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Agrobacterium mediated transformation of *DREB1A* gene for improved drought tolerance in rice cultivars (*Oryza sativa* L.)

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Abstract

Rice productivity is greatly affected by different environmental stresses such as drought, salt loading, and low temperature. Among these, drought stress is considered as a serious threat to effect rice productivity. Improved *Agrobacterium* mediated transformation system offers an alternative approach to develop transgenic rice cultivars with enhance drought tolerance. In this regards, dehydration response element binding factors (DREB) plays an important role in regulating gene expression in response to drought stress. In the present study, *AtDREB1A* gene under 35s *CaMV* promoters (pBIH Binary vector) was incorporated through *Agrobacterium* mediated transformation system in to two local rice cultivars i.e. JP-5 (*Japonica* type) and KSK-282 (*Indica* type), using calli as target plant tissue. The different parameters like lethal dose of hygromycine, cefotaxime level, and acetosyringone level were optimized for both rice genotypes. The transgenic plantlets (T_0) were confirmed by PCR (28% and 16 % for JP-5 and KSK-282, respectively) and the integration pattern of the transgene was tested by Southern blot analysis of genomic DNA. The T_1 transgenic plants showed normal growth and the expression of the transgene were confirmed by RT-PCR. The T_1 transgenic plants overexpressing *AtDREB1A* gene survived after exposure to drought stress condition over a period of one week under greenhouse condition. The net result of our study is the development of an efficient *Agrobacterium* mediated transformation system and overexpression of *AtDREB1A* transcription factors that resulted in improved drought tolerance in two rice cultivars. The transgenic lines developed in our study might prove better candidates for developing drought tolerant local rice cultivars in the future.

Keywords: *Agrobacterium* mediated transformation, Constitutive promoter, Dehydration responsive element, Drought tolerance, Rice.
Abbreviations: ABA_Abscisic acid; BA_6-benzylaminopurine; CMV_Cauli flower mosaic virus; DREB_Dehydration responsive elements binding factors; NAA_Naphthaleneacetic acid; RM_Regeneration media; 2,4-D_2,4-Diphenoxyacetic acid.

Introduction

Plants are seriously affected by exposure to various adverse environmental stresses such as drought, salinity, high temperature, cold, and light (Kim et al., 2013). Among these abiotic stresses, drought is a widespread environmental threat seriously affecting plant growth and development (Shinozaki and Yamaguchi-Shinozaki, 2000). Drought effected area is increasing worldwide and over 20 % of rice growing area worldwide is suffering from drought stress. Drought stress is becoming an increasingly severe problem limiting rice productivity in many regions and even mild drought stress can significantly reduce rice yield (Swamy and Kumar, 2013). Plants have developed a complex network of stress signaling and regulation of gene expression mechanism to respond and adapt to the stress condition with suitable functional changes (Tang et al., 2012). Plants perceived specific stress signals with the onset of stress conditions resulting in the activation of various signaling modules including hormones, signal transducers and various transcription factors. The signals are further transduced by the signaling components to induce the expression of various stress-related genes and the resultant cellular damage is neutralized by the discrete product of the expressed genes through various physiologic and metabolic processes (Shinozaki and Yamaguchi-Shinozaki, 1996; Lata and Prasad, 2011). Among these, transcription factors are key regulators of changes in the gene expression and environmental stress responses (Shinozaki et al., 2003). The transcription factors involved in abiotic stress tolerance are classified into different family's i.e ABRE/ABF (ABA-responsive element-binding protein/ABA-binding factor), AP2/ERF (APETALA2/ethylene responsive factor), bZIP (basic leucine zipper), MYC/MYB (Myelocytomatosis oncogene/Myeloblastosis oncogene), CUC (NAC) and various protein kinases (Shinozaki and Yamaguchi-Shinozaki, 2000; Todaka et al., 2012). Both ABA-dependent and ABA-independent processes are involved in activation of the transcription factors during stress responses. Among the transcription factors, the *DREB* genes of AP2 family, have been identified for their function in the ABA-dependent pathway by recognizing ABA-responsive elements (ABREs) containing a 9 bp (TACCGACAT) *cis* acting core motif (DRE) (Choi et al., 2000; Yamaguchi-Shinozaki and Shinozaki, 2006). The DREB transcription factors have a main role to induce the expression of number of genes involved in drought stress and genetic manipulation of the genes encoding DREB transcription factors displayed remarkable improvement in plant response towards drought stress (Liu et al., 1999; Udvardi et al., 2007). Overexpression

of *DREB* genes that regulate expression of multiple genes from various pathways is an important way of achieving tolerance to drought stress in plants. Several genes encoding DREB TFs have been incorporated into rice plants resulting in drought stress tolerance with either constitutive or inducible promoters (Lata and Prasad, 2011).Transgenic rice plants over-expressing various DREB genes showed strong tolerance to drought stress (Dubouzet et al., 2003; Ito et al., 2006; Datta et al., 2012; Ravikumar et al., 2014). Several transgenic rice lines were generated over-expressing *OsDREB1A* and *OsDREB1B* from rice and *DREB1A*, *DREB1B* and *DREB1C* from *Arabidopsis* under the control of constitutive promoter ubiquitin and 35s *CaMV* resulting in tolerance toward drought stress (Ito et al., 2006; Oh et al., 2009). Gene manipulation approaches involving varieties of genetic transformation methodologies have been employed to enhance the tolerance of crop plants toward multiple stresses (Kasuga et al., 1999). The standard rice transformation protocol for generating a large number of transformants, however, is limited to only a few cultivars, indicating that experimental parameters for rice transformation have not been fully optimized (Ozawa et al., 2009). Many rice varieties especially *indica* type are recalcitrant to transformation and are not efficiently transformed by *Agrobacterium.* In the present study, *AtDREB1A* gene under *CaMV* 35s promoter was successfully transferred into JP-5 (*Japonica* type) and KSK-282 (*Indica* type) local rice cultivars. The transgenic plants showed enhanced tolerance toward drought stress with normal plant growth and development.

