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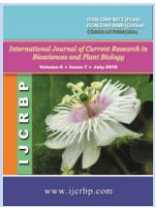

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Developmental Defects in Pusa Basmati 1 Transgenic Rice Plants Harboring Antisense *OsiEZ1* Gene

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ABSTRACT

OsiEZ1 is a member of evolutionary conserved Polycomb Repressive Complex 2 (PRC2) group of proteins which participate in stable maintenance of repressive states of genes on which they act upon. It belongs to Enhancer of Zeste (E(Z)) class, the proteins which carry the SET domain having histone methyltransferase activity. The PRC2 complex components and other SET-domain containing proteins act in a multitude of developmental processes in plants. The prominent aspects of plant life controlled by them include vegetative to floral transition, maintenance of meristem identity, vernalization; and gametophyte and seed development. To ascertain the role of *OsiEZ1* gene in plant development, antisense transgenics were constructed in *Oryza sativa* L. subspecies *indica* (var. Pusa Basmati 1) to partially suppress the functions of the endogenous gene. Some significant phenotypic changes were observed among a fraction of the R_1 and R_2 generation of *OsiEZ1* antisense transgenic plants, with respect to the wild-type plants. The phenotypic differences observed were curling of leaves and multiple shoot formation at young seedling stages. Some of the transgenic plants (both in R_1 and R_2 generations) exhibited lethal phenotypes with profuse root growth, multiple shoot formation, dwarfism and leaf-senescence progressing from the tip to the base of the plant. Our preliminary studies definitely indicate that *OsiEZ1* plays some important role in controlling early development in Pusa Basmati 1 rice plants.

Introduction

Proper development of any plant or animal depends upon the correct spatial and temporal expression of genes. Various physiological and developmental processes require switching on or switching off of particular genes in particular tissues for defined periods. The establishment and maintenance of expressed and repressed states of genes are most crucial requirements for appropriate functioning of the living system (Satijn and Otte, 1999; Guitton and Berger, 2005a; Pien and Grossniklaus, 2007; Schwartz and Pirrotta, 2007). Polycomb Repressive Complex 2 (PRC2) is a class of Polycomb Group

(PcG) of proteins which are required for stable maintenance of repressive chromatin states of genes, thus they act as negative regulators of target gene expression (Tie et al., 2001; Ringrose and Paro, 2004; Wang et al., 2006; Whitcomb et al., 2007). PRC2 complexes are composed of four distinct classes of proteins, named as enhancer of zeste (E(Z)), Suppressor of zeste12 (Su(Z)12), Extra sex combs (ESC) and nucleosome remodeling factor 55 (Nurf55). E(Z) proteins along with Su(Z)12, ESC and Nurf55 form the PRC2 complex in *Drosophila* (Czermin et al., 2002; Muller et al., 2002) , which is essential for maintaining HOX (homeobox) gene silencing during development (Simon and Tamkun, 2002; Ringrose and Paro, 2004). PRC2 is present in mammalian system as well with EZH1/2 (E(Z) components), SU(Z)12, EED (ESC homolog), and Rbap46/p48 (Nurf55 homologs) being the constituents of the complex (Cao et al., 2002; Kuzmichev et al., 2002; Kuzmichev et al., 2004). In *Arabidopsis*, there are three E(Z) homologs namely MEDEA (MEA), Curly Leaf (CLF) and Swinger (SWN) (Ng et al., 2007), three SU(Z)12 homologs: FIS2 (Fertilization Independent Seed 2), VRN2 (Vernalization 2) and EMF2 (Embryonic Flower 2) (Chanvivattana et al., 2004), one ESC homolog: FIE (Fertilization Independent Endosperm) (Ohad et al., 1999), and five Nurf55 homologues: MSI 1-5 (Multicopy Suppressor of IRA1) (Kohler et al., 2003a). Rice contains two E(Z) homologs: OsClf and OsiEZ1, two ESC members: OsFIE1 and OsFIE2, two Su(Z)12 members: OsEMF2a and OsEMF2b (Luo et al., 2009) and four MSI1 homologs. Hence, combinatorially distinct PRC2 complexes could be formed in plants while swapping the individual partners. PRC2 complex proteins participate in controlling seed development (Chaudhury et al., 1997; Luo et al., 1999; Kohler et al., 2003a; Kohler et al., 2003b; Wang et al., 2006), vegetative to floral transition (Bouveret et al., 2006; Calonje et al., 2008; Jiang et al., 2008), vernalization (Gendall et al., 2001; Schonrock et al., 2006; Wood et al., 2006; Schmitz et al., 2008), and proper gametophytic development in *Arabidopsis* (Johnston et al., 2008). The enzymatic activity associated with E(Z) proteins is histone 3 lysine 27 (H3K27me3) trimethylation (Czermin et al., 2002; Muller et al., 2002).

