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Optimization of factors affecting *Agrobacterium* mediated transformation in sugarcane through axillary buds with *AVP1* gene against drought and salinity

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Abstract:

Drought and salinity have severely affected the growth and sucrose contents of Sugarcane. Advances in genetic engineering promise to recover these losses in sugarcane. The present study was conducted to develop transgenic sugarcane against drought and

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salinity. Agrobacterium tumefaciens mediated transformation of sugarcane variety CP77-400 with EHA105 strain; plasmid pgreen0029 containing AVP1 gene driven by CaMV35S promoter was employed. Important Factors for successful transformation through Agrobacterium tumefaciens were optimized including concentration of Agrobacterium, infection time, co-cultivation days, cefotaxime doses and kanamycin concentration. An efficient protocol for sugarcane transformation mediated by A. tumefaciens through axillary buds was established. The OD ($_{600nm}$) = 0.4, infection time for 20 minutes along with co-cultivation for 3 days, 50 µM acetosyringone, 50 mg L^{-1} kanamycin and 500 mg L^{-1} cefotaxime concentrations gave maximum transformation efficiency. Fully developed transgenic plants were shifted to glass house and regenerated plantlets were used as plant material for DNA isolation. The transformed plantlets produced bands of the expected size 630 bp. Hence the alien gene was stably transformed to the plant genome.

Key words: *Agrobacterium tumefacien*, *AVP1* gene, Auxiliary buds, Transgenic, sugarcane.

Introduction

Sugarcane (*Saccharum officinarum* L.) is the second major cash crop of Pakistan after cotton and is mainly cultivated in tropical and subtropical region for sugar, power generation, paper making, livestock feed, chipboard, cane wax, fertilizer, bio-ethanol, syrup, mulch and other pharmaceutical products [1, 2]. Several remarkable economic damages were reported for low cane and sugar yields viz., salinity, drought, pests and diseases [3, 4. Sugarcane, being a typical glycophyte and hence shows stunted growth or no growth under salinity, with its yield falling to 50% or less than its true potential. Salinity at the root zones of sugarcane decreases the sucrose yield through its effect on both biomass and juice quality and quantity [5, 6]. Genetic improvement through conventional breeding becomes more

difficult due to several constrains such as narrow gene pool, complex genome, low fertility, and long breeding or selection practices. Moreover, modern varieties have a variable chromosome number (2n=100–120) and bear rare flower [7]. Abiotic stresses, particularly drought and salinity, are complex phenotypic and physiological phenomenon in plants. The Arabidopsis gene *AVP1* encodes a vacuolar pyrophosphatase that functions as a proton pump on vacuolar membrane and there over-expression could increase proton electrochemical gradient in vacuole, thereby activating vacuolar membrane-antiporters including Na+/H+, which helps in sequestration of Na+ into the vacuole [8]. Furthermore, over-expression of *AVP1* gene increases auxin transport and enhances auxin mediated root development as well as biomass, which result higher water absorption and retention capacities [8, 9]. Other groups have also demonstrated that over-expression of similar genes encoding vacuolar membrane-bound pyrophosphatase (H + PPase or H + pump) can increase both salt- and drought tolerance in heterologous systems, including rice [10], tobacco [11], cotton [12] and maize [13]. Therefore, Arabidopsis vacuolar proton pump (*AVP1*) gene has been chosen for introducing in sugarcane as the over-expression of this gene in the model plants showed to impart salt and drought tolerance. The time constraints and regeneration rates could be overcome by using axillary buds, as it require short period of time for explants preparation, transformation and direct regeneration because axillary buds give rise to shoots without the involvement of explants differentiation. On the other hand, use of axillary buds regeneration causes minimal genetic changes which are routinely used for mass multiplication of sugarcane [14, 15]. Thus, axillary bud is an alternative, viable target tissue for higher regeneration and gene manipulation. Sugarcane is a vegetatively propagated crop and its stable transformants can be clonally multiplied. While the co-culture

of *Agrobacterium*-mediated with other explants or callus requires regeneration of transformed cells which is genotype-dependent, painstaking, labor intensive, time consuming and complicated with somaclonal variation and epigenetic changes. The reported protocol is simple, efficient, reproducible, genotype-independent, cost-effective and selection of target tissues, which are both competent for transformation and regeneration to recover fertile plants with minimal time.

