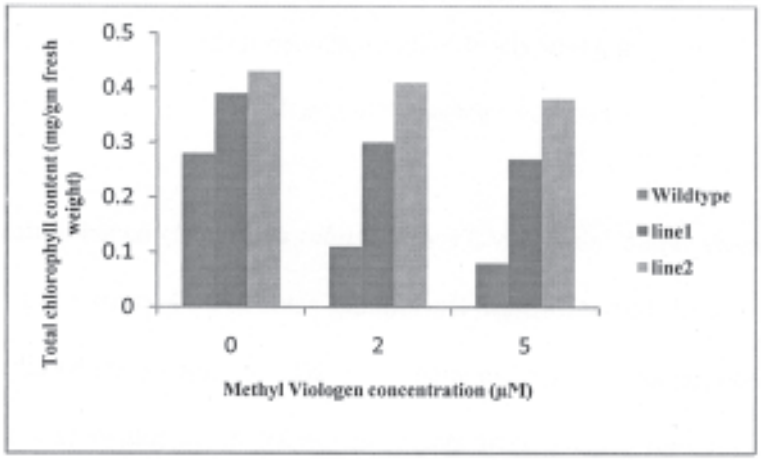

Fig. 5. Total chlorophyll content in the wildtype and two *cod A* transgenic plants exposed to methyl viologen induced oxidative stress.

with 30°C temperature and 85% relative humidity. After two or three weeks the plants were transferred to battery containers in the green house and allowed to establish.

PCR analysis of *cod A* putative transgenic plants

DNA from transformed and untransformed tobacco plants was extracted by C-TAB Method [4]. PCR amplification was carried out in thermocycler (PTC-100™ from MJ research, INC.) using gene specific primers (P_1-5' TCGACAACATCGAGAACCTG 3' ; P_2-5' TGGAGAGCACGACTTCATTG 3') with the following thermocycle profile 94°C for 4 min, 94°C for 1 min (Denaturation), 55.7°C for 1 min Annealing), 72°C for 2 min (Extension), GO TO step 2 for 35 more cycles, 72°C for 8 min, 4°C forever. The PCR product (799 bp) of *cod A* transgenic plants, wild type and the plasmid (*pGAH/cod A*) was analyzed on the 0.8% agarose gel.

Table 1. Effect of methyl viologen (2 µM and 5 µM) on cell viability (TTC reduction) of *cod A* transgenic plants and wilt type tobacco plants.

Plant type	Methyl viologen (µM) 0	2	5
Wild type	0	25.5	30.9
Line 1	0	11.8	13.9
Line 2	0	6.3	11.1

Assessment of stress responses in transgenic plants expressing *cod A*

In vitro developed *cod A* transgenic plants and wild type plants were transferred to pots and maintained under green house conditions.

Cell viability

Methyl viologen (MV) is an effective generator of ROS. The cell viability of methyl viologen treated leaf discs was assessed using property of TTC (Triphenyl tetrazolium chloride) reduction by respiratory enzymes, which converts colorless TTC to red formazon. The red color was measured at 485 nm. The red color intensity is an index of chlorophyll activity and cell viability. Leaf discs were incubated in methyl viologen ((2µM and 5µM) for 5 h and were exposed to higher light (1500 µ Ein / m^2 / sec) for 3 h and then after recovery of 8 h the TTC reduction was recorded by taking absorbance at 485 nm in spectrophotometer.