OsiEZ1 belongs to E(Z) class of proteins, which harbors SET domain. SET domain derives its name from three chromatin modifying proteins in *Drosophila*: **S**uppressor of variegation 3-9 (Su(var)3-9), **E**nhancer of zeste (E(z)) and **T**rithorax (Trx) (Tschiersch et al., 1994; Stassen et al., 1995; Alvarez-Venegas and Avramova, 2002). SET domain proteins are lysine methyltransferases known to methylate specific lysine residues on various protein substrates, predominantly histones (Dillon et al., 2005). Severe morphological abnormalities are associated with the mutants of key *PRC2* components in *Arabidopsis* (Ohad et al., 1999; Guitton and Berger, 2005b; Makarevich et al., 2006). Female gametophytic development is severely affected. Female gametophytic mutations affecting the initiation of seed development include *fertilization-independent endosperm* (*fie*), *medea* (*mea*), and *fertilization-independent seed2* (*fis2*) and *multicopy suppressor of IRA1* (*msi1*) (Ohad et al., 1996; Chaudhury et al., 1997; Kohler et al., 2003b). In *Arabidopsis*, MEA, FIE, FIS2 and MSI1 together form the repressive PRC2 complex named FIS2 complex, and loss of any subunit of this complex causes fertilization-independent seed development and seed abortion. Vernalization is a term describing the promotion of flowering after exposure to cold. The role of many proteins have been established in mediating the vernalization response in *Arabidopsis* including PRC2 components, VRN2, SWN, CLF, FIE, MSI1, (Wood et al., 2006). Several other aspects of flowering are also controlled by PRC2 proteins. A PRC2 complex containing CLF, FIE and EMF2 repress *FT* (*Flowering Locus T*) expression during vegetative development to repress the floral transition (Jiang et al., 2008). Leaf morphology is also controlled by PRC2 components. In *clf* (E(Z) class member) mutants in *Arabidopsis*, rosette leaves are short and curly (Goodrich et al., 1997). Role of PRC2 group genes are being investigated in rice as well. T-DNA insertional mutants of three polycomb group genes have been analyzed. No morphological changes were reported in

homozygotes of *Osclf* and *Osfie1* mutants compared to the wild-type. However phenotypic differences have been found in *OsEMF2b* mutant with fewer florets and abortive florets being two striking features (Luo et al., 2009).

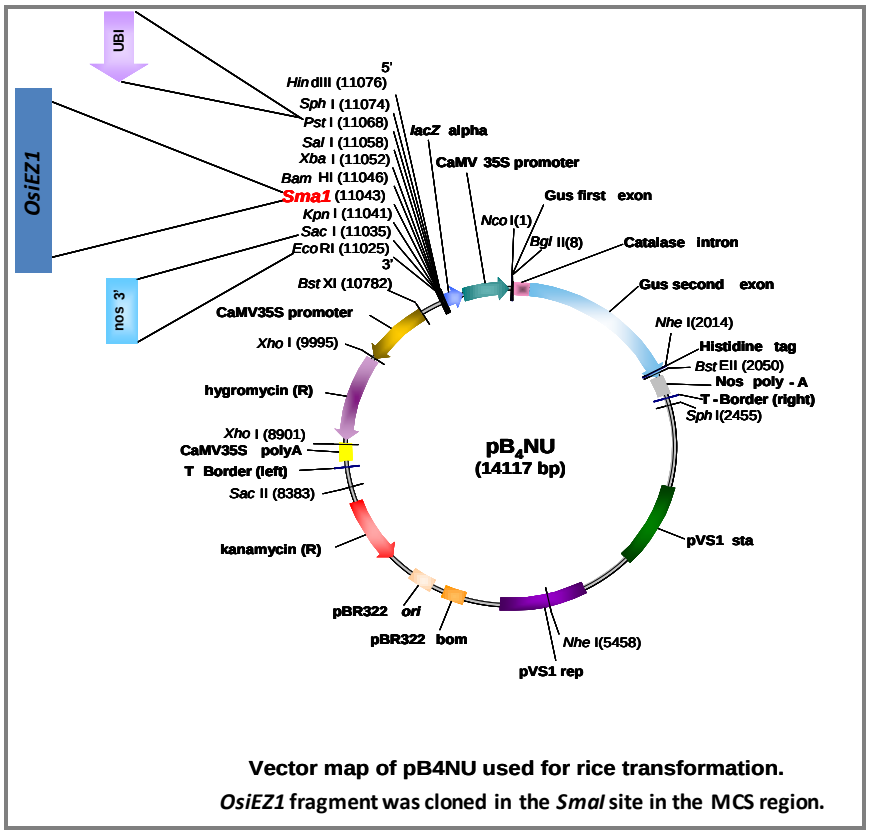
OsiEZ1 gene has been shown to express fairly in a variety of rice tissues including root, shoot, rachis and leaf but expressed more in flower. Also the expression seemed to be developmentally regulated during early stages of plant life (Thakur et al., 2003). The gene also expresses well in panicle, ovary, anther and endosperm (Luo et al., 2009). It was, hence, thought to further investigate the role of the gene using antisense transgenic approach which would most likely bring down the titer of sense transcript and shed some light on the processes in which the gene is involved. *Oryza sativa* L. subspecies *indica* (var. Pusa Basmati 1) was used to generate the antisense transgenics for this study.

Materials and methods

Cloning of gene segment

Vector used for rice transformation

Plant expression vector pB_4NU was used for *Agrobacterium*-mediated rice transformation. The transgene expression is driven by monocot-specific *ubiquitin* promoter. The vector imparts resistance to kanamycin and hygromycin antibiotics.



