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Genetic transformation of lowland rice variety GR11 for drought tolerance and its ratification for upland paddy cultivation

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Abstract

In the current investigation experiments were performed to produce high yielding transgenic rice plants, suitable for upland paddy cultivation in Gujarat. *DREB 2A* gene isolated from drought tolerant rice variety was placed in a binary vector driven by 35S promoter of cauliflower mosaic virus (CaMV) and a 5'non-coding region (5'-UTR) of rice alcohol dehydrogenase (*ADH*) gene acting as translational enhancer. Three to four weeks old proliferating calli of GR 11 variety of rice were used for transformation with *Agrobacterium* strains *LB 4404* harbouring *pRION_DREB 2A* plasmid. *NPT II* selectable marker gene in putatively transformed plants confers resistance to kanamycin which is used for selection of positively transformed GR 11 callus. *In vitro* regeneration of the transformed callus produced T_0 lines of rice harbouring *DREB 2A* cassette. The frequency of transformation was 10%. Stable integration of transgene conferring tolerance to drought in T_0 rice plants were verified using PCR, Reverse transcription PCR (RT PCR), Real Time PCR (absolute and relative quantification). The transgenic copy number in T_0 cisgenic lines were calculated as one to three copies of stably integrated *DREB 2A* gene per copy of genome of GR 11 rice variety.

Key words: *In vitro* regeneration, *indica* rice, *Agrobacterium tumefaciens*, genetic transformation, cisgenic plants

Introduction

Rice (*Oryza sativa* L.) has been in cultivation for thousands of years in almost all cradles of civilization, now with advancement of molecular biology it is considered as a model plant of cereal species. Rice is consumed as staple food in many parts of the world, including many developing countries in Asia, Africa, and Latin America (Mew et al. 2004). With the advent of Green revolution, rice yield witnessed a quantum leap in past three decades, although this increase in food production did not eliminate poverty and hunger, they did help to avert famine and prevent a greater disruption of the food supply in Asia (Datta 2009). With the projected increase of global population by 2050 to 9 billion, increase in water scarcity, decrease in arable land, threat of new emerging pathogens and pests and the adverse effects from climate change will extant great challenges for rice breeders and agricultural scientists to maintain and improve the yield of rice crop (Khush 1999).

Currently more than 50% of total rice growing area is under rainfed upland conditions. This area suffers from different degrees of drought during one or the other critical phases of growth. Moisture stress at the grain filling stage results in unfilled/chaffy grains which ultimately lower down the rice productivity (Sharma and Rao 2006). The slow progress in developing drought-resistant rice varieties directly reflects to the lack of a specific method for screening the large numbers of genotypes in breeding programs (Zeigler and Puckridge 1995). Thus genetic engineering can be used as a powerful and novel tool to complement the traditional methods of plant improvement to expedite faster development of stress tolerant varieties of rice (Hiei et al. 1997).

Many agronomically valuable rice genotypes are recalcitrant to *in vitro* manipulation because of their poor callus production and regeneration ability (Lee et

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al. 2002; Ozawa et al. 2003). Hence two critical steps to be mastered for transformation of plants are transfer of foreign DNA into the plant cell and regeneration of plants from transformed cells (Komari et al. 1998). Transgenic rice was first produced through direct gene transfer methodologies in late 1980s and early 1990s. Methods employing the soil bacterium *Agrobacterium tumefaciens* were developed in the mid-1990s. Rice has been transformed by polyethylene glycol (PEG), protoplast, electroporation, micro projectile bombardment (direct gene transfer) and Agrobacterium-mediated methodologies (Zaidi et al. 2006; Curtis 2010). In most of the above methods various transgenic events of rice were developed, raising considerable concerns on their commercialization; due to presence of exogenous genes from other species which could impact safety and health (Hou et al. 2014). Cisgenesis hence have great potential to overcome the major bottleneck of traditional breeding and transgenic approaches by isolating only the gene of interest from the donor plant (wild relative), which is then inserted into the recipient in one step. As no other genes are transferred, this method avoids linkage drag. This can enhance the breeding speed, particularly if several genes from different relatives must be combined into an elite variety (Schouten et al. 2006). The ectopic over expression of *DREB* transcription factors in homologous and heterologous plant systems has shown improved stress tolerance in plants, indicating a fair degree of conservation in DRE/DREB regulon system across different plant systems (Mallikarjuna et al. 2011).

In the present investigation GR 11 rice variety was taken as recipient of transformation. In Gujarat it is considered as the master card for rice cultivation with high grain yield ranging from 5500-6000 Kg/ha (Patel et al. 2010). This variety grows in irrigated transplanted lowland regions of middle and south Gujarat where there is a low chance of drought. To make it suitable for up land cultivation, transformation of GR 11 rice variety was performed with *DREB 2 A* gene isolated from AAU-DR1 a drought tolerant rice genotype of Gujarat with parentage of Sathi 34-36 (traditional variety with drought tolerance) and Dadri Kolam (upland local variety) to produce a Cisgenic line of GR 11 rice variety.

Materials and methods

Plant material

Mature seeds of GR11 and AAU-DR1 rice varieties were used as commencing material for this study. Seeds of rain fed drilled (upland) variety of rice (AAU-DR1) were chosen as plant material to isolate the gene of interest (*DREB 2 A*) due to its implicit ability to tolerate water stress.