Chlorophyll estimation

Take the known weight of leaf discs of both transgenic

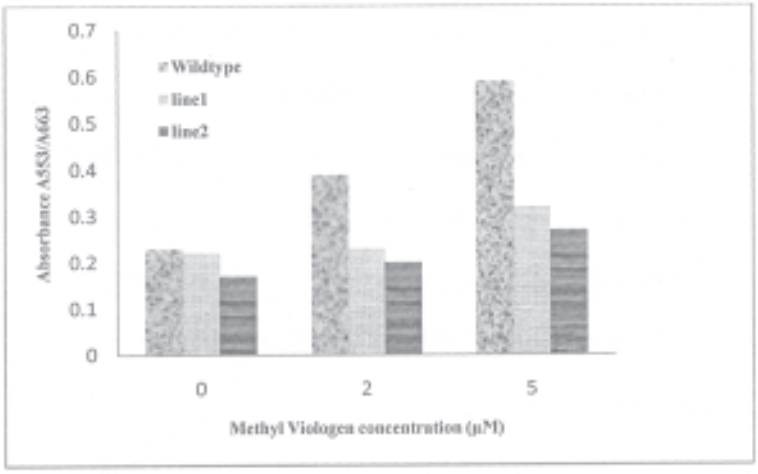

Fig. 6. Pheophytin to chlorophyll ratio (A_{553} / A_{663}) estimated in leaf disc exposed to methyl viologen induced oxidative stress in two transgenic and wildtype plants.

and wild type plant, incubate them on Methyl Viologen (2 µM and 5 µM) for 5 h and expose them to high light (1500 µEin / m^2 / sec) for 3 h. Take 10 ml of DMSO : Acetone (1 : 1) into the test tubes and incubate these stressed leaf discs overnight at room temperature, all the pigments will be extracted into the solution. Decant the colored solution into a measuring cylinder and make up the volume to 25 ml with DMSO : Acetone mixture. Record the absorbance at 645, 652 and 663 nm. Estimate chlorophyll content by using standard formula. In addition, the pheophytin content (degradative product of chlorophyll) was determined based on the absorbance at 553. The ratio A_{553} / A_{663} is a reflection of increase in pheophytin content with concomitant decrease in chlorophyll content. The higher ratio is an indicator of chlorophyll damage.

Results and Discussion

Vector confirmation

Confirmation of the presence of gene of interest (*cod A*) was done in the binary vector *pGAH* / *cod A* in *Agrobacterium* EHA 101. The restriction digestion analysis was carried out using the restriction endonucleases *EcoR I* and *Hind III*. The digested fragment released was 3.1 kb. The 0.8% agarose gel showed the release of the insert in lane 2, 4 and 6 and lane 1, 3 and 5 represents the undigested plasmid DNA (Fig. 2). The putative transformants were selected on the tobacco morphogenetic media containing 100µg / mL kanamycin and hygromycin 30 µg/mL and were further subcultured on morphogenic MS media and rooted on rooting MS media as mentioned earlier (Plate 1A). The rooted plants were initially transferred to 1 : 1 mixtures of vermiculite and sand and were prehardened under normal room conditions. The prehardened plants were transferred to pots for further observations and seeds were collected by bagging to avoid cross pollination (Plate 1B).

PCR analysis of *cod A* putative transgenic plants

The genomic DNA isolated from wild type and *cod A* transgenic tobacco plants were subjected to PCR analysis using *cod A* specific primers. The PCR product was analyzed on 0.8% agarose gel. The analysis showed the presence of 799 bp *cod A* fragment in the plasmid construct and in the transgenic tobacco lines 1, 2, 3, 4, 6 and 7 but not in the control tobacco lines (Fig. 3). These PCR positive plants (line 1 and line 2) were used for further physiological and biological analysis and they were allowed to attain maturity. The seeds were collected from these plants and stored.

Assessment of stress responses in transgenics expressing *cod A* gene

The relative tolerance to oxidative stress in transgenics expressing either *cod A* gene was examined by assessing cell viability (TTC test) and chlorophyll degradation.

Cell viability

Two lines of *cod A* gene line 1 and line 2 are selected for the stress studies. At 2 μM of methyl viologen (MV) the per cent reduction in cell viability was as high as 25.5% in wild type, whereas it was only 11.8% and 6.3% in line 1 and line 2 transgenic respectively (Fig. 4). At 5μM methyl viologen, per cent reduction in cell viability for wild type was 30.9% and for *cod A* transgenic is 13.9 and 11.1% in line 1 and line 2 respectively (Table 1) [5, 6] and also demonstrated that GB-accumulating transgenic tomato plants were more tolerant to MV-induced oxidative stress than WT plants.