Results

Effect of growth regulators on callus induction and regeneration

Our result clearly revealed that different concentrations of 2,4-D significantly affect the callus induction frequency and the response of both the genotypes was found different (Table 2 and Fig. 2). The ANOVA result further showed that 2,4-D×genotype interaction have significant effect on callus induction in both rice genotypes at 5% probability level. Subsequently, regeneration of plantlets was achieved through organogenesis from seed derived calli. Green shoot bud formation was observed after 2-3 weeks from the green patches on the calli. The green buds were further elongated and differentiated into multiple green shoots within a week. Significant differences were observed between the responses of the two genotypes towards different regeneration media. The JP-5 genotype showed maximum regeneration efficiency (84.03%) on RMIII (regeneration media) whereas the genotype KSK-282 showed maximum regeneration efficiency (25.66%) on RM II. Overall, the ANOVA results showed that regeneration efficiency and plantlet regeneration was highly affected by the genotype as well as the culture media ($P \leq 0.05$) (Table 2 and Figure 2).

Determination of lethal dose of hygromycin

Lethal dose of hygromycin was determined by exposure of the calli to three different concentrations of hygromycin. Significant differences were observed among the growth of calli on different concentrations of hygromycin. Most of the calli survived on low concentration of hygromycin (25 mg/l) while increasing the level of hygromycin to 50 mg/l showed necrosis of the calli and thus can provide more stringent condition for transgenic plants selection. Moving above this concentration (75 mg/l) leads to complete necrosis or death of the calli (Table 3).

Effect of different level of acetosyringone

Acetosyringone concentration of 100µM in co-cultivation media resulted in higher transformation efficiency and plantlet regeneration in both JP-5 (44%) and KSK-282 (26.66%). Higher concentration of AS (300 µM) resulted in decrease of the percentage of hygromycin resistance calli with 6% and 5% decrease observed in JP-5 and KSK-282 resp. Both the rice genotypes responded differently to three concentrations of acetosyringone with JP-5 having highest transformation efficiency compared to KSK-282. ANOVA results demonstrated that both acetosyringone and variety have significant effect on transformation efficiency and plantlet regeneration while their interaction showed non-significant effect (Fig 3).

Elimination of Agrobacterium in selection media using Cefotaxime

Three different concentrations of Cefotaxime were tested for elimination of bacterial proliferation in selection media. A substantial increase in transformation efficiency and regeneration potential were observed, when transformed calli were subjected to lower concentration (300 mg/l) of Cefotaxime. The *Agrobacterium* was fully eliminated at 300 mg/l concentration of Cefotaxime and no overgrowth was recorded throughout the experiment.

Regeneration and molecular analysis of putative transgenic plants

The regenerated plantlets with well-developed roots and shoots were transferred into test tube containing tap water for 4 days and were subsequently shifted to glass house for acclimatization (Fig 4). The putative transgenic plants (T_0) were confirmed by PCR and southern blotting. PCR confirmation of the transgenic plantlets reveals net transformation frequency of 28% and 16% for JP-5 and KSK-282 respectively (Fig 5). The transformation efficiency was calculated as percentage of PCR positive plantlets to the total number of calli inoculated in the co-cultivation experiment. The size of amplified *AtDREB1A* gene in transgenic plantlets was found 632bp, thereby confirming the incorporation of the target gene (Fig 6). Southern blot hybridization of the transgenic lines showed one and two copy of the transgene integrated into the genome (Fig 7). Reverese transcriptase-PCR analysis of T_1 trangenic lines confirmed the expression of *AtDREB1A* transgene under 35S *CaMV* promoter (Fig 8).

Drought tolerance analysis

The growth of both transgenic and wild type plants was normal by measurement of fresh weight of the plants for four consecutive weeks. The fresh weight (2 plants/sample) was found similar for both transgenic and wild type plants after one, two, three and fourth weeks (Table 5). The four weeks old transgenic lines (JP-1, JP-5-2, JP-5-3, JP-5-4, JP-5-5, KSK-282-1, KSK-282-2, KSK-282-3, KSK-282-4) and the non-transgenic wild type plants were subjected to drought stress by withholding water for one week followed by re-watering for one week. Under the drought stress condition of one week, the non-transgenic wild type plants started leaf rolling and wilting on the 3rd day of drought exposure

Table 1. Media used for rice tissue culture and transformation.