Drought induction

The seeds were germinated in the small size plastic bags containing soil, sand, vermicompost and FYM (Farmyard manure) in the ratio of 1:1:1:1 at 32°C for 7 days in greenhouse at 80% relative humidity. The germinated seedlings were then transferred to polyhouse. After four weeks growth in polyhouse, seedlings of drought resistant variety (AAU-DR1) were subjected to progressive drought by withholding water. After ten days of drought stress, leaves of both control and drought imposed seedlings were sampled and submersed in *RNAlater* solution in ratio of 1 leaf: 4 RNAlater (Qiagen) w/v, snap chilled in liquid N_2 (–196^{o}C) and stored at - 80 ^{0}C until the time of RNA isolation.

Sequencing of DREB 2A gene

RNA extraction

Total RNA extraction from drought imposed seedlings of AAU-DR1 rice variety was done using RNeasy Plant Mini Kit (Qiagen). The cDNA was synthesized from 1.5 µg of total RNA using Superscript III reverse transcriptase enzyme (Invitrogen) in combination with 50 µM oligo (dT) $_{20}$ primers.

DREB 2A gene amplification

The primers (*DREB 2A* F: gttacatatgatggag cgggggagg, *DREB 2A* R: atgcgagctcctaatagg agaaaagg) for directional cloning were designed based on the coding sequence (CDS) of *DREB 2A* gene, accession number: AF300971 (Dubouzet et al. 2003). The restriction recognition sites are incorporated in 5'end of each primer pair respective to the multiple cloning sites (MCS) of binary vector *pRION-101 DNA*, such that the isolated gene would be ligated to the vector in right orientation in relation to its promoter and enhancer leading to its expression. The 5'sequence of forward and reverse primers was extended to incorporate restriction sites of *KpnI and SacI* respectively (underlined). After PCR with cDNA template of AAU-DR1, the 850 bp product was purified and cloned to TA cloning vector *pTZ57R/T* (Fermentas) and sequenced using vector specific primers M13_F gtaaaacgacggccagtga and M13_R caggaaacagc tatgacc using ABI PRISM® 310 Genetic Analyzer

(Applied Biosystems, USA), with BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA).

Genetic transformation and Molecular analysis of putative transformants

DREB 2A gene construct

Directional cloning of *DREB 2A* gene to transformation vector was done by sequential digestion of cloning vector harboring gene (*pTZ57R/T*) and binary vector *pRI ON 101* DNA (Takara bio.) with *KpnI* and *SacI* restriction enzymes. Ligation was performed with subsequent purified restriction products. The plasmid *pRI ON 101- DREB 2A* (with gene) cloned in *E. coli* DH5-α was mobilized into *Agrobacterium* helper strain LB4404 using freeze and thaw method of transformation (Chen et al. 1994). The developed transgene cassette consists of *DREB 2A* gene driven by 35S promoter of cauliflower mosaic virus (*CaMV*) and a 5'non-coding region (5'-*UTR*) of rice alcohol dehydrogenase (*ADH*) gene acting as translational enhancer (Fig. 1).

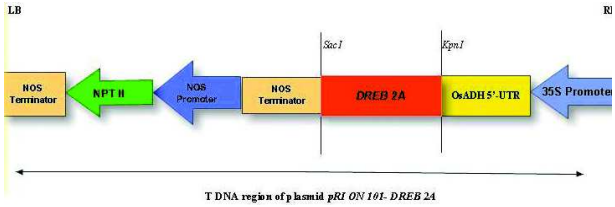

Fig. 1. The Structure *DREB 2A* gene construct: RB, LB: T-DNA border sequences, 35S promoter: Cauliflower mosaic virus (*CaMV*) promoter for expression of target gene in plant, *OsADH 5*': UTR 5' non-coding region of Rice Alcohol dehydrogenase gene (translational enhancer), *DREB 2A*: Gene of interest, KpnI and SacI: Restriction sites for directional cloning, NOS-terminator: Terminator for *DREB 2A* gene, NOS-promoter and NOS-terminator: Promoter or terminator for expression of selectable marker gene in plant, *NPTII*: Selectable marker gene in plant

Transformation of callus

The *DREB 2A* construct was introduced into GR 11 rice variety (*Oryza sativa* L. ssp. *indica*) by *Agrobacterium*-mediated transformation. The callus culture and transformation procedures were as per the protocol of Hiei and Komari, 2008. For transformation proliferating calli was incubated for 10 min at 25^{0}C in 1.0 ml of bacterial suspension (Optical density (OD) = 0.3 at 660nm). The calli were then transferred to a co cultivation medium constituting MS (Murashige and Skoog 1962) full strength + 2, 4-D (3 mgL^{-1}) + CH (1 gL^{-1}) + Proline (600 mgL^{-1}) + Glutamine (500 mgL^{-1}) + 2% Sucrose + 100 mM of acetosyringone + 0.9% and incubated in dark for 3 days at 25^{0}C. The co cultured calli was repeatedly washed with sterile water for 5 times then rinsed with antibiotic wash solution having 250 mgL^{-1} cefotaxime to kill *Agrobacterium* cells. The washed calli were cultured in first selection medium containing 30 mgL^{-1} kanamycin and 250 mgL^{-1} of cefotaxime for 14 days incubation under continuous light. Survived calli were placed in second selection medium constituting NAA (0.5 mgL^{-1}), BAP (0.1 mgL^{-1}) and 2, 4-D (1 mgL^{-1}) along with basic components of first selection medium for 14 days. The actively proliferating kan+ calli were transferred to shoot regeneration media for incubation under continuous illumination (5,000 lux) at 30^{0}C for two weeks. Regenerated shoots were transferred to root induction medium (MS half basal (A, B & F) + Kanamycin (30 mgL^{-1}) + sucrose 2 % + Agar 1 %, Activated charcoal 0.25 %) for two weeks. The T_0 plantlets with vigorous rooting were taken for acclimatization in green house (primary hardening) followed by secondary hardening in polyhouse.

