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GENERATION OF DROUGHT TOLERANCE IN *INDICA* RICE BY INTRODUCING *ZAT12* GENE

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ABSTRACT

The introduction of *ZAT12* gene for drought tolerance into rice cv. 'PR 116' was carried out through particle bombardment. Callus was induced on MS medium supplemented with 2,4-D (2.5 mg L^{-1}) + kinetin (0.5 mg L^{-1}) + proline (560 mg L^{-1}) + sucrose (30 g L^{-1}) + agar (8 g L^{-1}). The embryogenic scutellar calli (10-12 days old) were incubated on osmoticum medium for 4 hr followed by bombardment with isolated *ZAT-12* DNA (1 µg µL^{-1}). Next day morning bombarded calli were transferred to the recovery medium for 3 days to revive the growth followed by transfer onto the selection medium containing kanamycin (40 ppm). Percent selection recorded in genotype 'PR116' was 43.1% with the number of independent transgenic plants as 27. PCR analysis confirmed the presence of transgene *ZAT12* in 16 independent plants. These plants were grown to maturity in transgenic glass house. All the plants exhibited normal growth and development.

Key words: Abiotic stress tolerance, genetic transformation, *Oryza sativa,* transgenic

INTRODUCTION

Rice is one of the most important cash crops with > 90% rice cultivated and consumed in Asia. In 2010, approximately 154 million ha were harvested worldwide of which 48 million ha (31%) were harvested in Southeast Asia alone (FAOSTAT, 2012). Rice is prone to many abiotic and biotic stresses with drought being the major constraint to production and yield stability (Evenson *et al.*, 1996). Drought alone affects more than 70 million ha rice area worldwide (Hossain *et al*., 2000). Water deficit has increasingly become frequent in recent years even in irrigated areas due to falling water table. Rice is more susceptible to damage from water deficiency at crucial growth stages. Level of drought at vegetative stage causes moderate reduction in yield but can eliminate yield entirely if it coincides with the pollen meiosis or fertilization (O'Toole, 1982). Drought also affects the process of starch deposition in pollen grains which normally begins almost 3 days before anthesis, thereby contribute to the reduced anther dehiscence (Pantuwan *et al*., 2002). Genetic improvement for adoption of varieties to drought through conventional approaches is slow because of low heritability of yield and yield related attributes under stress, the inherent variation in field and the limitation that there is usually one experimentally droughted crop per year (Ribaut *et al*., 1997). Plants develop certain physiological and biochemical strategies to adapt to the adverse conditions (Pastori and Foyer, 2002). Abiotic stress regulations are controlled by a battery of genes which are largely governed by specific transcription factors. The members of AP2/EREBP, bZIP, zinc finger, and MYB families have been reported for their regulatory roles in plant stress or defense responses (Seki *et al*., 2003; Shinozaki *et al*., 2003). It is well documented that activation of

a specific transcription factor often results in expression of many functional genes related to stresses. Several genes encoding various transcription factors involved in complex network of environmental stress regulation are known by genome-transcriptome analysis (Riechmann *et al*., 2000). Zinc finger proteins (ZFPs) form a relatively large family of transcription regulators in plants (Kiełbowicz-Matuk, 2012; Rai *et al*., 2012). Of many stress responsive genes, the C_2H_2 type of ZFPs (also known as TF IIIA-type or krupple like) differ widely in structure and functions ranging from binding to DNA or RNA or protein (Gamsjaeger *et al*., 2007) and responses to various biotic and abiotic stresses (Davletova *et al*., 2005; Sun *et al*., 2010).

ZAT12, a member of stress-responsive C_2H_2 type ZFP is reported to control the expression of many stress-activated genes in plants mediated via signalling by reactive oxygen species (Davletova *et al*., 2005; Sun *et al*., 2010). Genes encoding stress activated transcription factors also control downstream gene(s) expression resulting in parallel increase in stress resistance (Kiełbowicz-Matuk, 2012). Altering the constitutive expression of genes encoding C_2H_2 type transcription factor resulted in improved stress tolerance in *Arabidopsis*, rice, tobacco and petunia (Huang *et al*., 2007). Using a fusion between ZAT12 promoter and the reporter gene luciferase, Davletova *et al*. (2005) demonstrated that ZAT12 expression is activated at the transcriptional level under varied abiotic stresses in *Arabidopsis*. By ZAT12 gain- and loss-of-function lines, these workers assign ZAT12 to have function during oxidative, osmotic, salinity, high light, and drought stress. Drought stress is the most important constraint limiting rice production in most rain-fed systems worldwide. The present work employs particle gun mediated transformation of rice var. 'PR 116' using a binary vector pBinAR harboring a multiple stress tolerant ZAT12 gene from *Brassica carinata* with an aim to develop drought tolerant transgenic rice plants.