Vector map of pB4NU used for rice transformation.
***OsiEZ1* fragment was cloned in the *SmaI* site in the MCS region.**

OsiEZ1 segment cloned for antisense production

The 3'-region (1960 base pair) of *OsiEZ1* cDNA harboring the SET domain-coding segment was cloned in plant expression vector pB_4NU in the MCS region at *Sma*I site using blunt-end cloning. The segment was earlier cloned in pBK-CMV vector (Stratagene) between *Eco*RI and *Xba*I sites, and was kindly provided by Dr. Jitendra K. Thakur, who deduced the entire sequence of the gene from *Oryza sativa* L. subspecies *indica* (var. Pusa Basmati 1) (Thakur et al., 2003). The segment was

transferred from pBK-CMV to pB_4NU vector. The cloning in pB_4NU was verified by digestion using *Eco*RI and *Bam*HI. The components of the reaction mix have been given below.

Components	Volume (µl)
Autoclaved MQ water	9.0
10X Buffer A (Roche)	2.0
Plasmid	8.0 (~400 ng)
*Bam*H1	0.5
*Eco*R1	0.5
Total volume	20.0

The reaction mix was incubated at 37°C for 3 h and then resolved on 1% agarose gel. The positive clones in pB_4NU confirmed by the above digestion reaction were further analyzed to determine the sense-antisense orientation of the insert *OsiEZ1*.This was confirmed by digestion using *Sac*I. The components of the reaction mix are given the table below.

Components	Volume (µl)
Autoclaved MQ water	15.0
10X Buffer A (Roche)	2.0
Plasmid	2.0 (~500 ng)
*Sac*1	1.0
Total volume	20.0

The reaction mix was incubated at 37°C for 3h and then resolved on 1% agarose gel. The positive clone obtained in antisense orientation was then transformed into *Agrobacterium tumefaciens* strain AGL1 for *Agrobacterium*-mediated rice transformation.

Preparation of *E. coli* competent cells

A single colony was picked from a freshly streaked plate of XL1-MRF' *E. coli* and inoculated into 25.0 ml of Luria-Bertani (LB) medium (Pronadisa, Madrid, Spain) with 25.0 µl of Tetracycline and incubated at 37°C, 200 rpm, overnight. Next day, 300 µl of this primary culture was subcultured into fresh 25 ml of L.B medium with 25 µl of Tetracycline and incubated at 37°C at 200 rpm for 1 to 1.5 hour till the absorbance at 600 nm reached 0.5-0.6. The culture was then pelleted in a pre-chilled centrifuge tube at 5,000 rpm for 5 minutes at 4°C. The pellet was resuspended in 5 ml of pre-chilled 0.1 M $MgCl_2$ and incubated in ice for 20-30 minutes. Cells were again pelleted at 5,000 rpm for 5 minutes at 4°C and the pellet was resuspended in 5 ml of pre-chilled 0.1 M $CaCl_2$ followed by incubation in ice for 20-30 minutes. The cells were again pelleted and finally suspended in 500 µl pre-chilled 0.1 M $CaCl_2$ and incubated in ice for 2 hours to develop competency. The suspended cells were then directly used for transformation or stored at -80°C after adding 20% glycerol.

Chemical transformation

An aliquot of 50-100 µl of competent cells was taken for a single transformation reaction, mixed with an appropriate volume of plasmid or ligation product and incubated in ice for 30 minutes. Heat-shock treatment was then given alternately at 42°C for 35 seconds and at 4°C for 45 seconds, 4-5 times, followed by incubation in ice for 10 minutes. Later, 500 µl of L.B. broth was added to the mix and the contents were incubated at 37°C for 1 hour at 200 rpm. The revived product was then plated on L.B. agar plates containing suitable antibiotic and incubated at 37°C overnight.

Plasmid isolation

Single colony was inoculated into L.B. medium with suitable antibiotics and incubated at 37 °C overnight at 200 rpm. Next day, the cells were pelleted at 5,000 rpm for 5 minutes. The pellet was suspended in 100 µl of Lysis solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-Cl, pH 8.0) containing 100 µg/ml of RNase followed by incubation in ice for 10 minutes. Then, 150 µl of freshly prepared Lysis solution II (0.2 N NaOH, 1 % SDS) was added, mixed well by inverting the tube and incubated at room temperature for less than 5 minutes. Subsequently, 200 µl of pre-chilled Lysis solution III (3M CH_3COOK, 1.8 M Glacial Acetic Acid) was added to the suspension and incubated in ice for 10 minutes. The contents were

then centrifuged at 13,000 rpm for 10 minutes at 4°C and, to the upper aqueous layer, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was added, mixed well and centrifuged again at 13,000 rpm for 15 minutes at 4°C for removing the proteins. The upper aqueous layer was taken and mixed with equal volume of chloroform:isoamyl alcohol and centrifuged at 13,000 rpm for 10 minutes at 4°C. Subsequently, plasmid DNA was precipitated with an equal volume of isopropanol, followed by washing of the pellet twice with 70% ethanol and then air dried. After that, the pellet was dissolved in 20 µl of 10 mM Tris-Cl.