Physiological analysis of the putative rice transformants (T_0 Plants)

Soil moisture content (SMC)

Soil moisture content of control and treated varieties of susceptible (GR11) and T_0 generation (cisgenic lines of GR 11) of rice plants were measured by drying method (Jackson, 1973).

Relative water content (RWC)

Leaf relative water content (RWC) was measured in control and treated varieties of susceptible (GR11) and T_0 generation (cisgenic lines of GR 11) of rice plants. Fully expanded leaves were excised and fresh weight (FW) was recorded. The leaves were then soaked for 4 hours in distilled water at room temperature under a constant light, and the turgid weight (TW) was recorded. After drying for 24 hours at 80°C, total dry weight (DW) was recorded. RWC was calculated according to the method of Barrs and Weatherley, 1962.

Leaf rolling index

On the tenth day of water stress treatment, leaf rolling index (LRI) of control and treated varieties of susceptible (GR11) and T_0 generation (cisgenic lines of GR 11) of rice plants were scored at 13.00 h using a 0 to 5 scale with 0 being no rolling, 1 being the first

evidence of rolling and 5 being a closed cylinder according to the method of O'Toole and Cruz's, 1980.

Molecular analysis of the putative rice transformants (T_0 Plants)

Confirmation of putative transformants using End Point PCR

The DNA isolated from rice transgenic plants (T_0) plants were primarily screened for the presence of the T DNA using *DREB2A* gene specific primers designed from CDs sequence of *DREB 2A* gene (SEQ Dreb2A F: TCAAATTGTGCTTACCGCGGTGTC, SEQDreb 2AR: CACGTTTCAAGCCATTGCTCCCTT). A 25 µl mixture of 50 ng of template DNA, 10 x PCR - 2.5 µl, 10 mM dNTP mixture - 0.5 µl, 50 mM $MgCl_2$ - 0.75 ml, Forward and reverse primer (10 pmoles/ml) - 0.8 ml each, *Taq* DNA polymerase (5U/µl) - 0.3 µl, Nuclease free water - 17.35 µl was prepared for the PCR assay. Initial denaturation cycle of PCR was at 94^{o}C for 5 min, followed by 35 cycles of 94^{o}C for 40 sec, 55^{o}C for 30 sec and an extension temperature of 72^{o}C for 1 min., and final extension of 72^{o}C for 10 min.The PCR products were analyzed in 2 % agarose gel.

Reverse Transcriptase (RT)-PCR analysis of putative transformants

Total RNA from T_0 generation, GR 11and AAU-DR1 rice varieties were isolated using RNeasy Plant Mini Kit (Qiagen). First strand cDNA was synthesized from 1.5 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) enzyme, 2 µl of 50 µM oligo $(dT)_{20}$ primers were used in a 20 µL reverse transcription reaction. The gene specific primers for RT PCR were designed to amplify a 200 bp amplicon (RTDreb2AF: AGGCGCCTATGGCTAGGATCATTT, RTDreb2AR: TATAGTGGCCGGCCCATTAGACAT), plasmid *pRI ON_DREB 2A* was used as positive control. A 25 µl mixture of 50 ng of template DNA, 10 x PCR - 2.5 µl, 10 mM dNTP mixture - 0.5 µl, 50 mM $MgCl_2$ - 0.75 µl, Forward and reverse primer (10 pmoles/µl) - 0.8 µl each, *Taq* DNA polymerase (5U/µl) - 0.3 µl, Nuclease free water - 17.35 µl was prepared for the PCR assay. Initial denaturation cycle of PCR was at 94^{o}C for 5 min., followed by 35 cycles of 94^{o}C for 40 sec., 55^{o}C for 30 sec., 72^{o}C for 1 min., followed by final extension of 72^{o}C for 10 min.The PCR products were ascertained in 2 % agarose gel.

Relative quantification of transgene using Real Time PCR

Transgenic zero (T_0), AAU-DR 1 and GR 11 rice seedlings of were subjected to progressive drought by withholding water for a span of 10 days, till development of physiological conditions of drought in GR 11 control variety. RNA isolation was followed by cDNA synthesis of respective samples using Superscript III reverse transcriptase (Invitrogen) enzyme. The relative quantification was used to measure the expression levels of *DREB 2A* gene. The Real time PCR primers were designed to amplify a 200 bp amplicon of target gene (F-AGGCGCC TATGGCTAGGATCATTT, R-TAGTGG CCGGCCCA TTAGACAT) and endogenous gene *tubulin* (F-ACTA CCAGCCACCCTCTGTTGTC, R-GCACAAACG CACGCTTAGCA) acted as the reference control in quantitation study. The calculation of fold levels of expression was according to the $2^{-\Delta\Delta\ CT}$ (Livak and Schmittgen 2001) method. The fold levels in up regulation and down regulation of *DREB 2A* gene was normalized by endogenous control *tubulin*. Two-step RT-qPCR was performed in fast optical 0.1 ml, 96-well reaction plates (MicroAmp™, Applied Biosystems, Cheshire, UK) using the ABI 7500 Fast, Real time PCR (Applied Biosystems, Foster City, USA) and Quantifast SYBR Green I detection chemistry (Qiagen, Germany). The reaction volume (20 µl) contained 2 µl of a cDNA diluted 4-fold, 10 µl of Fast SYBR Green Master Mix and 0.5 µM each of forward and reverse primers.