Type of Media	Culture time	Media Composition
Callus Induction Medium	12 days	N6 Salts (Chu et al., 1976), 2,4-D (2 mg/l, 3 mg/l and 4 mg/l), Casein hydrolysate 0.3 g/l, Myoinositol 0.1 g/l, Sucrose 3%, Gellan Gum Agar 0.3%, pH 5.8
Callus Maintenance Medium	04 days	N6 Salts, 2,4-D 2 mg/l, Vits., Casein hydrolysate 0.3 mg/l, Myoinositol 0.1 mg/l, Sucrose 3%, Gellan Gum Agar 0.3%, pH 5.8
Regeneration media	3-4 weeks	MS medium (Murashige and Skoog, 1964), Vitamins, 0.1 gm/l Myoinositol, Sucrose 3%, Gellan Gum Agar 0.3%, pH 5.8, Kinetin 0.5 mg/l, NAA:BAP (RIM I=1:2, RIM II=1:3 and RIM III=1:4)
Bacterial suspension medium	2 days	LB medium, sucrose 3%, Acetosyringone 100 µM, pH 7
Pre-induction medium	1 day	MS Salts (Murashige and Skoog, 1964), Sucrose 3%, Acetosyringone100 µM, pH 7
Infection Medium	5-10 mins.	Pre-induction medium ($O.D_{600}$ of 0.4)
Co-cultivation Medium	1-3 days	N6 salts, 2,4-D 2 mg/l, Casein hydrolysate 0.3 g/l, Myoinositol 0.1 g/l, Sucrose 3%, Acetosyringone (100µM, 200µM, 300µM), Gellan Gum Agar 0.3%, pH 5.8
Washing Medium	10 minutes	MS basal medium, Sucrose 30 g/l, Cefotaxime 500 mg/l, pH 5.8
Selection Medium	3-4 weeks	MS salts, 2,4-D 2 mg/l, Hygromycin (75mg/l), Sucrose 30%, Cefotaxime (300mg/l, 500mg/l, 700mg/l), Gellan Gum Agar 0.3%, pH 5.8
Regeneration Medium	3-4 weeks	MS basal medium, Sucrose 3%, BAP, NAA (RIM III=JP-5 and RIM II=KSK-282) Kinetin 0.5 mg/l, Hygromycin 25 mg/l, Gellan Gum Agar 0.3%, pH 5.8
Rooting Medium	2 weeks	MS Salts, Sucrose 3%, NAA 1mg/l, Hygromycin 25mg/l, Gellan gum agar 3%, pH 5.8

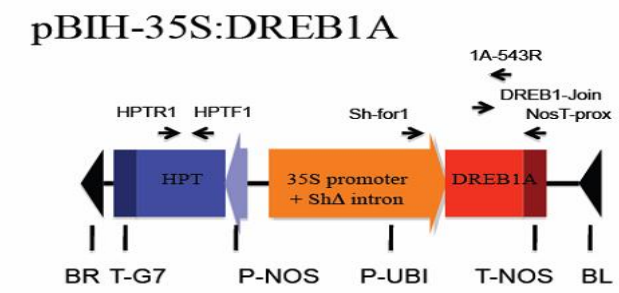

Fig 1. Schematic diagram of the structure of the T-DNA region in the plasmid pBIHCaMV35SDREB1A. LB left border, T-G7 terminator, hpt hygromycin phosphotransferase gene, P-NOS promoter, *35S*CaMV promoter, DREB1A gene, TNOS terminator of nopaline synthase gene, RB right border.

whereas the transgenic plants remained healthy and showed normal growth. After one week of drought stress, the wild type plants showed complete wilting and drying whereas the transgenic plants showed minor symptoms of drought induced damage. After re-watering, transgenic plants recovered after one week of stress while the controlled non transgenic plants died (Table 6). The *AtDREB1A* overexpression plants showed significantly increased drought tolerance in contrast to the wild type plants. The survival rate of transgenic plants were significantly higher (100%) compared with the wild-type control (0%) after drought stress. These results suggest that *AtDREB1A* overexpression under the 35S *CaMV* constitutive promoter enhanced the tolerance in rice and were found stably integrated as apparent from the RT-PCR results.

Discussion

In our study, we have optimized an effective protocol for *Agrobacterium* mediated transformation of *AtDREB1A* gene in two rice genotypes and the transgenic rice cultivars obtained were tested for their tolerance toward drought stress. The optimization of tissue culture conditions is the preliminary step for an efficient *Agrobacterium* mediated transformation experiment (Hiei and Komari, 2008). In this regards, different factors like type of explants, genotype and nutrient media supplemented with different concentrations of hormones play a crucial rule in the success of transformation (Yookongkaew et al., 2007; Sahoo et al., 2011). Firstly, we tested three different concentrations of 2,4-D for an efficient callus induction from seeds of rice. The growth regulator 2,4-D alone have been previously tested as an effective hormone for embryogenic callus induction (Rashid et al. 2001; Shahsavari et al., 2010). The two rice genotypes showed different callus induction frequency on media supplemented with three different concentrations of 2,4-D i.e. JP-5 and KSK-282 showed better callus induction frequency on concentration of 2 mg/l and 4mg/l respectively. Previous studies revealed that different genotypes have different response toward callus induction (Lin and Zhang, 2005; Ge et al., 2006). Subsequently, the seed derived calli were subjected to different hormonal combinations represented by RMI, RMII and RMIII (NAA:BAP) for regeneration. Previous studies demonstrated that synergic or antagonistic effects among plant growth regulators are very effective in tissue culture for regeneration (Ge et al., 2006). The regeneration media RM III was found effective for JP-5

Table 2. Callus induction and regeneration frequency of two rice cultivars (JP-5 and KSK-282).