Automated DNA sequencing

The cloned DNA fragments were sequenced from both ends in an automated DNA sequencer (ABI, 3700 DNA Sequencer), using Thermosequenase Dye Terminator cycle sequencing kit (Amersham, International Inc., UK) as per manufacturer's instructions.

Preparation of *Agrobacterium* competent cells, transformation and plasmid isolation

A single colony of *Agrobacterium tumefaciens* from a freshly streaked plate was inoculated into 25.0 ml of YEM medium (0.2 g/l $MgSO_4$.7H2O, 0.5 g/l KH_2PO_4, 1.0 g/l NaCl, 1.0 g/l yeast extract, 10.0 g/l mannitol) containing 100 µg/ml rifampicin. This primary culture was incubated at 28°C ± 1°C at 200 rpm overnight. Next day, 2 ml of this primary culture was subcultured in 25.0 ml of fresh YEM containing suitable antibiotic. The growth was checked till the absorbance reached 0.4-0.5. The cells were then pelleted at 4,000 rpm, at 4°C, and resuspended in 1.0 ml of chilled 10 mM $CaCl_2$. For transformation, 1 µg of plasmid was transformed into 100 µl of competent cells and incubated at 37°C for 5 minutes. To this, 700 µl of YEM was added and kept for recovery at 28°C ± 1°C, 200 rpm, for 14-16 h. The recovered cells were plated on YEM plates containing 100 µg/ml rifampicin and 50 µg/ml Kanamycin and the plates were incubated at 28°C for 2-3 days till the appearance of colonies. The transformed colonies were confirmed by PCR and restriction digestion. For plasmid isolation, single colonies were inoculated into 5 ml of YEM medium with suitable antibiotics and incubated for 2 days at 200 rpm at 28°C ± 1°C. The cells were pelleted and plasmid was isolated following the same protocol as for bacterial plasmid isolation, with the exception that lysozyme (20 µg/ml) was added to the Lysis solution I used during isolation.

Agrobacterium-mediated rice transformation

Inoculation of seeds

Dehusked seeds of Pusa Basmati 1 were surface-sterilized with 0.1% mercuric chloride (Qualigens) and few drops of teepol, for 10 minutes, followed by several washes with autoclaved Milli-Q water (Millipore Water Purification Systems, France). Seeds were soaked overnight in sterile Milli-Q water in dark. The seeds were then blotted on sterile tissue and inoculated on 2 MS medium (Murashige and Skoog, 1962) containing 30 g/l sucrose, 2 mg/l 2, 4-D and 4 g/l phytagel, pH 5.8. The plates were incubated at 28°C ± 1°C in dark for 21 days for callus initiation. The calli were then subcultured on 2MSCA (2MS medium, 1 g/l casamino acid, pH 5.8) for 4 days in dark. Same day, *Agrobacterium* strain, AGL1, containing the desired construct was inoculated in YEM broth containing suitable antibiotics and incubated at 28°C ± 1°C, 200 rpm, in dark for 3 days. A day prior to co-cultivation, 1 ml from this primary culture was inoculated into 50 ml YEM medium containing suitable antibiotics and incubated at 28°C ± 1°C, 200 rpm, in dark.

Co-cultivation

The *Agrobacterium* culture was pelleted at 4,000 rpm for 15 minutes at 4 °C. The pellet was then resuspended in 25 ml of filter-sterilized (0.2 µm pore size, miilipore) AAM medium (Toriyama and Hinata, 1985) containing 400 µM Acetosyringone, pH 5.2. The embryogenic calli were suspended in this *Agrobacterium* solution and kept for shaking at 80-90 rpm for 30 minutes. Then, *Agrobacterium*

suspension was discarded and the calli were placed on sterile tissue paper to absorb excess of bacterial suspension. These calli were then transferred to 2MSAS (2MSCA containing 10 g/l glucose and 400 µM Acetosyringone, pH 5.2) and incubated at 28°C ± 1°C in dark for 3-4 days.

Selection

On the 3rd or 4th day depending on the growth of *Agrobacterium* around the calli, the calli were washed with autoclaved Milli-Q water containing Cefotaxime (250 mg/ml) in a sterile flask at 125 rpm for 35-45 minutes. These washes were repeated till a clear wash through was obtained. After washing, the calli were blotted on sterile tissue paper to remove excess water and then plated on selection medium, MSCHCA (2MSCA containing 250 mg/l Cefotaxime and 50 mg/l Hygromycin) and incubated at 28°C ± 1°C in dark for 15-20 days. These calli were subsequently subjected to 3-4 selections on MSCHCA for 15 days each.

Regeneration

The healthy proliferating calli were then transferred to MSRNH medium (MS salts and Vitamins, 30 g/l, 1 g/l casamino acids, 1 g/l benzyaminopurine, 250 mg/l cefotaxime, 50 mg/l hygromycin, 4 g/l phytagel, pH 5.8) in Cassablanca jars till the shoots emerged from calli. The boxes were kept in culture room, with conditions maintained at 28°C ± 1°C with 16 h/8 h light/dark cycle. When shoots attained a length of 5-6 cm, they were transferred to rooting medium, MSBH (MS salts and vitamins, 20 g/l sucrose, 2 g/l phytagel, 50 mg/l hygromycin, 250 mg/l cefotaxime, pH 5.8) in culture tubes and were incubated in culture room till proper initiation and formation of roots. After root proliferation, the plants were transferred to Rice Growth Medium or Yoshida's medium (Yoshida et al., 1976) for 10-15 days.

