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Efficient Regeneration and Genetic Transformation of Sugarcane with *AVP1* Gene

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Abstract: *Agrobacterium* mediated genetic transformation is considered to be more efficient and reliable method than direct gene bombardment in sugarcane. In the present study an efficient regeneration from callus and genetic transformation was established by using *Arabidopsis Vacuolar Pump (AVP1)* gene conferring tolerance against drought and salinity in local cultivar of sugarcane CP77-400. *Agrobacterium* strain EHA105 harboring pGreen0029 vector containing *AVP1* and kanamycin resistant gene *Npt II* driven by 35S CaMV promoter was employed. Three weeks older calli of CP77-400 were infected with 0.2 optical density of *Agrobacterium*, 100 uM acetosyringone and co-cultivated for 4 days. The transformed plantlets were selected on 150 mg L^{-1} of kanamycin and it was found that cefotaxime concentration 250 mg L^{-1} was effective in controlling *Agrobacterium* overgrowth as well as to enhances regeneration and transformation efficiency in sugarcane. The transgenic plants were acclimatized in glass house and both *AVP1* and *Npt II* genes in transgenic plants of sugarcane were confirmed at genomic level through DNA amplification using Polymerase Chain Reaction (PCR). The evaluation of transgenic and non transgenic plants showed that transgenic plants more resistance to high salt concentration while the non transgenic plants were died at high salt stress.

Key words: *Agrobacterium* · Transformation · *AVP1* · Sugarcane · Salinity

INTRODUCTION

Sugarcane is an important agro-industrial crop that belongs to the family Poaceae. It is an octaploid species possessing complex genome (2n=80~270) with x=10 basic number of chromosomes. [1]. It is mainly cultivated in tropical and subtropical regions of the world and is used for sugar, power production, paper making, feed for live stocks, chipboard, cane wax, fertilizer, bio-ethanol, syrup and mulching [2]. The major production constraints are both biotic and abiotic stresses such as salinity, drought, pests and viral diseases [3, 4]. It is difficult to overcome these losses caused in sugarcane by traditional methods. Advances in genetic engineering enabled scientists to isolate and transform desired traits into crops [5]. However, *Agrobacterium*-mediated genetic transformation in sugarcane was considered to be more efficient than direct bombardment and electroporation method in monocot and dicots. Moreover, genetic transformation system is affected by a number of distinct factors such as cultural conditions, acetosyringone concentration, co-cultivation period, concentration of different antibiotics and different explants sources [6, 7]. Limited reports are available on successful *Agrobacterium* mediated transformation in sugarcane [8-15]. Various factors that affect *Agrobacterium* mediated transformation have been well investigated in many monocots like rice [16-18], maize [19-21], banana [22], barley [23] and wheat [24]. The *Arabidopsis AVP1* gene that code for a vacuolar *pyrophasphatase* protein perform its function as proton pumping on vacuolar membrane conferring tolerance against drought and salinity and it

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also acts as a antiporter that helps in Na^+ cation uptake from the cytoplasm to vacuole. It's over expression improve vegetative growth of roots as a result higher water absorption and retention from the soil occurred [25, 26]. However, to obtain genetically modified sugarcane it is necessary to optimize a reproducible and efficient regeneration protocol for successful transformation that will produce transgenic plants and reduce the chances of somaclonal variation as well as epigenetic changes. The present study demonstrates an efficient genetic transformation system through *Agrobacterium* in sugarcane.

MATERIALS AND METHODS

Indigenous CP77-400 sugarcane cultivar was obtained from sugarcane program, Crop Sciences Institute (CSI), National Agricultural Research Centre (NARC), Islamabad. Apical leaves surrounding from apical meristem were excised from the young disinfected stems of sugarcane grown in the field. Leaf rolls were peeled cylindrical pieces approximately 3-5 mm in diameter under sterile conditions. For surface sterilization of explants two treatments were employed. In the treatment I, explants were decontaminated with the help of 80% Clorox solution having 2-3 drops of Tween-20 for 20 minutes. In the second step the explants were rinsed three times with double distilled water to remove the traces of Clorox. Slices were cultured in already optimized callus induction medium containing 3mg/l 2,4-D to induce callus as described by Ali *et al*. (2010) [27]. The culture plates were kept in room temperature at 26±2°C under dark conditions. The cultures were transferred to fresh callus induction medium (CIM) after 2-3 weeks for long-term maintenance. Data of callus induction was routinely recorded. In order to achieve the regeneration from callus an already optimize media was used [27].