Callus induction			Regeneration (%)		
2,4-D (mg/l)	JP-5 (%)	KSK-282 (%)	Regeneration media	JP-5 (%)	KSK-282 (%)
2	41.33±2.40a (86.11)	32.00±1.15b (66.66)	RM I	20.67±1.20c (43.05)	08.66±1.75e (18.05)
3	36.33±1.76ab (75.69)	35.33±0.88ab (73.61)	RM II	27.67±0.95b (57.64)	25.66±1.76b (53.46)
4	30.00±1.15b (62.50)	36.67±1.76a (76.39)	RM III	40.33±0.88a (84.03)	14.66±0.82d (30.55)

Mean values followed by the same letter with a column shows no significant diff ($p \leq 0.05$).

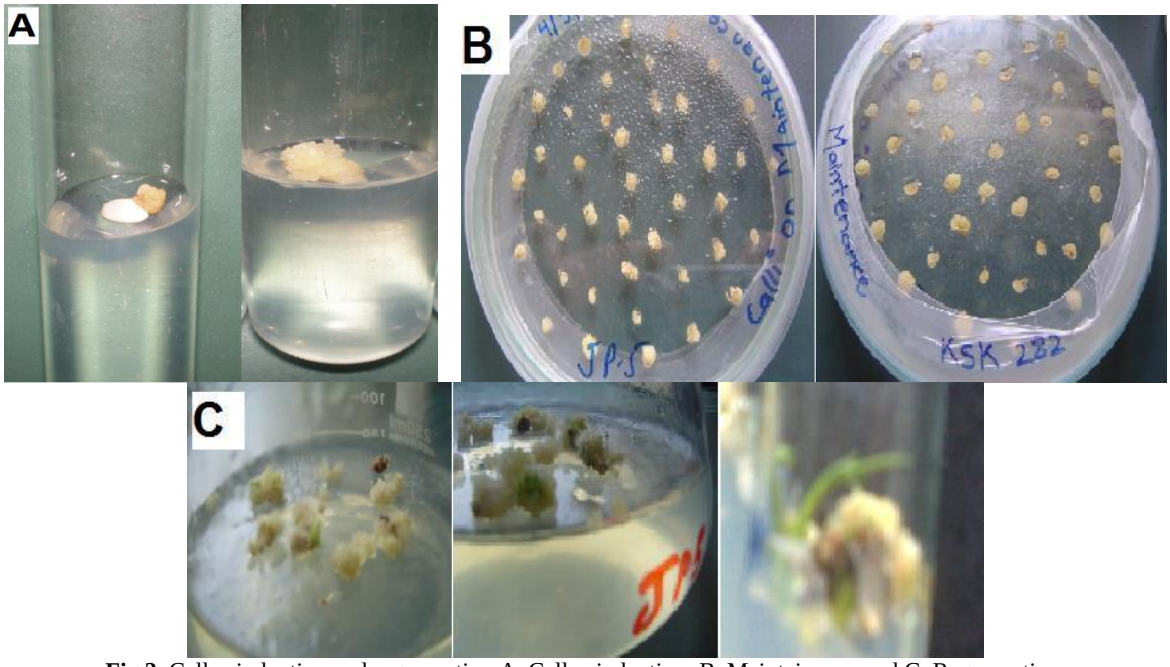

Fig 2. Callus induction and regeneration A. Callus induction, B. Maintainance and C. Regeneration.

derived calli with regeneration efficiency of 85% and maximum plantlets were produced. Our results were found in agreement with previous studies (Lee et al., 1999; Yang et al., 2000). KSK-282 genotype showed maximum regeneration efficiency of 32% and high numbers of plantlets were produced on RM II. Regeneration of an intact plant from a transformed callus represents a major constrain in the genetic transformation of rice with *Agrobacterium* (Rachmawati et al., 2004; Kumar et al., 2005). Hygromycin concentration of 50 mg/l was found optimum for the selection process, providing more stringent selection in which minimum calli were escaped whereas it has less negative effect on plantlet regeneration. Hygromycin at 50 mg/l was also used for the first successful *Agrobacterium*-mediated transformation of *Indica* and *Javanica* rice cultivars (Dong et al., 1996; Rashid et al. 1996). We have been successful in eliminating *Agrobacterium* at a lower dose of 500 mg/l after infection. A high concentration of cefotaxime was found to inhibit regeneration (Nauerby et al., 1997; Rashid et al., 1996). Various studies examined the importance of acetosyringone to *Agrobacterium* co-cultivation procedures (Rashid et al., 1996; Gould, 1997; Ali et al., 2007). The efficiency of *Agrobacterium* mediated transformation varies greatly with different concentrations of acetosyringone and there interaction with different genotypes. In our study, co-cultivation media supplemented with 100 µM acetosyringone revealed maximum transformation efficiency for both the rice genotypes i.e. 44% for JP-5 and 26.66% for KSK-282 (Fig. 3). Our results are in agreement with that of previous studies (Ali et al., 2007; Bernal et al., 2009). However, increase in acetosyringone concentration resulted in lower transformation efficiency as reported by previous study (Amoah et al., 2001). The PCR analysis led to the confirmation of the transgenic population with 28% efficiency for JP-5 and 16% for KSK-282 cultivars. Transformation efficiency varies with the genotype, type and age of explants (Hiei et al., 2008). All the PCR positive transgenic plants subjected to southern blot analysis were found to be one or two copy number integrated into their genome. We have successfully introduced DREB1A gene in two rice cultivars JP-5 (*Japonica*) and KSK-282 (*Indica*) by *Agrobacterium* mediated transformation. The transformation protocols for rice are highly genotype dependent particularly in *indica* type cultivars and development of protocol for *Agrobacterium* mediated gene transformation will pave the way for transgenic rice development in the future. The expression of transgene in T_1 transgenic plants were confirmed by RT PCR analysis. The T_1 transgenic plants showed normal growth and no significant differences were observed between transgenic and non-transgenic control plants after weighing their fresh weight for four consecutive weeks (one week interval) (Table 5). Oh et al., 2009 revealed stunted growth of transgenic rice plants after overexpression of the transgene with 35s *CaMV* promoter. DREB transcription factors play a key role in the ABA-independent stress tolerance pathways that induce the expression of numerous stress related genes in plants (Lata and Prasad,