Growth under greenhouse conditions

The plants were then transferred to pots containing a 1:1 mixture of soil and organic manure in the greenhouse maintained at 28°C ± 2°C, 16 h light/8 h dark conditions, 70-75% relative humidity. The seeds were harvested after drying the plants and stored in a cool and dry place.

Histochemical staining for GUS activity in transgenic plants

GUS activity was checked in different transgenic lines by incubating the leaves in GUS histochemical buffer (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6.3H_2O$, 0.1% Triton X-100 and 1 mM X-gluc) at 37°C for 24 h. The tissue turned blue due to the activity of β-glucuronidase on X-gluc as substrate. The leaves were then transferred to the destaining solution (acetone:ethanol in 1:3 ratio) for chlorophyll leaching and then a note was made of the lines exhibiting positive GUS activity.

Gene-expression analysis by Real-Time PCR

For quantitative real-time PCR analysis, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. First strand cDNA was synthesized using 1.5 µg of total RNA employing the cDNA Archive kit (Applied Biosystems) according to the manufacturer's instructions. Primers for real-time PCR analysis were designed using primer design software 'Primer Express 2.0' (PE Applied Biosystems). To ensure that each pair of primers amplified the desired cDNA fragment, each pair was checked using the BLAST searches that they pick the desired gene only. The diluted cDNA samples were used as template and mixed with 200 nM of each primer along with SYBR Green PCR Master Mix (Applied Biosystems, USA) for real-time PCR analysis, using ABI Prism 7000 Sequence Detection System and Software (PE Applied Biosystems, USA). Following conditions were used for PCR: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C in the 96-well optical reaction plate (Applied Biosystems, USA). The specificity of the PCR reactions was verified by the melt curve

analysis. The relative mRNA levels of the individual genes in different RNA samples were normalized with respect to internal control gene *Ubiquitin5*. Three technical replicates were used for real-time PCR analysis.

Real-Time PCR calculation

Relative mRNA level of the test gene with respect to the transcript level of *Ubiquitin5* gene (used as control), in a particular experimental tissue, was calculated by the formula:

$$\text{Gene/Ub5} = 2^{(\text{CT Ub5} - \text{CT test gene})}$$

The mean Gene/Ub5 values, obtained from the biological replicates, were plotted along Y-axis versus the experimental tissues along X-axis. 'CT' stands for threshold cycle value.

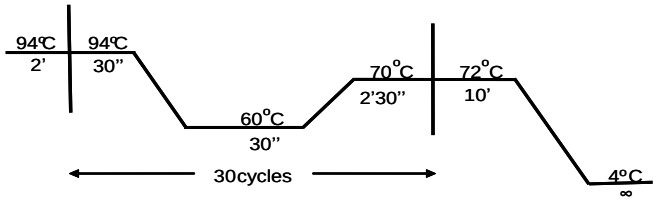
Isolation of RNA for RT-PCR and first strand cDNA synthesis

RNA was extracted from leaf tissues using RNeasy Plant Mini Kit (Qiagen) according to the instructions given in the manual. On-column DNase1 treatment was given with RNase-free DNase set (Qiagen, Germany) to remove any DNA contamination. cDNA was synthesized for various RT-PCR reactions using Transcriptor First Strand cDNA synthesis kit (Roche), following manufacturer's instructions. 1 µg of RNA was used for each reaction. The reaction was set in a DEPC-treated PCR tube with 1 µg of total RNA and 50 pmol/µl of respective primer.

PCR conditions for amplification of *OsiEZ1* fragments in transgenic plants

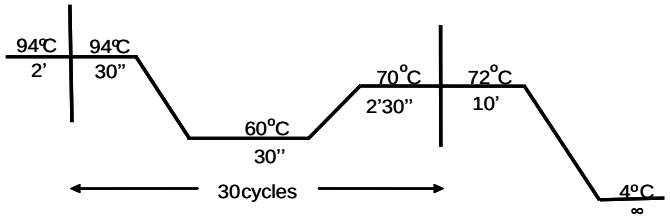
The antisense (*OsiEZ1*) transgenic plants obtained were confirmed by RT-PCR. First strand cDNA synthesis was carried out with either gene-specific forward primer or reverse primer, using RNA of leaf tissue from wild type and transgenic plants. Second strand was synthesized using gene-specific primers designed from the region used for antisense constructs. The components of the reaction mix and the reaction conditions are given below.

Components	Volume(µl)
Autoclaved MQ	18.25
10X Buffer	2.50
25 mM $MgCl_2$	1.5
10 mM dNTP	0.5
20 µM 5' primer	0.5
20 µM 3' primer	0.5
DNA	0.75
T*aq* DNA Pol	0.5
Total reaction volume	25.0



Further, high expression of antisense transcript from transgenic rice plants harboring antisense construct for *OsiEZ1* was analyzed by RT-PCR. First strand cDNA syntheses was carried out using either forward or reverse primer, designed from the region used for raising transgenics, using RNA from leaf tissues. Second strand cDNA syntheses were performed using both forward and reverse primer. The components of the reaction mix and the reaction conditions are given below.