Transformation Strain and Vector: Agrobacterium super virulent strain EHA105 harboring plasmid construct pGreen0029-having *AVP1gene* was used for genetic transformation. The vacuolar pump gene used in this study was taken from *Arabidopsis thaliana* and *Neomycin phasphotransferase* gene (*NPT II*) as a selectable marker under the control of CaMV 35S promoter. *Agrobacterium* cells were cultured on Luria broth (LB) medium and subsequently maintained on agar plates containing 50mg L^{-1} kanamycin sulfate and 50 mg L^{-1} rifampicin.

Genetic Transformation and Co-Cultivation: Single colony was taken from fresh cultured plate and dissolved in falcon tube containing 10 ml yeast beef extract (YEB) liquid medium. The falcon tube was incubated at 28°C to obtain maximum bacterial culture. After 30 hours, culture was centrifuged at 5000 rpm for 10 minutes to get pellet. The pellet was dissolved in MS liquid and optical density of the culture was maintained through dilution of culture. Freshly cut calli were dipped in this suspension, blotted on sterile flter paper and subsequently transferred to regeneration medium. *Agrobacterium* suspension culture of optical densities 0.2, 0.4 and 0.6 were used at the time of infection. Different time periods of Co-cultivation media (2, 3 and 4 days) supplemented with acetosyringone (50, 75 and 100 uM) followed by cefotaxime at the rate of 250, 500, 750 mg L^{-1} to identify the best transformation event.

Molecular Analysis: Total genomic DNA was extracted from fresh leaves of putative transgenic and non transgenic sugarcane plants using little modify protocol of Sambrook *et al*. 1989 [28]. The desired amplified fragment of *AVP1* gene was obtained with a set of forward and reverse primers through polymerase chain reaction (PCR). The amplified product was visualized on 1% agarose gel.

Salt Stress Trials: The resulted three transgenic plants and three non transgenic plants (control group) were tested under four different event of salt stress (50, 100, 150 and 200 mM). The plants were grown in pots under glass house at 16/8 (light/dark) at 25 ± 2 °C, after 3 weeks of germination the plants were subjected to salt stress for 12 days. Initially low salt stress 50 mM was applied for 3 days to both transgenic and control groups. The salt stress was increased up to 100mM for next three days, 150 mM for next three days and so on. After salt treatments, the plants were shifted to normal growth conditions and data was recorded.

RESULTS AND DISCUSSION

Effect of Bacterial Optical Density on Transformation: Prior to *Agrobacterium* infection optimum optical density is pre-requisite, high optical density of *Agrobacterium* results in browning, lower cell recovery, overgrowth and low transformation efficiency that ultimately leads to death of desired explants [29]. Hitherto, there is no any appropriate report available related to *Agrobacterium*

tumefaciens concentration used in callus transformation of sugarcane. In the present study, three different levels of optical densities i.e. 0.2, 0.4 and 0.6 at 600 nm were applied for the best range of experimental infection, kanamycin selection and subsequently on transformation efficiency of sugarcane. The results (Fig. 1) showed that optimum optical density 0.2 used for callus infection yielded 84% transformation frequency while on other hand the minimum 48% transformation was achieved on 0.6 O.D. Similar trend was also reported by Zhangsun *et al*. 2007 [12]. At 0.6 O.D, excessive bacterial growth was observed. As a result callus totipotency declines and death of callus occurred. On other hand in monocot like wheat maximum transformation efficiency was obtained at O.D range of 0.75-1.0 [30, 31]. Their findings are contrary to our results. The reason for this deviation might be due to the differences in crop, genotype, explants source and strain used.