Materials and methods

Explants Preparation and Sterilization

Axillary buds ranging from 0.5-2 cm size of freshy stalks of CP77-400 were used. All these explants were obtained from 4 to 8 months old stock grown in the field of sugarcane program at National Agricultural Research Centre Islamabad. The young fresh and regenerated excised buds from the spindle were partially trimmed off with minor natural nutrient (stem segment) and then washed vigilantly under running tap water for 15 minutes each for two to three times to wash off exterior dust and contaminants. After this, explants were shifted to laminar hood. The buds were once again rinsed with commercial bleach 100% Clorox (Sodium hypochlorite 5.25 %) with two to three drops of Tween-20 as a wetting agent for 10 minutes. This treatment was done twice to reduce the chances of contamination and infectivity. After performing these measures, explants were washed two times in sterile water for 10 to 20 minutes and kept on sterile tissue paper for drying.

Regeneration of Shoots from Axillary Buds

To initiate direct culture regeneration from axillary buds, the axillary buds were inoculated on MS media (Murashige and Skoog, 1962) containing (MS + 0.1 mg L^{-1} BAP + 0.1 mg L^{-1} Kin+ 0.1 mg L^{-1} GA3 + 0.1 mg L^{-1} NAA 0.25 mg L^{-1}) for

primary shoot regeneration. After 3 weeks of culture initiation, the primary shoots developed from the axillary buds were excised and trimmed the dead leaves and brownish tissue at the bottom. One primary shoot was cultured per tube in the secondary shoot regeneration medium containing (MS salts, BAP 1 mg L^{-1}, Kinetin 1 mg L^{-1} and GA_3 1 mg L^{-1} supplemented with 3% sucrose and 0.2% phytagel as described by [16]. The primary shoot produced a clump of 5–7 secondary shoots in about 3 weeks. The secondary shoots were then multiplied on the same media for another four to five cycles each of 3 week duration. All cultures were maintained at temperature range of 25$\pm$ 1 ^{0}C under 16 hours of photoperiod with 2200 lux light intensity. After a period of 3-4 weeks the emerging shoots were transferred into respective media.

Agrobacterium Strain and Culturing

The EHA105 strain [17] harboring pGreen0029 vector with kanamycin resistance gene driven by cauliflower mosaic virus *CaMV35S* promoter and *Nos* terminator were used for explants infection. *Agrobactrium* culture was taken from fresh glycerol stock and was streaked on the plate containing yeast beef extract (YEB) media; single colony was taken from fresh plate and was dissolved in falcon tube containing 10 ml YEB broth. The falcon tube was kept in incubator at 28 ^{0}C to obtain maximum bacterial growth. Thirty 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.

Agrobacterium Infection

EHA105 strain grown in YEB media at three different levels of optical densities 0.2, 0.4 and 0.6 were used as in time of infection. The axillary buds were slightly wounded with a sterile hypodermic syringe needle and surgical scalpel tip by

prickling method up to 2–5 times and the injured explants were immersed in infection suspension for 10-30 minutes.

Co-cultivation of Buds with *Agrobacterium*

The infected axillary buds of CP77-400 sugarcane cultivar were transferred to another plate containing co-cultivation media covered with and without filter paper. The infected buds were inoculated on co-cultivation media with the addition of phenolic substance like acetosyringone ranging from 50-100 uM. Plates were covered tissue paper and parafilm and kept in dark conditions for 2 to 4 days.

Washing of Axillary Buds

The co-cultivated axillary buds were washed two to three times with distilled water, followed by three different concentrations of cefotaxime at rate of 250, 500 and 750 mg L^{-1}. The resulted washed materials were blotted on plate containing autoclaved filter paper for 15 minutes. Dried buds were inoculated on selection media to check the best transformant event.

Screening of Axillary Buds and Secondary Shoots Regeneration

After 2 to 3 weeks, small putative primary shoot emerged from transformed bud by considering as a clone. Preliminary sensitivity tests were conducted to evaluate the best selection pressure with different level of kanamycin i.e. 50, 100, and 150 mg L^{-1} to confirm the initial transformation.

Regeneration, Multiplication and Acclimatization of Transformed Plants

In order to minimize the chimeric and mosaics two to three weeks older higher regenerated primary shoots selected from primary shoots extra leaves, dead brown and damage tissues were trimmed off at the basal part and then sub-cultured on

selective secondary shooting media followed by 3 to 4 cycle of micro-propagation. Putative transgenic shoots with 3-5 cm height were separated and sub-cultured on selective rooting media. The resulted transgenic rooted plants from jars were transferred to polythene bags containing the sand and soil mixtures. After shifting, the plants were covered with another polythene bags to maintain the humidity and temperature.

Molecular Detection of Transgene

The resulted putative sugarcane plants fresh green leaves were selected to isolate total genomic DNA following the procedure of CTAB [18] to perform polymerase chain reaction (PCR) with a set of forward and reverse primer, the desired fragment of *AVP1* gene was amplified. The PCR product was visualized on 1% agarose gel with help of UV gel documentation system.