Components	Volume (µl)
Autoclaved MQ	18.0
10X Buffer	2.50
25 mM $MgCl_2$	1.5
10 mM dNTP	0.5
20 µM 5' primer	0.5
20 µM 3' primer	0.5
DNA	1.0
T*aq* DNA Pol	0.5
Total reaction volume	25.0



Primer sequences

For confirmation of *OsiEZ1* antisense plants by RT-PCR

5' PRIMER	5'- ATAGGATCCAGTGCTACCATATTGTCTGAATCAGAAG-3'
3' PRIMER	5'- ATTGGTACCTTTAAACTTTGTGCGCTCGGTGG-3'

For Real-Time PCR of wild-type and transgenic antisense *OsiEZ1* R_2 plants, designed from the:

(1) Region of the gene present inside the inserted cassette

3'EZ1-F	5'-TCGAGTTGGTATCTATGCAAAGGA-3'
3'EZ1-R	5'-TGGTCAGGTCCATAGCGGTAA-3'

(2) Region present outside the inserted cassette

5'EZ1-F	5'-CGTGGATCTTCCTGGACAAAA-3'
5'EZ1-R	5'-TGACAATTGGGTCGTAGTAAATTCTC-3'

Results and discussion

Cloning of *OsiEZ1* 3' fragment in antisense orientation in plant expression vector pB_4NU and rice transformation

OsiEZ1 is a member of E(Z) class of PRC2 proteins. It harbors the SET-domain and hence qualifies under SET-domain containing proteins as well. The *OsiEZ1* cDNA of rice was isolated and characterized and was shown to be represented as a single copy in the rice genome (Thakur et al., 2003). The transcript level of the gene was found to be highest in rice flowers, undetectable in developing seeds of 1-2 days post-fertilization but increased significantly in young seeds of 3-5 days post-fertilization (Thakur et al., 2003). *OsiEZ1* expression complemented a *set1Δ Saccharomyces cerevisiae* mutant impaired in telomeric silencing (Thakur et al., 2003). To further deduce the role played by *OsiEZ1* in rice development, the transgenic approach was utilized in this investigation. The 3' region of *OsiEZ1* gene, of 1960 base pairs length, was used as the insert sequence for rice transformation. This region of the cDNA sequence codes for SANT, CXC and SET-domains (Fig. 1). This region was cloned into the plant expression vector pB_4nU, and the cloning was confirmed by digestion of the transformed plasmids with *Eco*RI and *Bam*H1 (Fig. 2). The pB_4NU vector harbors the ubiquitin promoter which drives the expression of the inserted transgene. The transformed vector was hence checked for the cloning of the insert in sense or antisense orientation with respect to the ubiquitin promoter. Digestion of these transformed plasmids, were performed with *Sac*I, which differentiated between sense and antisense clonings. The sense clones released a fragment of 1070 bp, and antisense clones released a fragment of 900 bp after digestion (Fig. 3). The plasmid harboring the transgene in the antisense orientation was used for *Agrobacterium*-mediated rice transformation (see Materials and Methods for details).

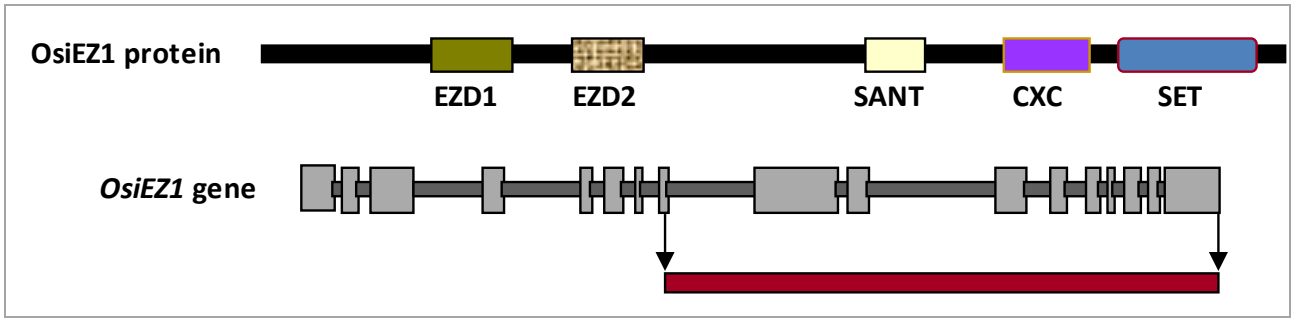


Fig. 1: Protein and gene structure of OsiEZ1. The region used for generating antisense construct has been shown as brown-coloured box below the gene, and encompasses a part of exon 8 and full portions of exons 9 to 17. The corresponding region in the protein consists of the segment spanning SANT, CXC and SET domains. Conserved domains in the protein have been marked. The exons and introns in the gene have been represented by boxes and line segments, respectively.