Determination of transgene copy number using Real Time PCR

A standard curve experiment was performed using Real Time PCR for determination of copy number. Gene specific primer was designed to amplify 200 bp of *DREB 2A* insert ((F-AGGCGCCTATGGCTA GGATCATTT, R -TAGTGGCCGGCCCATTAGACAT). Genomic DNA was isolated from T_0 (cisgenic) lines, AAU-DR 1 and GR 11 rice variety by protocol described in Zaidani et al., 2005. Plasmid *pTZ57R/T* (Fermentas) with *DREB 2A* insert was isolated using Qiagen plasmid mini kit. Real time PCR assay was performed in fast optical 0.1 ml, 96-well reaction plates using the ABI 7500 Fast Real Time PCR (Applied Biosystems, Foster City, USA), employing Quantifast SYBR Green I detection chemistry (Qiagen).

Standard curve for transgene copy number was generated using known amounts of Plasmid DNA

harboring *DREB 2A* gene (Size: 3736bp). The quality and quantity of plasmid DNA to prepare standard dilution series were measured in NanoDrop Spectrophotometer (Thermo scientific). Five dilution series from 10^{-1} to 10^{-5} were prepared for standards and were subsequently used generate standard curve for copy number calculation. The transgene copy number in an unknown sample was calculated by interpolation from standard curve CT values of known amount of standard DNA concentrations. Since one copy of rice genome contains 0.5 pg DNA (Bennett 1995), total rice genome copies (Y) in template DNA (50-75 ng) used for Real Time PCR is 1-1.5 X 10^5 copies. Thus the copies corresponding to each CT value of transgenic event (Z) was calculated as the ratio of amount calculated using standard curve (X) to the total amount target DNA (Y) used in Real-Time PCR. The formulae used for copy number detection of transgenic *DREB* gene in putative rice transformants was Z = X/Y (Ahmad et al. 2005).

Results

Sequencing of DREB 2A gene

The *DREB 2A* gene from draught stressed rice variety AAU-DR 1 was amplified, subsequently cloned and sequenced. The sequence analysis using Nucleotide BLAST revealed the most identical homology match with two complete CDS sequence from *Oryza sativa* with accession numbers NM_001048642.1 and AF300971.2 (Table 1). Out of the two best hits AF300971.2 locus with description '*Oryza sativa* DRE binding protein 2 mRNA, complete CDS' is the sequence from which gene amplification primers were designed.

Generation of Cisgenic plants

Proliferating scutellar calli derived from mature dehulled, healthy seeds of GR 11 rice variety were used as target tissue for *Agrobacterium* mediated transformation. Transgenic cell lines with insertion of the *DREB 2A* gene were generated using steps given by Hiei and Komari, 2008. The binary transformation vector *pRION 101 DNA* (Takara bio.) used in the study has a kanamycin resistant gene (Neomycin phosphotransferase), *NPT III*, as the selection marker for *E. coli,* Rhizobium (*Agrobacterium*) and a mutant type kanamycin resistant gene, *NPT II,* as the selection marker for plant under the control of the *35S* cauliflower mosaic virus (*CaMV 35S*) promoter. A total of 250 scutellar derived calli were employed in transformation. Assortment of putative transgenic calli was carried out in selection media constituting 30 mgL^{-1} kanamycin and 250 mgL^{-1} of cefotaxime,

Table 1. BLAST N results which holds homology with sequenced insert

S.No.	Accession	Description	Total score	Query coverage	E value	Max indent
1	NM_001048642.1	*Oryza sativa Japonica* Group Os01g0165000 mRNA, complete cds	584	100%	1e-163	99%
2	AF300971.2	*Oryza sativa* DRE binding protein 2 mRNA, complete cds	584	100%	1e-163	99%
3	AK067313.1	*Oryza sativa Japonica* Group cDNA clone:J013106O15, full insert sequence	579	100%	5e-162	99%
4	AK121956.1	*Oryza sativa Japonica* Group cDNA clone:J033107M13, full insert sequence	573	100%	3e-160	99%
5	JQ341059.1	*Oryza sativa Indica* Group cultivar Pokkali dehydration responsive element binding protein 2A (DREB2A) RNA, complete cds	523	88%	3e-145	100%
6	HM807364.1	*Oryza sativa Indica* Group DREB-like protein (DREB1) gene, complete cds	523	88%	3e-145	100%
7	FN556368.1	*Oryza sativa Indica* Group mRNA for AP2 domain containing protein (ap2 gene), cultivar Nagina 22, clone 4	523	88%	3e-145	100%
8	AK376344.1	*Hordeum vulgare* subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv3121G09	265	92%	2e-67	83%
9	XM_003569037.1	Predicted: *Brachypodium distachyon* dehydration-responsive element-binding protein 2A-like (LOC100845995), mRNA	320	97%	4e-84	85%
10	EF672101.1	*Avena sativa* putative DRE-binding protein DREB2 (DREB2) mRNA, partial cds	292	96%	8e-76	84%

allowing selective growth of Kan+ callus. A total of 25 independent Kan+ Cisgenic (T_0) lines were generated amounting to 10 % of genetic transformation frequency (Fig. 2).