Table 3. Determination of lethal dose of hygromycine for calli selection.

Hygromycin (mg/l)	Genotype	Calli survived (14 days)	Calli survived (24 days)	Range (5%)
25	JP-5	18.66±0.67	13.00±1.00	a
	KSK-282	14.66±0.88	09.33±1.45	
50	JP-5	12.00±0.57	09.00±1.15	b
	KSK-282	08.00±1.53	05.00±1.00	
75	JP-5	03.33±1.20	02.66±0.66	c
	KSK-282	02.33±0.33	00.00±000	

Mean values followed by the same letter within a column shows no significant differences (p≤0.05).

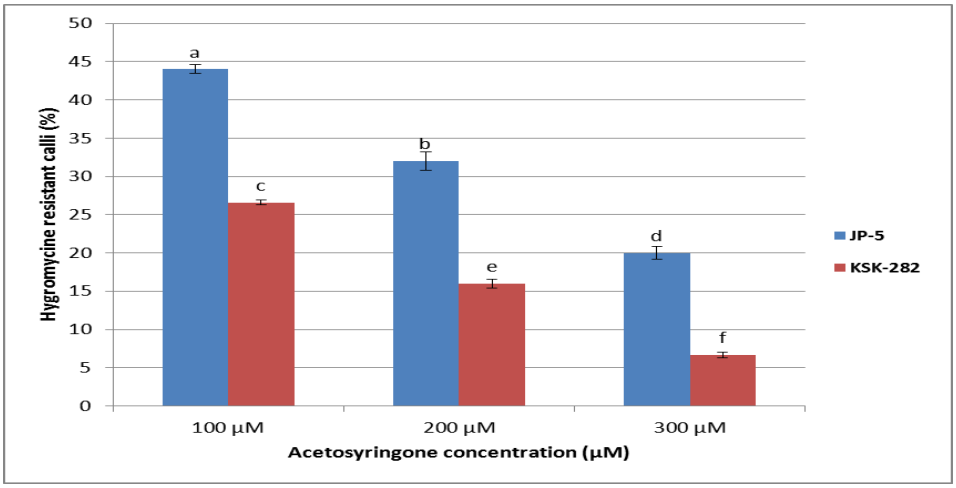

Fig 3. Percentage of hygromycine resistant calli recovered after co-cultivation on selection media. Means that do not share a letter are significantly different.

2011). We have developed transgenic plants of two rice cultivars JP-5 (*Japonica*) and KSK-282 (*Indica*) overexpressing *DREB1A* gene. The transgenic lines were tested for drought tolerance in T_1 generation at vegetative stage. Previously, numerous studies have carried out drought stress analysis of transgenic rice plants at the vegetative stage (Tang et al., 2012, Datta et al., 2012). Overexpression of transcription factors (AP37 and AP59) in rice under the control of the constitutive promoter resulted increase tolerance to drought at the vegetative stage (Oh et al., 2009). Some transcription factors (ABFs or AREBs), involved in drought stress, function mostly in vegetative stage (Kim et al., 2004; Fujii et al., 2011). The 4 weeks old transgenic rice lines were subjected to drought stress condition for one week and then re-watered for the next one week. The transgenic lines completely recovered after drought stress and demonstrated high tolerance whereas the transgenic wild type plants start wilting and completely died (Table 5). The survival rate of transgenic rice plant was significantly higher as compared to wild type plants. Previously, over-expression of several *DREB* genes in various plant species like rice (Oh et al., 2005, Chen et al., 2008, Datta et al., 2012, Ravikumar et al., 2014), Arabidopsis (Liu et al., 1998, Qin et al., 2007, Matsukura et al., 2010), wheat (Pellegrineschi et al., 2004, Shen et al., 2003) etc has resulted in plants more tolerant to drought stress.

Materials and methods

Plant materials

Mature seeds of two local rice cultivars namely JP-5 and KSK-282 collected from Crop Sciences Institute (CSI), National Agriculture Research Centre, Islamabad, were used as starting material in the present study.