MATERIALS AND METHODS

Generation of transgenic plants

Seeds of *Indica* rice variety 'PR 116' were dehusked manually and treated with 0.1% bavistin for 4 hr, followed by washing with sterile distilled water. The seeds were sterilized by 0.1% $HgCl_2$ for 6-8 min. and then rinsed with sterile distilled water. These seeds were cultured on callus induction medium (CIS) i.e. MS media supplemented with 2,4-D (2.0 mg L^{-1}), kinomycin (0.5 mg L^{-1}) containing thiamine HCl (0.1 mg L^{-1}), pyridoxine HCl (0.5 mg L^{-1}), nicotinic acid (0.5 mg L^{-1}), myo-inositol (100 mg L^{-1}), proline (560 mg L^{-1}), sucrose (3.0 g L^{-1}) and agar (0.8 %). After 7-10 days incubation on callus induction media at 25$\pm$1°C, the scuteller calli were excised and arranged in the centre of target plate (60 x 50 mm) containing osmosticum medium i.e. CIS media supplemented with mannitol (0.4 M).

Particle gun bombardment

Bombardment of embryogenic calli (Fig. 1 A, B, C) was performed using 'Bio Rad Gun' (PDS-1000/He systems).Tungsten particles (0.7 µm) coated with DNA were accelerated towards the tissue placed at 9 cm away from macrocarrier launch assembly with rupture discs withstanding 1100 psi pressure. After bombardment the petri dishes were sealed with parafilm and incubated at 25±2ºC under diffused light. After 16 hr, the bombarded calli were transferred onto MS medium for 2 d. The calli were subcultured on selection medium containing hygromycin (30 mg dm^{-3}) for two cycles of 2 weeks each and the resistant calli were regenerated into plantlets. Putative transgenic calli were transferred to shoot regeneration MS medium supplemented with benzylaminopurine (BAP 2.0 mg dm^{-3}) + kinetin (0.5 mg dm^{-3}) + naphthalene-acetic acid (NAA; 0.5 mg dm^{-3}) + hygromycin (30 mg dm^{-3}) + sucrose (30 g dm^{-3}) + agar (8 g dm^{-3}). After 2 weeks, the surviving calli were again transferred to fresh shoot regeneration medium. Regenerated plantlets were rooted on basal ½MS medium + hygromycin (30 mg dm^{-3}). Putative transgenic plantlets were hardened by

placing them on absorbent cotton in test tubes initially for 1 week where plantlets resumed their growth. Plantlets were then directly kept in test tubes in tap water for another week where profuse rooting occurred and the plantlets got ready for transferring to the soil. Hardened plantlets were finally transferred to the soil in plastic pots and kept in transgenic glasshouse maintained at 32°C with relative humidity of 80% and 16 hr photoperiod.

Screening of putative transgenic plantlets

Putative transformants were screened by PCR analysis using rice genomic DNA from non-transformed and putative transgenic plants as template and *ZAT-12* forward (5' ACTAGTCAGAAG AAAAATGGTTGCGATA-3´) and reverse (5´- GGATCCGAAAAATTCAAAGAATGAGAC-3´) primers. The PCR was carried out in 0.02 cm^3 solution comprising 50 ng rice genomic DNA, 0.004 cm^3 of 5× PCR buffer (Promega, Madison, WI, USA) (pH 8.5), 1.2 cm^3 of 25 mM $MgCl_2$, 3.0 cm^3 of 1.0 mM dNTP mix, 1 U Taq DNA polymerase and 1 cm^3 of 20 µM of each primer. The PCR profile was an initial denaturation at 94ºC for 4 min. followed by 40 cycles of denaturation at 94ºC for 1 min., primer annealing at 54ºC for 2 min. and enzymatic extension at 72ºC for 1 min. and final extension at 72ºC for 7 min. followed by rapid cooling at 4°C.