Effect of Acetosyringone and Cefotaxime on Transformation Efficiency: Acetosyringone is an alcoholic compound that enhances the *Agrobacterium* infection by activating *vir* genes and T-DNA delivery system. *In vitro* application of different concentrations of Acetosyringone i.e. 50, 75 and 100 µM at the time of co-cultivation were evaluated to find out the best callus transformation event. When 100µM of Acetosyringone was used, 19% of putative calli were selected on kanamycin and transformation efficiency 76% was recorded as shown in (Fig. 2). It was observed that acetosyringone has influenced the transformation efficiency. Our results are not in commitment with the findings of Gould *et al*. 1991 in maize, rice [17, 32] and banana [22] axillary buds of sugarcane [11]. Enriquez-Obregon *et al*. (1998) [9] also reported that sugarcane release own phenolic compound so, there is no need of addition of acetosyringone in co-cultivation media. Cefotaxime is broad spectrum antibiotic commonly used in transformation media to eliminate excess of *Agrobacterium* [29, 33, 34]. In the present study three different concentration of cefotaxime (250, 500 and 750 mg L^{-1}) were investigated for the removal of excess of *Agrobacterium*. It was observed that 250 mg L^{-1} cefotaxime produced tremendous effect of controlling *Agrobacterium* overgrowth and thus produced maximum number of shoots regeneration as given in Fig 3. Our results are in line with the findings of (Kaur *et al*. 2008)[35] that used cefotaxime in media for multiplication and elongation of sugarcane shoots. High concentration

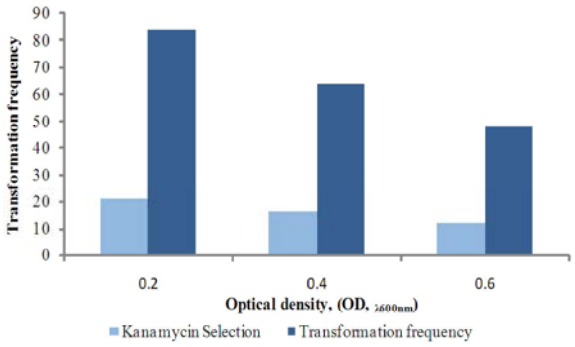

Fig. 1: Effect of optical density on kanamycin resistance shoots and transformation frequency.

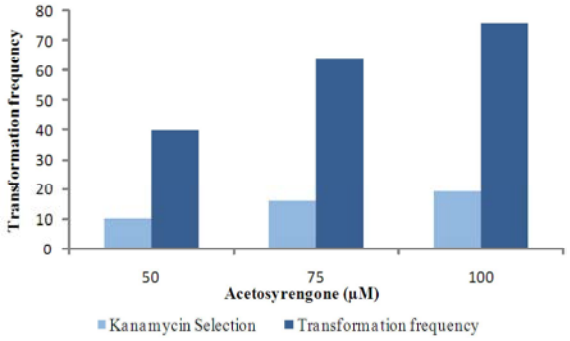

Fig. 2: Influence of acetosyringone on kanamycin resistant shoots and transformation frequency

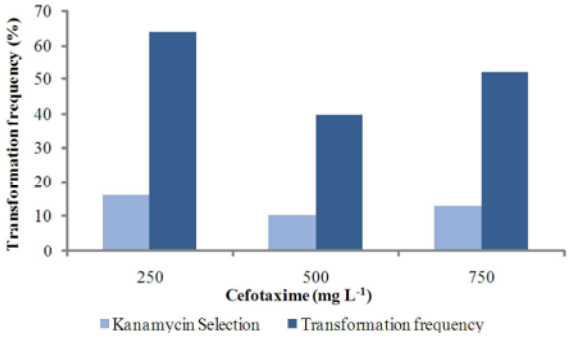

Fig. 3: Effect of cefotaxime on transformation frequency and kanamycin resistant shoots regeneration

up to 500 mg L^{-1} of cefotaxime was also used by Shaik *et al*. (2007)[36] in the transformation of sugarcane while Arencibia *et al*. (1998) [8] used 500 mg L^{-1} of cefotaxime in selection process to control bacterial overgrowth in sugarcane. Therefore, use of cefotaxime in regeneration medium can give a high frequency of green and healthy shoots. So, in regeneration media use of cefotaxime in tissue culture may be act as chlorophyll synthesis or may enhance totipotency. Further studies are required to

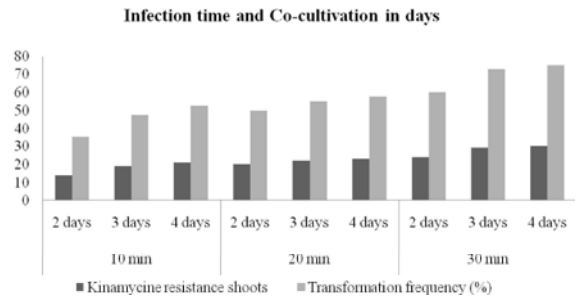

Fig. 4: Infection and co-cultivation time periods on transformation frequency of sugarcane

investigate what its actual mechanism. Use of Cefotaxime in regeneration media not only enhance the in *vitro* root induction and shoot elongation as well as to reduce the overgrowth of *Agrobacterium*. While on other hand contrary results were reported in tobacco [37].