Results

Optical Density

Optical density is a basic step to measure the growth of bacteria in the cell suspension with help of spectrophotometer. For plants transformation the optimum growth of *Agrobacterium* is a pre-requisite. Up till now, there is no suitable report on *Agrobacterium tumefaciens* concentration for any sugarcane cultivar transformation. Overgrowth of *Agrobacterium* cause browning, lower cell recovery, death of explants and ultimately results in low transformation efficiency (Fig 1a). The results showed that 0.4 O.D producing maximum number of kanamycin resistance plants and higher number of (80%) transformation efficiency (Table 1 and Fig 1b)

Effect of Infection and Co-cultivation

Infection and Co-cultivation times both are crucial steps in plant genetic transformation through *Agrobacterium*. In this

experiment three different co-cultivation time period i.e. 2, 3 and 4 days with three different infection times (10, 20, 30 min) were checked for generation of kanamycin resistance shoots and transformation frequency (Fig 2-3). The application of different time periods on transformation of sugarcane was thoroughly evaluated. Maximum kanamycin resistance plants with transformation frequency 75% were observed when infected for 20 minutes in the suspension culture of *Agrobacterium* and subsequently co-cultivated on 3 days

Effective Dose of Acetosyringone

Acetosyringone is a plant phenolic compound released after the wounding of plants. *In vitro* application of acetosyringone enhances the T-DNA delivery of *Agrobacterium*. In the present study three different levels of acetosyringone (50, 75 and 100 µM) were used for evaluating the transformation efficiency. The results showed that 50 µM acetosyringone was the best level for axillary buds. The transformation frequency was 72% was recorded (Table 3).

Screening of Kanamycin Resistance Shoots

Kanamycin sulfate is an aminoglycoside antibiotic commonly used as plant selectable marker in genetic transformation experiments. In the present study the best selection was evaluated by the application of different levels of kanamycin lethal doses (50,100, and 150 mg L^{-1}) these doses were applied on axillary buds of sugarcane cultivar CP77-400 (Table 4). Different concentrations of kanamycin were checked on buds and shoots (Fig. 1c).

Effect of Cefotaxime Concentration

In the present experiment three different concentration of cefotaxime (250, 500, and 750 mg L^{-1}) were investigated for the removal of excess of *Agrobacterium* growth response of axillary

buds towards shooting. It was observed that 500 mg L^{-1} cefotaxime produced significant effect on controlling the *Agrobacterium* and maximum number of regeneration frequency was observed. On other hand the use of 250 mg L^{-1} and 750 mg L^{-1} tremendous reduce the transformation frequency (Table 5).

Molecular Analysis

To detect the foreign gene in transformed plants total genomic DNA was extracted from green fresh emerged regenerated leaves of sugarcane plants acclimatized at the transgenic glass house (Fig. 1e). The stable integration of *AVP1*gene in plant genome was confirmed though polymerase chain reaction with specific set of primers designed manually (Fig. 4).

Discussion

In the present study three different levels of OD600= 0.2, 0.4 and 0.6 were applied for the best range of experimental infection and subsequent effects on transformation efficiency of axillary buds of CP77-400 cultivar of sugarcane. The OD600= 0.4 increased transformation efficiency up to 80 %. Our results are highly in line with [19] their results showed that OD600 = 0.2 ~0.4 were optimal to obtain high transformation efficiency and high cell survival. When OD600 ranged from 0.2 to 0.4, *A.tumefaciens* was in the first half period of logarithmic multiplication phase and has high infectionability. Whereas OD600 =0.6, the suspension has too much cell compete_for Oxygen resulting in decreasing of the infection ability. The finding are opposing nevertheless, higher or lower *Agrobacterium* densities were successfully used in another plants such as winter jujube [20], sugarcane [21], *Acacia crassicarpa* and *Vigna radiata* [22] at OD600= 1.0 [23] lily at OD600= 0.6 [24] and tomato at OD 600= 0.1 [11].

Transformation frequency rises when infection and co-cultivation time period increases up to their maximum limit. Co-cultivation period of longer than 3 days led to a reduction in transformation frequency due to leaching of bacterial overgrowth, whereas when their concentration increases up to 5 days, had showed complete suppression of shoot emergence in sugarcane [23]. Similarly results were obtained in melon [24], rice [25] citrus [26] and sugarcane [27]. Normal co-cultivation time has increased the transformation efficiency and longer co-cultivation periods frequently resulted in *Agrobacterium* over growth and subsequent death of explants. Co-cultivation for a period of 4 days produced the highest number of transgenic sugarcane plants [21], this difference might be due to in genotype and type of explants used.