Bacterial strain

AtDREB1A gene was introduced into expression vector and the recombinant binary vector, pBIHCaMV35SDREB1A, was maintained in *E. coli* DH51α strain and mobilized to *Agrobacterium tumefaciens* EH105 strains. The T-DNA region of the plasmid vector is given in Figure 1.

Callus induction and regeneration

Dehusked mature rice seeds of both rice varieties were surface sterilized with chlorox (50%) and were transferred onto N6-media (Chu et al., 1976) listed (Table 1) fortified with 2, 3 and 4 mg/l 2,4-D. The culture tubes were kept in 16/8 hrs photoperiod conditions at 25±2 ºC for 12 days. After the specific incubation period of 12 days, embryogenic calli induced from seed scutellum, were shifted to maintenance media. After subculturing, the calli were aseptically transferred to regeneration media I, II and III (Table 1) and were kept in light (16/8 hrs photoperiod) conditions for four weeks. Three independent experiments with different concentrations of hygromycin B (25mg/l, 50mg/l and 75mg/l) were carried out to determine the lethal dose for the transformation experiment.

Agrobacterium mediated transformation

The plasmid construct (Fig 1) was first maintained in *E.coli* and then mobilized into *Agrobacterium* strain EHA 105. Single colony was scrapped from selection plate of recombinant *Agrobacterium* strain EHA105 and suspended in LB liquid media containing kenamycin sulfate (250 mg/l). The suspension culture was incubated at 28 °C and 150 rpm for 2 days. The bacterial culture was centrifuged at 8000 rpm to form pellet and was re-suspended in pre-induction medium

Table 4. Determination of effective dose of cefotaxime for elimination of *Agrobacterium.*

Cefotaxime (mg/l)	Genotype	Agrobacterium contamination (%)	Range (5%)
25	JP-5	36.00±1.53	A
	KSK-282	29.33±0.33	
50	JP-5	10.67±0.33	B
	KSK-282	06.67±0.33	
75	JP-5	00	c
	KSK-282	00	

Mean values followed by the same letter within a column shows no significant differences (p≤0.05).

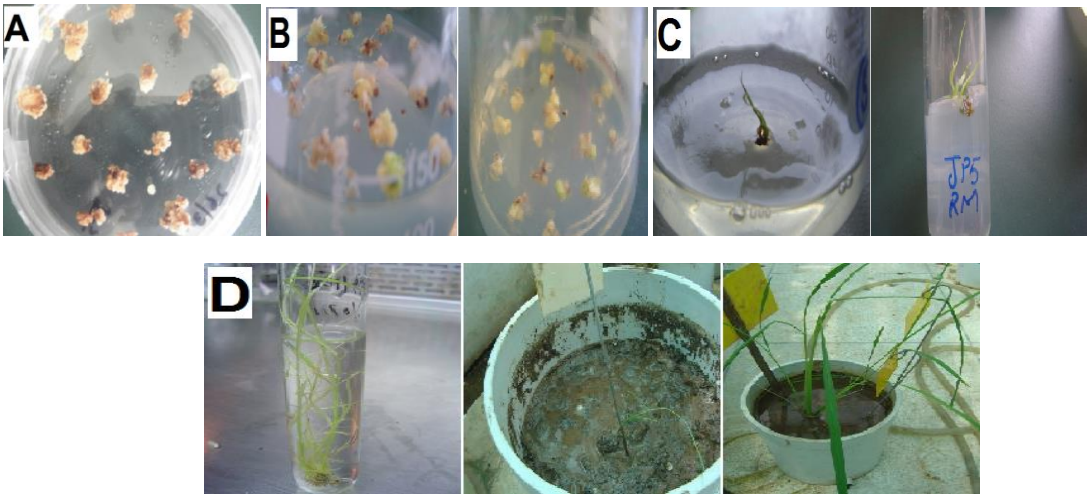

Fig 4. Different phases of transgenic plant development after co-cultivation A. Calli selection after co-cultivation B. Transformed calli with green spots C. Putative transgenic plantlet regeneration D. Acclimatization of transgenic plantlets under greenhouse condition.

Table 5. Fresh weight of transgenic and contolled plants (2 plants/sample) for four consecutive weeks in glass house at normal condition.

Time period after seed sowing	Fresh weight in gms (Control plants)	Fresh weight in gms (transgenic plants)
1 weeks	0.43±0.029	0.43±0.012
2 weeks	0.90±0.021	0.91±0.032
3 weeks	1.91±0.034	1.93±0.027
4 weeks	3.27±0.040	3.23±0.023