RESULTS AND DISCUSSION

To examine whether *ZAT12* has potential to enhance tolerance of rice plants to drought, we performed transformation of *ZAT12* gene in rice via particle gun (Fig. 1). For transformation a total of 10 experiments were carried out and a total 2750 calli were bombarded with *ZAT-12* gene (Table 1). After two cycles of selection, 301 calli were selected which were capable of growing on selection medium containing kanamycin (40 mg L^{-1}) (Fig. 1D). Further, 253 calli were transferred to regeneration medium in presence of kanamycin (40 mg L^{-1}) and were incubated under good light intensity (5000 lux). After three weeks of incubation, some of the calli exhibited the development of green spots and shoot primordia along with shoot regeneration. Complete plantlets were developed after 5 weeks incubation. Sixteen independent putative transgenic plantlets were regenerated (Fig. 1F,G). Sometimes 35.0% sub-cultured calli turned necrotic (brown). Histochemical GUS assay was carried out by randomly selecting the calli portions. GUS assay showed Gus expression in 54.3% calli. The regenerated plants were also tested for GUS expression. The GUS expressing tissues showed the development of blue colour in tissues; whereas, the non-transformed (control) were of the normal colour. A total of 27 independently regenerated plantlets were tested of which 18 were GUS positive. Plantlets regenerated via tissue culture are usually very fragile and most of these die if directly transferred to the field, mainly because of sudden transplantation shock. In case of *ZAT-12* putative rice transgenics confirmed by kanamycin

Table 1: Genetic transformation of rice variety PR 116 by bombarding scuteller-derived calli with *ZAT12* gene

Experiment	Calli bombarded (No.)	Calli selected on 40 mg L^{-1} kanamycin (No.)	Calli transferred to regeneration medium having 40 mg L^{-1} kanamycin (No.)	Independent regenerated plants (No.)
1.	320	28	26	2
2.	250	32	31	1
3.	290	24	22	1
4.	230	37	20	3
5.	250	27	25	2
6.	340	30	29	1
7.	300	33	23	2
8.	200	24	22	2
9.	260	29	28	1
10.	310	37	27	1
Total	2750	301	253	16

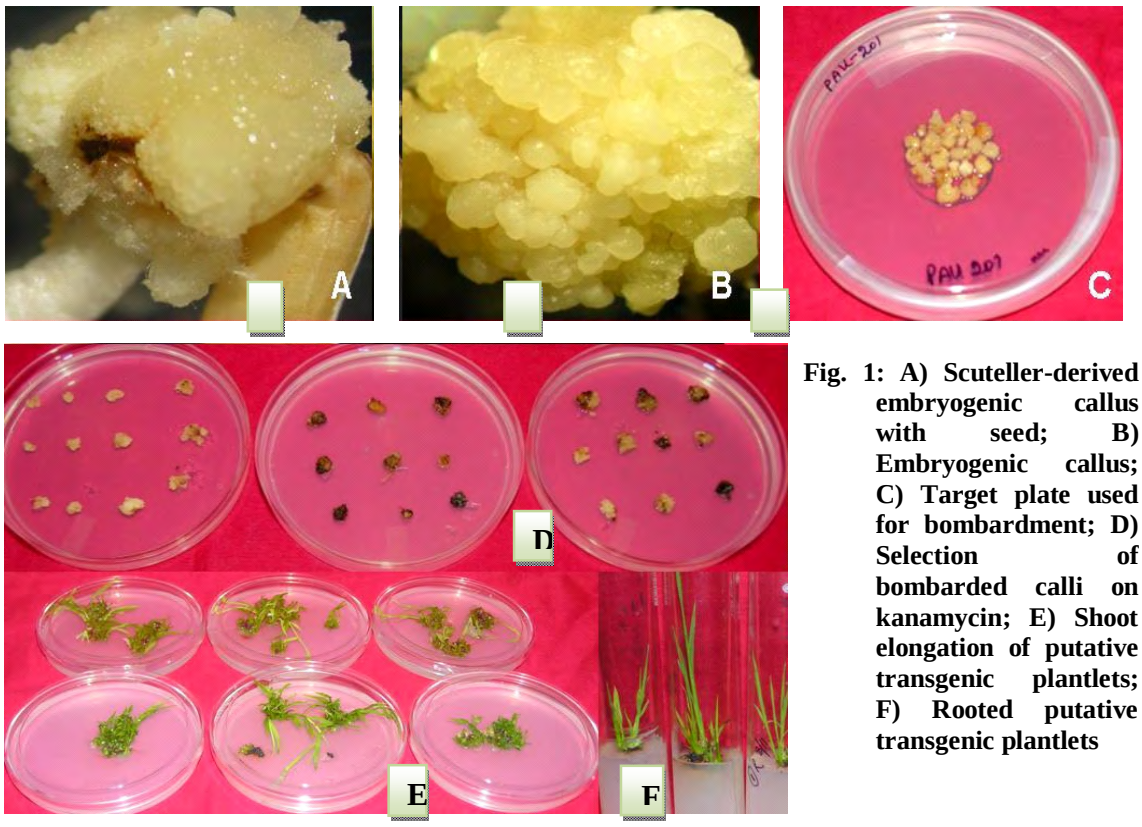

Fig. 1: A) Scuteller-derived embryogenic callus with seed; B) Embryogenic callus; C) Target plate used for bombardment; D) Selection of bombarded calli on kanamycin; E) Shoot elongation of putative transgenic plantlets; F) Rooted putative transgenic plantlets