Physiological analysis of T_0 generation

Physiological parameters were measured after ten days of withholding water (progressive drought) in T_0 and GR 11 rice seedlings. From 25 cisgenic lines that were generated 8 plants were randomly selected and subsequently named as $T_{0\text{-}1}$ to $T_{0\text{-}8}$. The results of soil moisture content (SMC), relative water content (RWC) and leaf rolling index (LRI) are furnished in Table 2. The SMC% of controls of both T_0 and GR 11 rice were 3.51% and 3.85% respectively, where as in case of stress treatment $T_{0\text{-}1}$ to $T_{0\text{-}8}$ lines had values in range of 1.56% to 0.95% and GR 11 control plant under stress registered 0.55%. In case of T_0, RWC% of leaves of control was 72.30% and that during stress in lines $T_{0\text{-}1}$ to $T_{0\text{-}8}$ were in range of 49.37% to 63.19%, on the other hand relative water content of leaves of GR 11 control was 71.54% and it got severely declined to 44.19% when stress was applied. In drought stress $T_{0\text{-}1}$ to $T_{0\text{-}8}$ lines rendered a modest degree of leaf rolling in range of 1.4 to 2.0 points, in contrast GR 11 seedlings experienced severe leaf rolling index of 5 under stress.

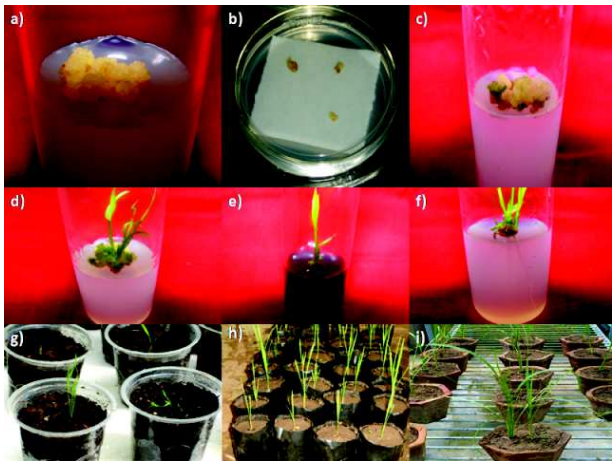

Fig. 2. Transformation of GR11 rice variety: a) Callus from GR 11 rice used for co-cultivation step, b) Co-cultivation of callus with *A. tumefaciens*, c) Shoot initiation of transformed callus in selection media, d) Multiple shoot proliferation from embryogenic calli of transformed rice, e) Regenerated shoot placed in rooting media, f) Root induction of shoots, g) In vitro regenerated transgenic rice plant in green house, h) Transgenic zero lines during secondary hardening, i) Transgenic zero lines with *DREB 2A* insert in pots

Molecular analysis of T_0 generation.

End point PCR analysis of genomic DNA isolated from T_0 plants for *DREB 2A* gene demonstrated that T DNA has been integrated into genome of selected T_0 plants, *pRI ON_DREB 2A* plasmid was used as positive control giving a characteristic band of 375 base pair (Fig. 3).

Twenty five transgenic plants were verified having *DREB 2A* gene insertion. Though stable

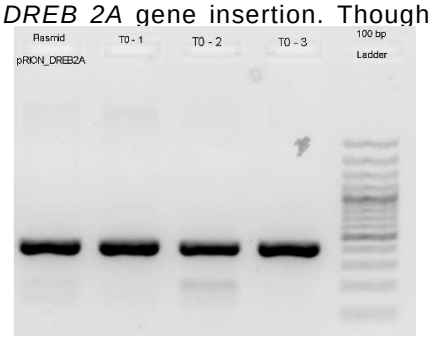

Fig. 3. Endpoint PCR of selected random T0 lines using gene specific primers

integration of transgene into the plant host genome is indicated by PCR analyses, expression of transgene is further confirmed by performing reverse transcription PCR (RT-PCR). Eight putatively transformed (T0-1 to T0-8) lines were randomly selected and analysed for effective transcription using DREB 2A gene specific primer giving a characteristic 200bp band. In all cases tested, the amplification product obtained by RT-PCR was identical to the one of the positive PCR plasmid control (pRI ON_DREB 2A) indicating that the DREB 2A gene was appropriately transcribed in the randomly selected cisgenic lines of GR 11 rice (Fig. 4).