Three different concentrations of acetosyringone (50, 75 and 100 µM) were used. Our results showed that 50 µM acetosyringone concentration enhanced transformation efficiency. Similar results were also reported in maize [28], rice [29, 30] and banana [31]. Our result is also supported by previous findings in sugarcane [27].

The low dose of kanamycin 50 mg L^{-1} was found significant for selection. The high dose of kanamycin showed negative effect on transformation efficiency. Brasileiro [32] reported that deleterious effect of kanamycin is due to its potential to inhibit protein synthesis by its binding to the 30S subunit of the ribosome thus blocking the formation of initiation complexes and decreasing the fidelity of translation. It may even be toxic to untransformed tissues by secreting inhibitors or preventing transport of nutrients to the living transformed cells [33]. Our results are in corroboration with [23, 34], they used kanamycin at 150 mg L^{-1}.

Cefotaxime is an antibiotic frequently used to get rid of excess *Agrobacterium* from infected explants. The 500 mg L^{-1} cefotaxime showed good effect on controlling the *Agrobacterium*

infection and yield the maximum regeneration. Our results are parallel to that of [35] used 500 mg L^{-1} of cefotaxime in sugarcane. The same concentration was also used by [36] in the transformation of drought and salinity tolerant gene GLY in sugarcane. [37] also used 500 mg L^{-1} cefotaxime in selection process to control bacterial contamination in sugarcane.

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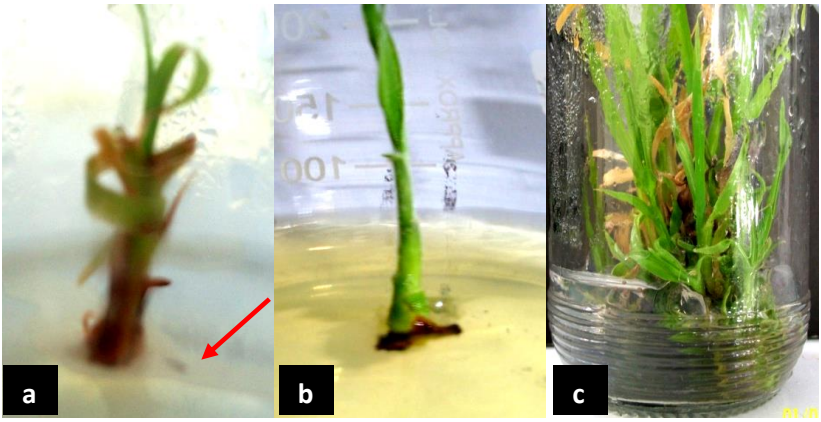
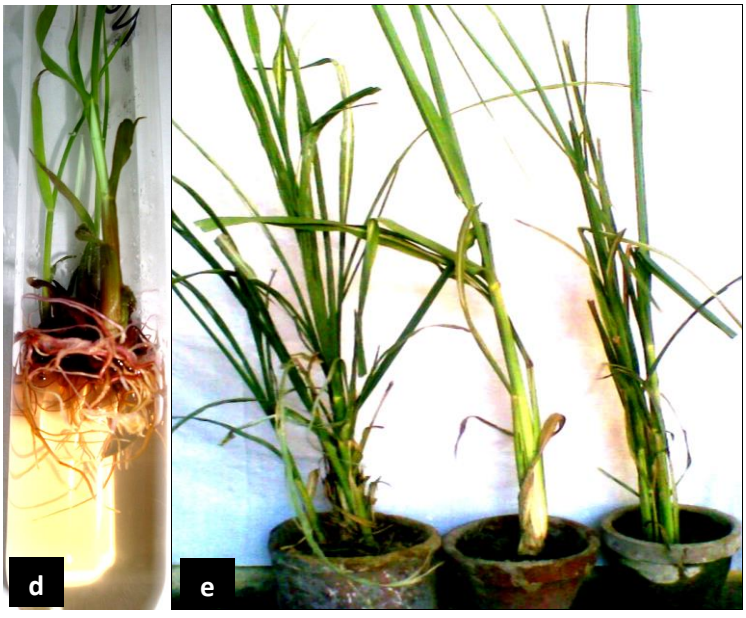


Fig.1. Effect of different factors and regeneration of axillary bud into multiple shoots a) 0.6 optical density result in overgrowth (as indicated with), browning and subsequent death of axillay bud b) primary shooting after Co-cultivation of axillary buds c) Putative primary shoots multiplication on selection media yellow shoots are non-transformed green shoot are transformed



d) Rooting of shoots. **e**) Acclimatization of transgenic plant after 4 to 5 round of micro-propagation.