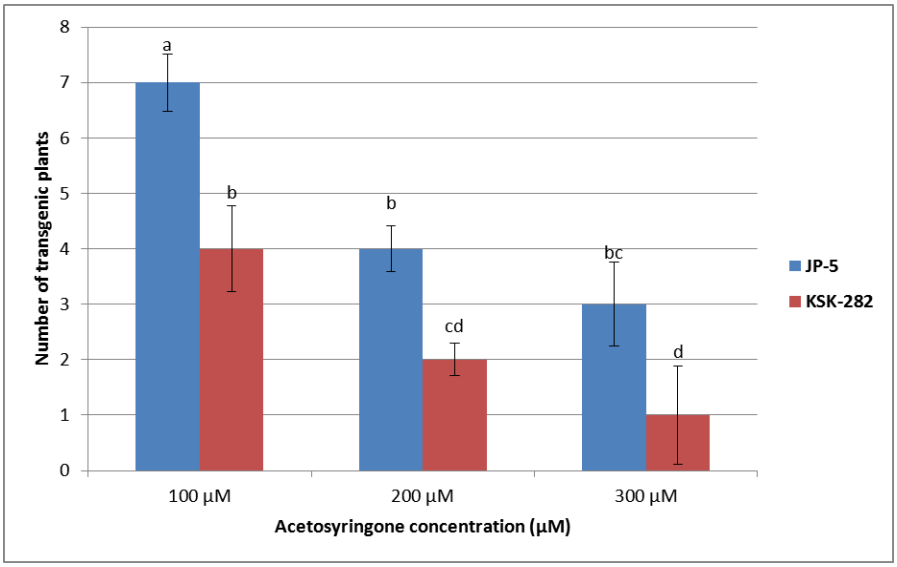

Fig 5. Transgenic plant recovered from each hygromycin resistant calli. Means that do not share a letter are significantly different.

Table 6. Reverse transcriptase-PCR analysis of transgenic rice lines and the survival rate of transgenic rice lines after one week of drought stress.

Transgenic lines	Reverse transcriptase-PCR analysis	Survival rate of transgenic rice lines after drought stress
Control	-	Died
JP-5 (1)	+	Survived
JP-5 (2)	+	Survived
JP-5 (3)	+	Survived
JP-5 (4)	+	Survived
JP-5 (5)	+	Survived
KSK-282 (1)	+	Survived
KSK-282 (2)	+	Survived
KSK-282 (3)	+	Survived
KSK-282 (4)	+	Survived

+ represent RT-PCR positive transgenic plants and – represent RT-PCR negative control plants

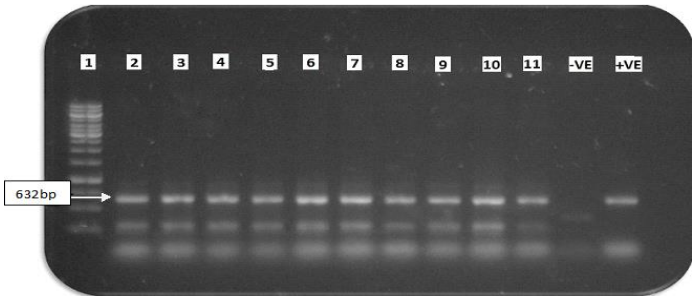

Fig 6. PCR Lane 1: Marker, Lane 1-6: Transgenic plants (JP-5), Transgenic plants (KSK-282), -VE: Control plant and +VE: Positive sample (Gene construct).

(Table 1) until it reaches to 0.4 OD_{600}. The rice calli were dried after immersion in infection medium for 5 minutes and transferred onto Petri plates containing co-cultivation media (Table 1). The efficiency of three different acetosyringone concentrations (100µM, 200µM and 300µM) was analyzed in the co-cultivation media. The calli were co-cultivated for 2 days at 25±2ºC under light (16/8 hrs photoperiod) condition. After co-cultivation, excess *Agrobacterium* was removed by washing the calli with antibiotic cefotaxime (500mg/l). The calli after drying were shifted to selection media containing hygromycin (75 g/l) and cefotaxime. Three different concentrations of cefotxime (300mg/l, 500mg/l and 700mg/l) were tested in the selection media. Petri plates were kept in growth chamber for about one months under light (16/8 hrs photoperiod) condition at 25±2ºC. The hygromycin resistance calli were calculated in percentage to the total number of calli inoculated. Hygromycin resistance cells that proliferated to white calli were removed carefully and transferred to regeneration media (Table 1), while untransformed calli were discarded. Regeneration media I (RMI) used for JP-5 and regeneration media II was used for KSK-282. The calli were shifted to growth chamber under light (16/8 hrs photoperiod) condition at 25±2ºC for about one month. The shoots developed were then transferred to rooting media (Table 1). When the roots have been well developed, plantlets were transferred to test tube containing tap water for acclimatization. Transgenic plantlets were transferred to pots in glasshouse under controlled condition and allowed to grow.

Molecular analysis

PCR analysis was performed to confirm the putative transgenic plants. DNA was extracted from leaves of putative transgenic rice plant by CTAB method (Sambrook et al., 2001). PCR analysis was performed using set of primer (*DREB1A* primers) targeting *AtDREB1A* coding sequence. The *DREB1A* primers 5'-CTACGCGTACGTACGATACA-3'F and 5'-TCGGCTTGGAGACTCCGAAT-3'R were used for amplification of 632bp fragment. The cycling parameters were pre-denaturation of 94 °C for 5 minutes and further 1 min for denaturation, annealing at 50°C for 30 sec, extension at 72°C for 1 min (35 cycles) and final extension at 72°C for 10 mins. PCR products were then analyzed by 1% gel electrophoresis inTAE buffer. The genomic DNA from putatively T_0 transgenic plantlets were subjected to Southern blot analysis. Approximately 10 µg of DNA from all the putatively transgenic and non-transgenic control plants were ristriction digested with the enzyme Sac1 and analyzed on 1% agarose gel. The restricted DNA was transferred to Nylon memberane (Amersham, Uppsala, Sweden) and hybridized with 633 bp PCR amplified non-radioactively labelled (Alkphos Direct Labeling and Detection system of Amersham Biosciences) DREB1A gene fragment. Reverese transscriptase-PCR analysis was performed for all the T1 transgenic lines to evaluate the expression of DREB1A gene. Total RNA was extracted from transgenic plants using RNA extraction kit (Invitrogen). RT-PCR analysis was carried out using Thermo script RT-PCR system (Applied biosystems). One sample of RNA was subjected directly to PCR without reverse transcription and served as the negative control while the plasmid DNA (pBIHCaMV35SDREB1A) was used as positive control. The same set of condition as described earlier for PCR was used. The amplified product was separated on 1% agarose gel and analyzed under UV-Transiluminator.