Real Time PCR analysis of relative quantification experiment was carried out using SDS 2.0.1 software (Applied Biosystems 7500 Fast Real Time PCR system) to obtain CT, ΔCT and ΔΔCT value of respective samples and target genes (Table 3). The amplification plot and RQ Vs sample graph showed the fold levels of *DREB 2A* gene regulation in relation to reference gene *tubulin* and calibrator sample GR

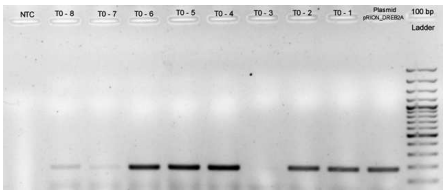

Fig. 4. RT-PCR analysis of putative transformants

Table 2. Physiological parameters measured during drought stress

S.No.	Genotypes	SMC Mean(%)	RWC Mean(%)	LRI
1	GR 11 (Control)	3.85	71.54	0
2	T_0 (Control)	3.51	72.30	0
3	T_{0-1} (Stress)	1.37	57.13	1.5
4	T_{0-2} (Stress)	1.45	59.34	1.6
5	T_{0-3} (Stress)	0.95	49.37	2.00
6	T_{0-4} (Stress)	1.56	61.76	1.4
7	T_{0-5} (Stress)	1.43	63.19	1.5
8	T_{0-6} (Stress)	1.41	58.25	1.4
9	T_{0-7} (Stress)	1.12	51.42	1.8
10	T_{0-8} (Stress)	1.20	52.16	1.6
11	GR 11 (Stress)	0.55	44.19	5

SMC = Soil moisture content, RWC = Relative water content and LRI = Leaf rolling index

Table 3. Relative quantification data sheet

Sample	Target name name	C_T mean	ΔC_T mean	$\Delta\Delta C_T$ mean
T 0	RT DREB 2A	34.98	1.156653	-0.33
GR 11	RT DREB 2A	35.45	1.487195	0.00
AAU DR 1	RT DREB 2A	34.94	1.226983	-0.26
T 0	Tubulin	33.83	-	-
GR 11	Tubulin	33.96	-	-
AAU DR 1	Tubulin	33.71	-	-

11. The results indicate T_0 lines with 0.100 fold change in expression (Fig. 5) compared to AAU-DR 1 (positive control) during drought stress in the quantitation study GR 11 rice was taken as reference sample.

Absolute quantification through Real Time PCR determined the transgenic copy number of cisgenic lines (Fig. 6). Standard curves generated from known amounts of Plasmid DNA harboring *DREB 2A gene* were used to calculate transgene copy number in eight randomly selected cisgenic (T_{0-1} to T_{0-8}) lines (Table 4). Amplification efficiency of standard curve was 102.6% (slope -3.23) with R^2 value of 0.99 which indicated successful design of qPCR assay. The transgenic copy number in T_0 lines ranged from 1 to 3 copies, which means 1 to 3 copies of stable integration of *DREB 2A* gene insert per copy of genome of GR 11 rice variety to produce cisgenic T_0 lines.

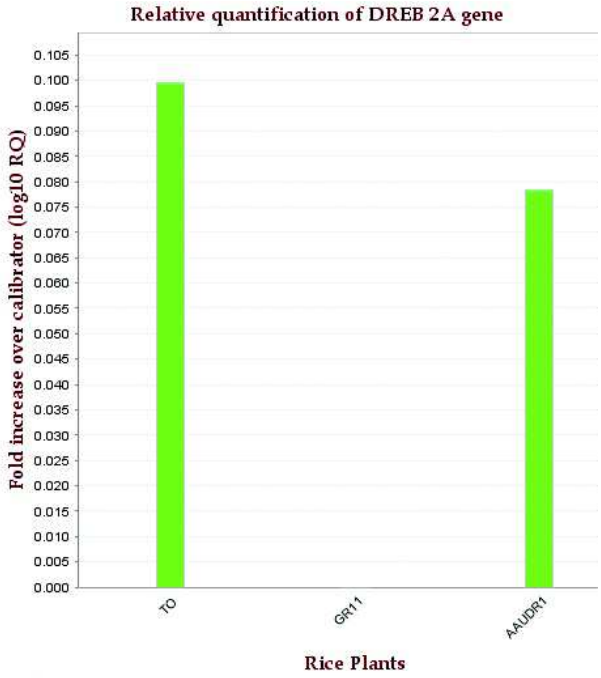

Fig. 5. Relative quantification of DREB *2A* gene

Table 4. Determination of transgene copy number through Real Time PCR

Rice lines	Genomic DNA (ng)	Genomic copy number (Y)	CT value	Real time PCR copy number (X)	Transgene copy number (Z)
T_{0-1}	20	4.75X104	29.92	5.91X104	1.0
T_{0-2}	20	4.75X104	27.67	1.18X105	2.0
T_{0-3}	20	4.75X104	29.68	5.80X104	1.0
T_{0-4}	20	4.75X104	25.12	1.77X105	3.0
T_{0-5}	20	4.75X104	25.75	1.45X105	3.0
T_{0-6}	20	4.75X104	27.13	1.18X105	2.0
T_{0-7}	20	4.75X104	30.32	5.74X104	1.0
T_{0-8}	20	4.75X104	30.14	5.65X104	1.0

Discussion

Water requirement of rice crop is comparatively higher than any other crop of the similar duration. In Gujarat rice is grown in 6.5 to 7.0 lakh hectares of land comprising 45% lowland rice and 55 % upland drilled rice. GR 11 is mega rice variety grown in regions of middle and south Gujarat with an average yield of 6000 kg/ ha. The regions of North Gujarat belonging to dry