Infection Time and Co-Cultivation of Sugarcane Calli: Beside of other basic steps of *Agrobacterium* mediated transformation, infection and co-cultivation time period of explants are crucial steps to achieve better transformation efficiency. In our results, the highest transformation frequency was 75% obtained on 30 minutes of infection and 4 days of co-cultivation period as shown in Fig. 4. Similar results were also obtained based on co-cultivation for a period of 4 days produced the highest number of transgenic plants of sugarcane [13]. On other hand, contrary results were found in melon [38], rice [39], citrange [40] and sugarcane [14]. These variations might be due to changes in explants used, infection and co-cultivation times and strain and construct used. Normal co-cultivation time increases transformation efficiency and longer co-cultivation periods frequently result in *Agrobacterium* overgrowth and subsequent death of desired explants. Co-cultivation period of more than 5 days leads to suppression of shoot emergence and transformation frequency was low or completely abolished [11].

Molecular Analysis of Alien Genes in Transformed and non Transformed Sugarcane Plants: To detect the presence of foreign *AVP1* gene in transformed plants total genomic DNA was extracted by using the method of Sambrook *et al*. 1989 [28] from the green fresh emerged regenerated leaves of sugarcane plants. The plants were acclimatized at the transgenic containment. The stable integration of *AVP1and Npt II* gene in plant

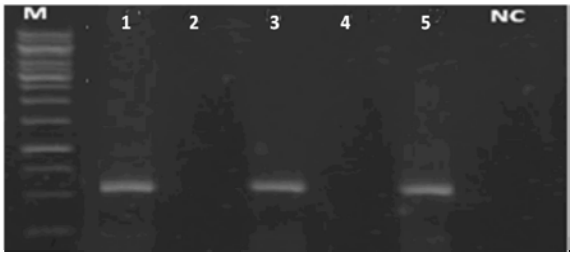

Fig. 5: a: Lane M= 1kb Marker, Lane1, 3= transgenic sugarcane plants, lane 2,4= non transformed, lane 5=positive control, NC= negative control

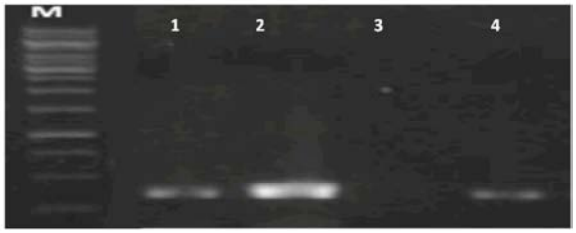

Fig. 5: b: Lane M= 1kb Marker, Lane 1and 4= kanamycin resistant plant, lane 2= positive control, lane 3= negative control

genome was confirmed though polymerase chain reaction (PCR) with specific set of primers (Forward 5-ATGGTGGCGCCTGCTTTGTTACCG-3 and Reverse 5-GAACAGAGGTAACAGCACCA-3) (Forward 5'-GAGGCTATTCGGCTATGACTG –3' and Reverse 5'-ATCGGGAGGGGCGAT ACCGTA-3' designed manually by retrieving NCBI database. The amplified product of *AVP1* gene was 630 bp and for *Npt II* gene 300 bp was observed as shown in Fig 5a and 5b, respectively.

Effect of Stress Trials on Transgenic and non Transgenic Plants: Salinity is one of the main abiotitic stresses which directly affect the yield of sugarcane. In present research work, transgenic sugarcane plants were produced and these plants were tested under different events of salts (50, 100, 150 and 200 mM). From the results we found that after 12days of salt stress treatment i.e. 50, 100 and 150 mM the transgenic plants remains green and healthy while the non transgenic plants showed the symptoms of wilting even at very low 50 mM of salt stress. The transgenic plants still survive at extreme at stress 200 mM while all non transgenic plants were died at this stress. The transgenic plants having foreign gene against abiotic stress significantly increased the performance of plants and it is one of the best method to remove hunger from the world [41]. The transgenic