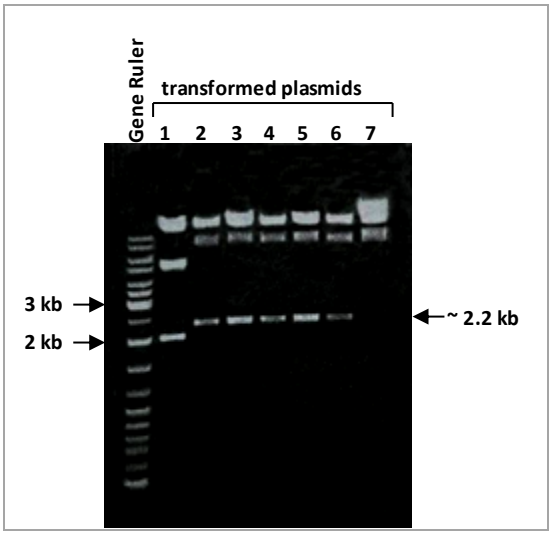


Fig. 2: Digestion of pB_4NU, containing 1960 bp long 3' region of *OsiEZ1* (along with an additional ~300 bp of the *Nos* terminator sequence in the construct), with *Eco*RI and *Bam*HI. Plasmids 2, 3, 4, 5 and 6 yield the desired fragment after digestion while plasmids 1 and 7 do not.

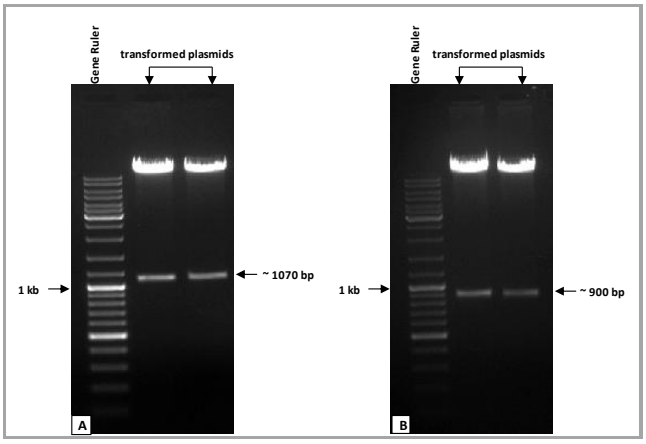


Fig. 3: Confirmation of cloning of 1960 bp long 3' region of *OsiEZ1* in pB_4NU, in sense (A) and antisense (B) orientation with respect to the Ubiquitin promoter, by digestion with *Sac*I.

Analysis of transgenic plants

GUS expression and RT-PCR

Seven independent transgenic lines (R_0 generation, named D-J) were obtained, out of which four were found to be GUS +ve (Table 1A). GUS expression in the leaf segments of few transgenic lines has been shown (Fig. 4). Since GUS expression in pB_4NU is driven by a separate CaMV 35S promoter, and transgene expression by ubiquitin promoter, some of the positive transgenic plants might not show GUS expression. The expression of the antisense transcript from the ubiquitin promoter was, hence, checked in few R_0 and R_1 transgenic lines by RT-PCR (Fig. 5). The RNA from leaf tissues of WT (wild-type) and transgenic rice plants were subjected to first strand cDNA synthesis using either of the forward or reverse gene-specific primers. The second strand cDNA syntheses were performed using gene-specific primers designed from the region used for transgenic raising. The desired PCR band was obtained in the transgenic lines strongly, when first strand cDNA syntheses were carried out using gene-specific forward primer. The faint PCR band in cDNA amplified from transgenic plants, using reverse primer in first strand cDNA synthesis reaction, points out towards the residual sense transcript in transgenic plants. The WT plant, as expected, did not show any band when forward primer was used for first strand cDNA synthesis, but showed the desired band, when reverse primer was used for first strand cDNA synthesis. The faint band in WT RP lane (inside red box in Fig. 5) showed the presence of endogenous transcript. Thus, the experiment confirmed that antisense transcripts were being produced from the ubiquitin promoter in transgenic plants, which once again proved that the cloning was successfully done in antisense orientation.

Phenotypic variations in R_1 transgenics

Certain specific phenotypic characters were observed in the R_1 transgenic plants obtained from different R_0 lines (Fig. 6, Table 1A). The R_1 transgenics in general, exhibited multiple shooting at very early seedling stages, when compared to the WT plants. The rice seedlings after two weeks of germination have been shown in figure 6. Plants marked as A and B had R_0 plant D as their parent, while plant marked C descended from R_0 plant E. The details of the transgenic lines obtained and proceeded further for the next generations have been listed in Table 1. The plant labeled as C, showed profuse rooting and multiple shooting, and eventually died in the Greenhouse two months after

germination. The R_1 plants from R_0 transgenic line H, also showed multiple shooting and leaf curling at early stages of development (about two weeks after germination), as shown in the lower panel of Fig. 6. The leaf-curling phenotype was found to be quite consistent among R_1 plants from different R_0 lines.