agro-climatic zone grows coarse varieties which is not as high yielding as GR 11 variety, giving only low to medium low productivity in range of 1000 to 1500 kg/ha (Patel et al. 2010). In the current study cisgenic T_0 lines of GR 11 rice variety was developed by inculcating *DREB 2A* gene isolated from drought tolerant rice variety of Gujarat AAU-DR1. Transcription factors (TFs) are important for maintaining expression of functional protein genes in the genome. Proteins enhance or repress the TFs of candidate genes in response to biotic or abiotic stimuli and developmental processes (Sharoni et al. 2011). The dehydration-responsive element binding (*DREB*) transcription factors and/or C-repeat *CRT* binding (*CBF*) transcription factors bind to *DRE* (A/GCCGAC) and/or the *CRT* (TGG CCGAC) core cis-acting sequences in promoters of stress-responsive genes and regulate their expression in an ABA-independent manner (Shinozaki and Yamaguchi 2000). Many *DREBs* and/or *CBFs* have been identified, isolated and characterized from several plant species (Agarwal et al. 2006). In this work DREB gene was amplified, cloned and sequenced; analysis of sequence gave 100 % query coverage and maximum identity of 99 % with Accession numbers AF300971.2 and NM_001048642.1 having locus descriptions of *Oryza sativa* DRE binding protein 2 mRNA, complete cds and *Oryza sativa Japonica* Group Os01g0165000 mRNA, complete cds. Sequence analysis affirm the presence of conserved dehydration responsive element binding protein sequence in both japonica and indica groups of rice of which AAU-DR1 belong to indica subspecies (Dubouzet et al. 2003).

Genetic transformation of GR11 variety with *DREB 2A* gene isolated from a drought tolerant AAU-DR1 rice *via Agrobacterium* mediated transformation was accomplished in this work. A total 25 independent T_0 rice lines were generated; physiological parameters (SMC%, RWC% and LRI) were taken into consideration to study the effect of drought stress on T_0 plants. In comparison to GR 11 rice, T_0 lines utilized minimum amount of soil moisture to that of GR 11 variety whose soil moisture content was entirely depleted during progressive drought treatment. Similar results were registered for relative water content and leaf rolling index pointing to conclusion that T_0 lines are drought tolerant due to constitutive expression of *DREB 2A* gene which is part of *AP2/EREBP* super-family of transcription factors that regulate the expression of stress-related genes in an ABA-independent manner and exist extensively in plants during stress (Riechmann et al. 2000).

Further ratification of *DREB 2A* gene expression in T_0 generation of rice plants was done through molecular techniques. The transgene concentration in genomic DNA is usually considered in the range of femtograms (10^{-15}) which cannot be detected in the form of band through agarose gel electrophoresis. PCR helps in increasing the concentration of particular sequence of DNA from femtograms (10^{-15}) to micrograms (10^{-6}) which can be easily be detected on a gel. PCR of T_0 lines gave 375bp amplification cisgenic lines selected via kanamycin selection medium. The results are in coherence with studies of Sakuma et al. 2006, which detailed on over expression of active form of *AtDREB 2A* causing up regulation of downstream drought inducible genes thereby improving drought stress tolerance in Arabidopsis. RT PCR analysis revealed *DREB 2A* gene was properly transcribed in the tested cisgenic lines. However 200 bp gene specific band were observed to have varying degrees of intensities signifying the change in expression levels of *DREB 2A* gene transcript in respective samples. Cisgenic line $T_{0\text{-}3}$ had much lower amplicon band intensity compared other lines, similar low band intensities were observed for $T_{0\text{-}7}$ and $T_{0\text{-}8}$ lines. The integration of transgene and its expression in $T_{0\text{-}3}$, $T_{0\text{-}7}$ and $T_{0\text{-}8}$ lines were comparatively low in comparison to transgenic lines $T_{0\text{-}1}$, $T_{0\text{-}2}$, $T_{0\text{-}4}$, $T_{0\text{-}5}$,

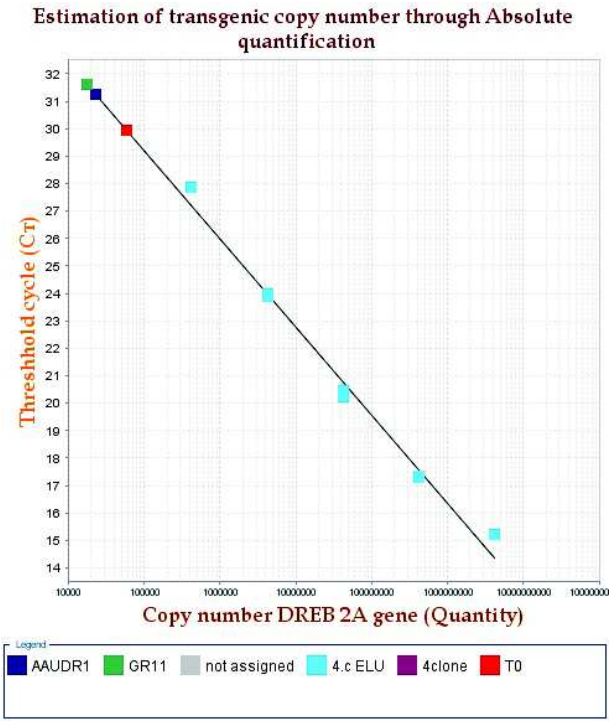

Fig. 6. Standard curve for copy number estimation of T_0 lines of rice

T_{0-6} having highest levels of expression of *DREB 2A* gene. The molecular data was supported by physiological data in which T_{0-3}, T_{0-7} and T_{0-8} cisgenic lines registered SMC % of 0.95, 1.12, and 1.20, RWC % of 49.37, 51.42 and 52.16 and LRI of 2.00, 1.8, and 1.6. The values indicate that T_{0-3}, T_{0-7} and T_{0-8} cisgenic lines have comparatively lower expression of *DREB 2A* gene which caused them to be more in stress, during induction of draught. The current finding were in congruence with the findings of Mallikarjuna et al., 2011; where in 30 T_0 rice plants expressing *OsDREB2A* were developed. RNA blot analysis revealed that the band intensity of the samples were weak during non-stress control conditions, were as high intense banding patterns were observed under osmotic and salt stresses.