Chlorophyll estimation

The chlorophyll retained in transgenic plants was higher especially in those expressing *cod A* gene (Fig. 5). Similar trend was also seen regarding the accumulation of pheophytin levels especially at 5 μM MV. In wild type plants, the pheophytin accumulated was high and the least was seen in plants expressing *cod A* gene (Fig. 6).

Results indicated that the targeted expression of glycine betaine synthe sis in subcellular location like chloroplast is more effective in obtaining higher degree of stress tolerance than any other organelles. Similar results were obtained in crops like *Arabidopsis thaliana* [7], *Brassica chinensis* [8] and *Solanum tuberosum* [9].

Conclusion

Abiotic stresses are the major constraints to realize the potential yields of our crop plants. Interestingly, under any abiotic stress (salinity, drought, high and low temperature and high and low light) the plant experiences oxidative stress. So, one of the option to reduce the damage caused by ROS is to increase the glycine betaine a compatible osmolyte accumulation in the plants by targeting the gene product to chloroplast.

Cod A overexpressing transgenic plants showed relatively high tolerance to MV induced oxidative stress compared to wild types. The chlorophyll damage in the transgenic plants was less under MV induced oxidative stress compared to wild type plants. The pheophytic/chlorophyll ratio as an estimate of chlorophyll damage was estimated. The ratio was less in all the transgenic plants compared to wild type plants. These results clearly indicated that the oxidative stress tolerance can be obtained by accumulation of glycine betaine by over expressing *cod A* gene into chloroplast.

References

1. Prasad KVSK, Saradhi PP (2004) Enhanced tolerance to photoinhibition in transgenic plants through targeting of glycine betaine biosynthesis into the chloroplasts. Pl Sci 166 : 1197—1212.
2. Chen HHT, Murata N (2011) Glycine betaine protects plants against abiotic stress : Mechanism and biotechnological applications. Pl Cell Environ 34 : 1—20.
3. Chen HHT, Murata N (2008) Glycine betaine : An effective protechtant against abiotic stress in plants. Trends in Pl Sci 13 : 499—505.
4. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning : A laboratory manual. Cold Spring Harbor, Ny : Cold Spring Harbor Laboratory Press.
5. Park EJ, Jeknic Z, Sakamoto A, De Noma J, Yuwansiri R, Murata N, Chen THH (2004) Genetic engineering of glycine betaine synthesis in tomato protects seeds, plants and flowers from chilling damage. Pl J 40 : 474—487.
6. Park EJ, Jeknic Z, Pino MT, Murata N, Chen THH (2007) Glycine betaine accumulation in chloroplasts is more effective than in cytosol in protecting transgenic tomato plants against abiotic stress. Pl Cell Environ 30 : 994—1005.
7. Sulpice R, Tsukaya H, Nonaka H, Mustardy L, Chen THH, Murata N (2003) Enhanced formation of flowers in salt-stressed *Arabidopsis* after genetic engineering of the synthesis of glycine betaine. Pl J 36 : 165—176.
8. Qing-bin Wang, Wen Xu, Qing-zhong Xue, Wei-ai

Su (2010) Transgenic *Brassica chinensis* plants expressing a bacterial *cod A* gene exhibit enhanced tolerance to extreme temperature and high salinity. Biomed & Biotechnol 11 : 851—861.

9. Ahmad R, Kim MD, Back KH, Kim HS, Lee HS, Kwon SY, Murata N, Chung WI, Kwak SS (2008) Stress-induced expression of choline oxidase in potato plant chloroplasts confers enhanced tolerance to oxidative, salt and drought stresses. Pl Cell Reports 27 : 687—698.