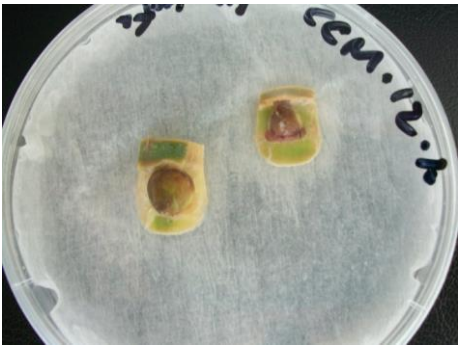


Fig.2. Co-cultivation of axillary buds with *Agrobacterium* on filter paper to overcome the over growth.

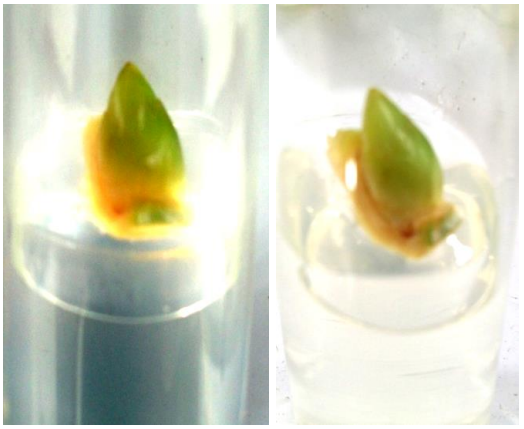


Fig.3. Regeneration of transformed axillary buds on culture initiation media.

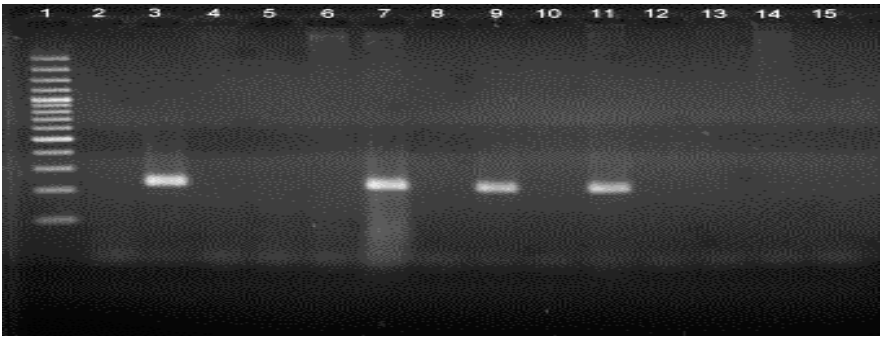


Fig.4. Molecular detection of alien gene through PCR.
Lane 1= Ladder I Kb
Lane 3, 7, 9 and 11= Transgenic plants

Lane 2= Negative control

Lane 4, 5, 6, 8, 10, 12, 13, 14 and 15= Non transgenic plants

Table-1. Influence of optical density on kanamycin resistance plants and transformation frequency

Type of Explants	No. of Buds infected	O.D (600nm)	Kanamycin selection	Transformation Frequency (%)
Axillary Bud	25	0.2	14	56
	25	0.4	20	80
	25	0.6	10	40

Table-2. Effect of co-cultivation and infection time on axillary buds of sugarcane

Explants	Infection time (min)	Co-cultivation (days)	Total no. of buds infected	Kanamycin resistance shoot	Transformation frequency (%)
Axillary Bud	10	2	40	13	32.5
		3	40	19	47.5
		4	40	14	35
	20	2	40	14	35
		3	40	29	72.5
		4	40	19	47.5
	30	2	40	19	47.5
		3	40	25	62.5
		4	40	18	45

Table-3. Effective dose of Acetosyringone on transformation efficiency

Explant	Total no. of explants	Acetosyringone (µM)	Putative kanamycin resistance	Transformation frequency (%)
Axillary Bud	40	50	18	72
	40	75	15	60
	40	100	10	40

Table-4. Effect of different levels of kanamycin on transformation efficiency

Explant	Total no. of explants	Kanamycin Conc (mg L^{-1})	Putative kanamycin resistance	Transformation frequency (%)
Axillary	40	50	28	70

Bud	40	100	20	50
	40	150	10	25

Table-5. Effect of different levels of cefotaxime on bacterial overgrowth and transformation efficiency

Explants	Total no. of explants	Cefotaxime conc. mg L^{-1}	Mean over Growth	Shoot regenerated	Transformation frequency (%)
Axillary bud	25	250	17	08	32
	25	500	08	17	68
	25	750	14	11	44