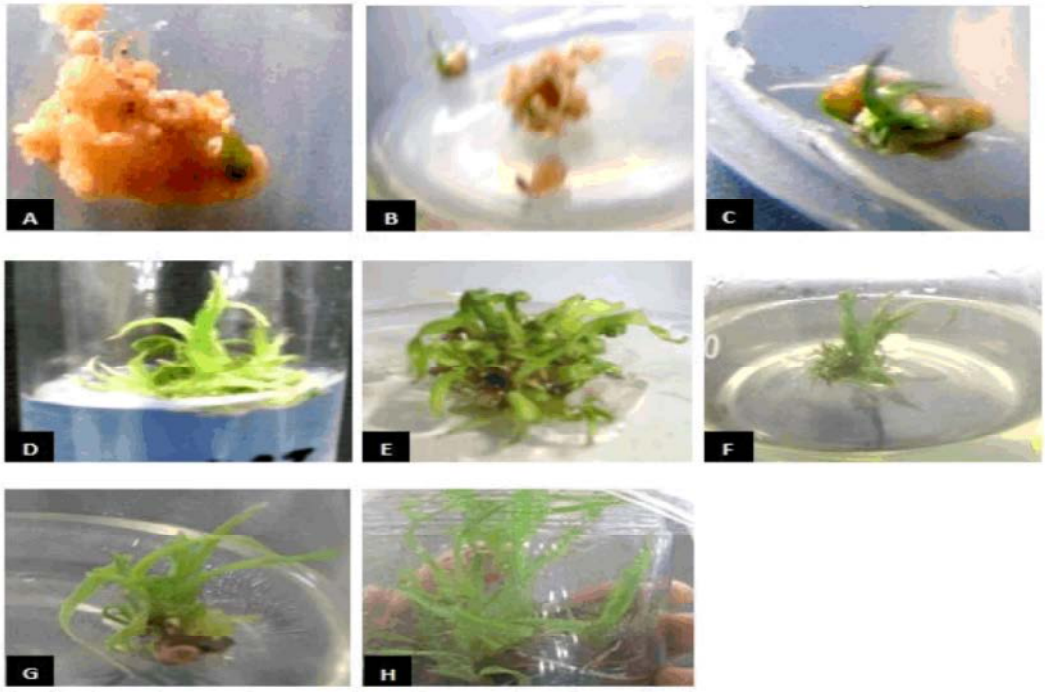

Fig. 6: (A, B & C) Early Shoot formation from callus on selection media (D, E & F) Secondary and multiple shoots on selection media.

Fig. 7: Regeneration of Profuse roots and acclimatization of transgenic plants in glass house.

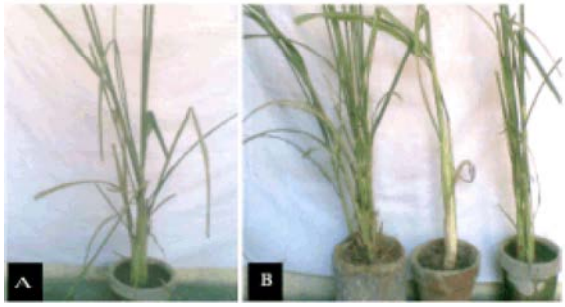

Fig. 8: Phenotypes of non-transgenic sugarcane (CP77-400) plants.

Arabidopsis plants having *AVP1* gene showed tolerance against salinity [26]. The extreme drought and salt stress caused increased root length and biomass in some plants species, which directly provide tolerance against drought and salt stress [42, 43].

CONCLUSION

It has been concluded that various parameters used for *Agrobacterium* mediated transformation of *AVP1* gene *in* sugarcane. Different parameter and methodology for *Agrobacterium* mediated genetic transformation of sugarcane are highly reliable and achieve reproducible results, despite minor differences were observed in transformation efficiency. As the presence of undesirable traits in sugarcane is one of the main causes of the loss in crop productivity, the generation of a drought and salt tolerance in indigenous sugarcane variety is a significant step in the genetic improvement of sugarcane plant. A reliable transformation methodology would make possible the production of transgenic sugarcane plants resistant to drought and salinity, herbicides, pathogens and pests, as well as the possibility of manipulating the biochemical pathways to allow the production of metabolites useful for food, pharmacy and industry.

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