Relative expression of *DREB 2A* gene in T_0 rice plants was compared with that of AAU-DR1, which is taken as reference sample during drought stress. The results show T_0 plants having 0.100 fold increase in its expression in comparison to that of reference sample (gene donor) inferring constitutive expression of coding sequence of *DREB 2A* driven by *35S CaMV* promoter and 5'-*UTR* of rice alcohol dehydrogenase (*ADH*) gene acting as translational enhancer. Expression profiling studies of *DREB 2* type gene from lettuce revealed that the expression of *LsDREB2A* was significantly increased by hyperosmotic and high salinity treatment, but not during cold, heat, nor abscisic acid (ABA) treatment (Kudo and Uno 2013). The findings of the current investigation corroborates with results of Cui et al. 2011 where, the rice *OsDREB2A* gene was isolated and expressed under the control of a stress-inducible promoter (*4ABRC*) to improve the abiotic stress tolerance of japonica rice variety TNG67. Transgenic lines over-expressing *OsDREB2A* were found to have improved survival rates under severe drought and salt stress conditions.

In transgenic research transformants at each stage must be analyzed because gene of interest or transgene is randomly inserted into the plant genome. In transgenic plants with multiple transgene copies integrated into one or more chromosomal locations often results in gene silencing. Hence estimation of transgene copy number is vital to the selection and cultivation of genetically modified (GM) plants (Flavell 1994; Vaucheret et al. 1998). In this study standard quantification assay using Real Time PCR was performed instead of conventional method of southern blotting. In comparison to southern blotting Real Time based quantification saves considerable efforts and time as southern hybridization is a labor intensive and relatively time consuming method (Ahmad et al. 2005). Southern analysis also requires relatively large amounts of plant material as starting material for genomic DNA extraction, which would be detrimental for plant involved in expression of transgene (Yang et al. 2005). There has been a recent trend towards application of quantitative Real Time PCR for determining copy number in transgenic plants, indicative of the difficulty in obtaining reliable quantitative data from Southern blot analysis, given the likelihood of unequal DNA loading and transfer and uneven backgrounds after hybridization and washing, insertion of more than one T-DNA copy at a single locus and the generation of DNA fragments of very similar sizes that are not resolved on the gels.

Quantitative Real time PCR thus allows quantitation of gene copy number as its offers the advantages of being very rapid and requiring little genomic DNA so that it is suitable for high throughput screening of transgenic plants very early in their seedling stage (Prior et al. 2006). Plants with one to three integration events in a genome commonly yield high levels of expression of the exogenous gene as shown in the study of Yang et al. 2005, where the copy numbers of GUS (β-glucuronidase) and HPT (hygromycin phosphortransferase) in primary rice transformants (T_0) were calculated by comparing quantitative PCR results of the GUS and HPT genes with those of the internal standard, SPS (sucrose phosphate synthase). In the present investigation Real time PCR based quantification was used due to its simplicity, efficiency and cost savings. Eight randomly selected cisgenic events of GR 11 rice variety had a *DREB 2A* transgene integration of 1 to 3 copies, showing stable integration of transgene to T_0 cisgenic lines.

In present study cisgenic T_0 rice lines were produced with constitutive expression of *DREB 2A* gene isolated from drought tolerant rice variety. This path was chosen to dulcify expression of gene which would otherwise be from heterologous plant systems (other than rice plant).As the actual set of target genes for each *DREB* paralog vary in different plants with different coding sequence (Nakano et al. 2006; Ito et al. 2007; Gao et al. 2009 and Qin et al. 2007). Sharoni et al. 2011, has identified 163 *AP2/EREBP* (*APETALA2*/ethylene responsive element binding protein) genes in rice. The study was carried out by analysing gene structures, phylogenies, domain duplication, genome localizations and expression

profiles. *OsAP2/EREBP* genes play an important role in the cross-talk of signalling pathways of different kinds of stresses. Matsukura et al., 2010, performed a comprehensive analysis of all five *DREB2*-type genes in rice (*OsDREB2s*: *OsDREB2A*, *OsDREB2B*, *OsDREB2C*, *OsDREB2E* and *OsABI4*) to determine which of them contribute to plant stress responses. The expression patterns of these genes were analysed under abiotic stress conditions, the sub cellular localisation and transcriptional activation activity of their translational products in protoplasts were also examined. Only *OsDREB2A* and *OsDREB2B* showed abiotic stress inducible gene expression. In addition, *OsDREB2B* showed nuclear specific localisation and the highest transactivation activity. *OsDREB2B* has functional and non-functional forms of its transcript which were similar to its orthologues in the grass family, and the functional form of its transcript was markedly increased during stress conditions. These results suggested that *OsDREB2* is a key gene that encodes a stress-inducible *DREB2*-type transcription factor that functions in stress-responsive gene expression in rice. The expression levels of *DREB2A* gene in cisgenic T_0 rice lines markedly increased during drought stress as shown by the present study making it a better candidate for upland paddy cultivation in Gujarat.

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