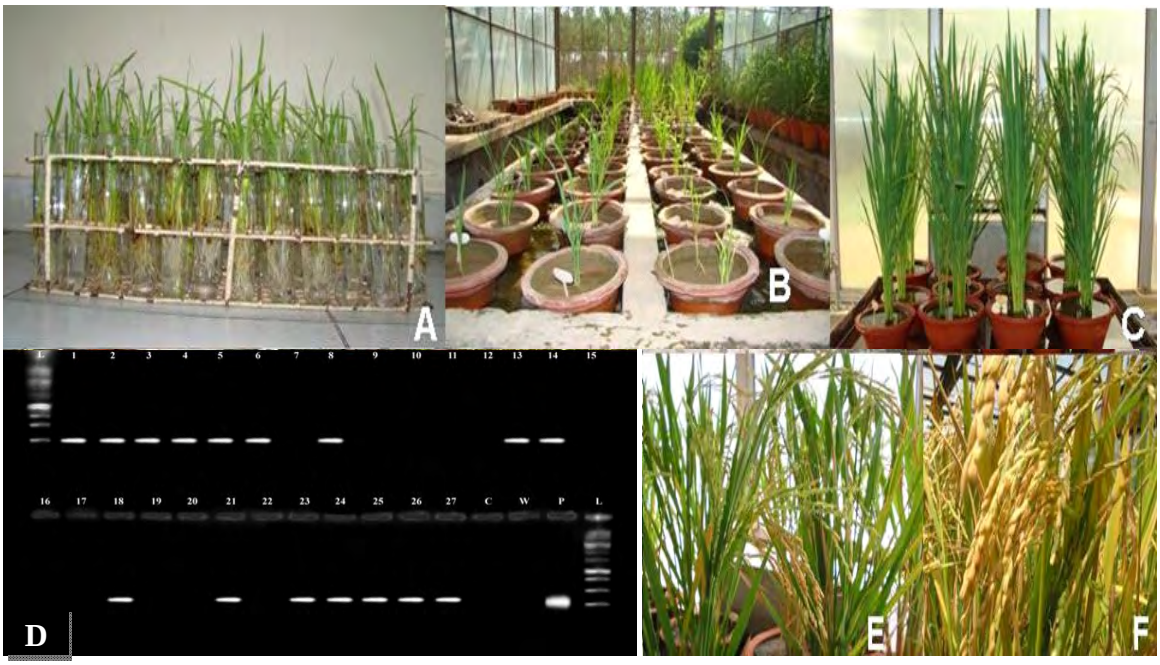

Fig. 2: A) Hardening of putative transgenic plants; B) Transfer of putative transgenic plants in glass house; C. Establishment of putative transgenic in glass; D. PCR amplification of *ZAT12* in transgenic rice plants; E-F. Transgenic plants showing normal pollen and seed set

resistance, GUS expression, 27 T_0 plants of variety PR 116 were hardened and transferred to soil in earthen pots and grown in transgenic glass house which exhibited normal growth and flowering. PCR analysis confirmed the presence of transgene of 550 kb in 59.2% independent plants when

total genomic DNA from various independent putative transgenic plants was used as template and end sequences of *ZAT-12* were used as primers in PCR analysis. All the plants exhibited normal growth and development (Fig. 2E,F). The average plant height was 161 cm, average number of tillers plant^{-1} were 8, average number of panicles 9 and percent fertility was 96.0, which were at par with the control. The transgenes did not exhibit any effect on regeneration, growth or morphology of the resultant transgenic plants (Fig. 1). These putative transgenic plants were screened using PCR which confirmed the presence of the transgene in T_o generation i.e. presence of 550 kb *ZAT* in 15 independent plants (Fig. 2D). Rai *et al.* (2013) demonstrated a proper integration of BcZAT12 gene in tomato cv. H-86. The transformed tomato plants over-expressed BcZAT12 transcripts and were tolerant to drought stress. Transgenic tomato line ZT1 and ZT5 were better adapted to drought and could be used for augmenting production of tomato in drought affected areas. Rice varieties differ greatly in their tolerance to drought. Genetically drought tolerance of rice is a complex trait under polygenic control and involves complex morpho-physiological mechanisms (Li and Xu, 2007). Improvement in yield under water deficit has been limited, in part because drought tolerance in rice is poor as compared to other cereals (Laftte and Bennet, 2003). In this situation genetic engineering can go a long way to get desired water stress tolerance by water use efficiency in commercial varieties of rice and may address many unsolved questions of crop design for abiotic stress tolerance in rice plants.

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