Drought tolerance analysis

In order to test the drought stress tolerance of T_1 transgenic lines during vegetative stage, the seeds of positive transgenic and wild-type plants (10 plants each, three repeats) were

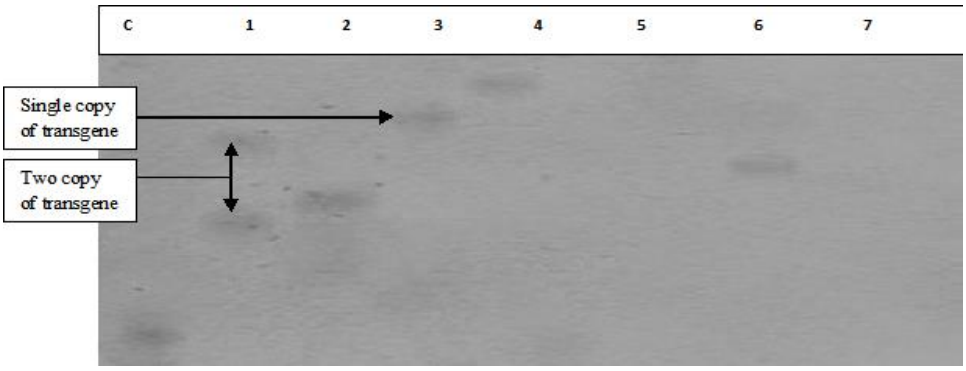

Fig 7. Southern blotting of the T0 transgenic rice plants with each lane representing the copy number of the transgenic lines. Lane 1: Positive control, Lane 2-4: Transgenic lines of JP=5, Lane 5: Negative control, Lane 6-7: Transgenic lines of KSK-282.

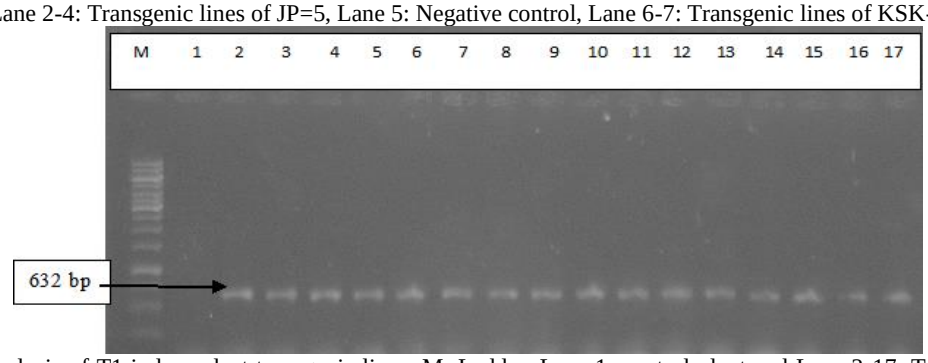

Fig 8. RT-PCR analysis of T1 independent transgenic lines: M: Ladder, Lane 1; control plant and Lane 2-17: Transgenic plants of JP-5 and KSK-282 (*AtDREB1A* amplified fragment of 632 bp).

grown in sandy soil in pots under green house condition. The growth of transgenic and wild type plants were monitored for four consective weeks. In this test, the fresh weight of transgenic and wild type plants (2 plants per sample) were compared after one, two, three and four weeks. After four weeks, drought stress testing was conducted by withholding watering of transgenic and wild type plants for one week followed by rewatering of the plants for one week. The growth of the plants were closely monitored during the period of drought stress and the survival rate of transgenic and wild type plants were recorded after drought stress condition.

Expiremental design and statastical analysis

All expirements was conducted in completely randomise design (CRD) with three treatments. Each treatment was repeated three times in the expirement and each repeat comprised of 48 and 25 explants in the tissue culture and transformation experiments respectively. The data was statistically analyzed by two factor factorial-CRD design using Minitab software. The means were compared by LSD (LSD and DMRT) using MSTATC. $p \leq 0.05$ were considered significant.

Conclusions

Our study optimized *Agrobacterium* mediated genetic transformation system in two local rice cultivars i.e. *japonica* and the difficult to transform *indica* rice cultivars. Stable transgenic line with normal plant growth and development were produced. The transgenic rice lines with strong constitutive expression of *AtDREB1A* transcription factor resulted in enhance tolerance toward drought stress. After fixing the trait in next generations, these lines might prove better candidates for developing drought tolerant local rice cultivars in the future.

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