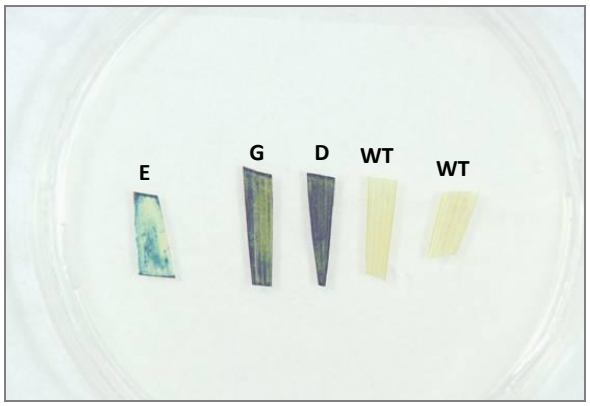


Fig. 4: GUS staining of leaf segments taken from WT and R_0 *OsiEZ1* antisense transgenic lines. Names of the individual lines have been mentioned above the leaf segments.

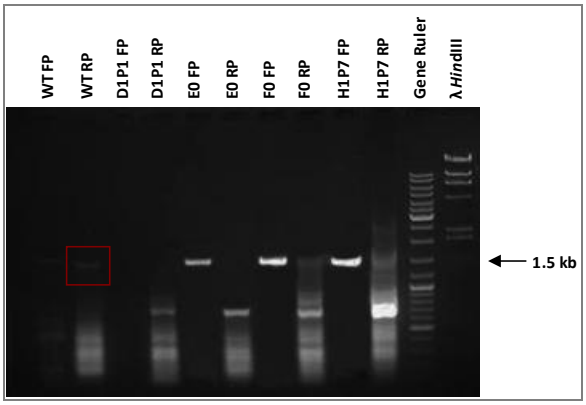


Fig. 5: Confirmation of *OsiEZ1* antisense plants by RT-PCR. First strand cDNA syntheses were carried out using either gene-specific forward primer (FP) or reverse primer (RP) from RNA of leaf tissues of wild type (WT) and transgenic plants. Second strand cDNA syntheses were performed using gene-specific primers designed from the region used for antisense constructs (Same as used in 1st strand cDNA synthesis). E0, F0 are R_0 transgenic lines obtained from transformed calli while D1P1 and H1P7 are R_1 plants obtained from D and H plants of R_0 generation. Presence of gene-specific PCR band at ~ 1.5 kb in FP amplified first strand cDNA in transgenic lines confirms the cloning in antisense orientation. A faint band in WT RP lane (inside red box) shows the presence of endogenous transcript. Bands observed below 900 bp in transgenic RP lanes are non-specific in nature.

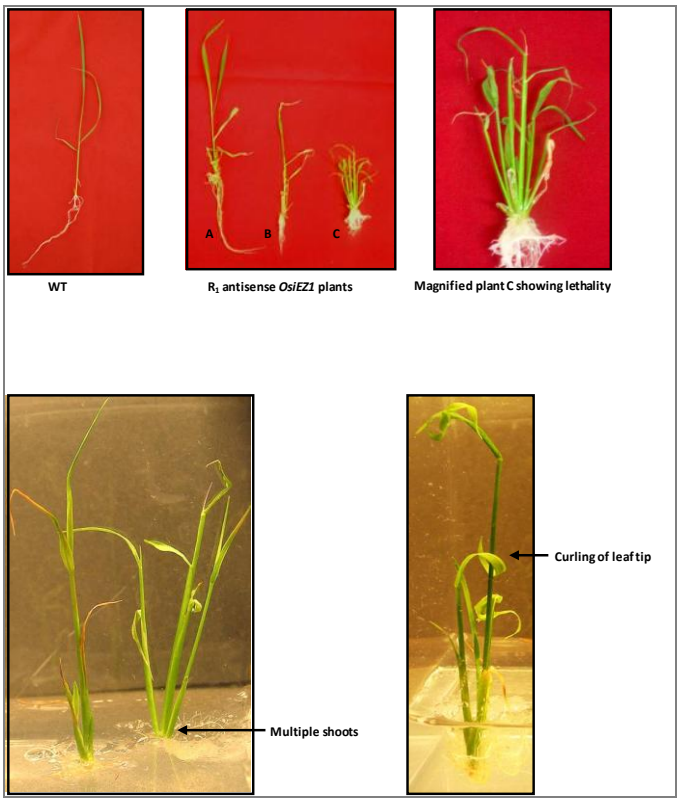


Fig. 6: Curling of leaf tips and multiple shoot initiation in antisense *OsiEZ1* R_1 transgenic plants. No such phenotype was observed in the WT seedlings.

Phenotypic variations in R_2 transgenics

The plants exhibiting lethal phenotype were again obtained in the R_2 generation of transgenics having plant D as the parent R_0 plant. The R_2 plants were derived from three different R_1 transgenic lines P1, P2 and P3. None of the descendents of P1 showed lethal phenotype, while out of 12 descendents both from P2 and P3, 2 and 4 plants exhibited lethal phenotype, respectively (Table 1B). The phenotypes of the R_2 plants with normal and lethal characteristics and the WT plant of same age (three weeks after germination) have been shown (Fig. 7). Multiple shoot formation, profuse rooting, and leaf senescence proceeding from tip to base, were the features associated with the plants showing lethality. The GUS expression was checked in the transgenic plants from the same parent, exhibiting normal or lethal phenotype to check whether the silencing was more in the plants with lethal characters. The leaf segments were taken from these plants and incubated in the GUS

buffer for identical duration (3 hours). Interestingly, plants with lethal characters showed more GUS expression than their normal counterparts (Fig. 8).

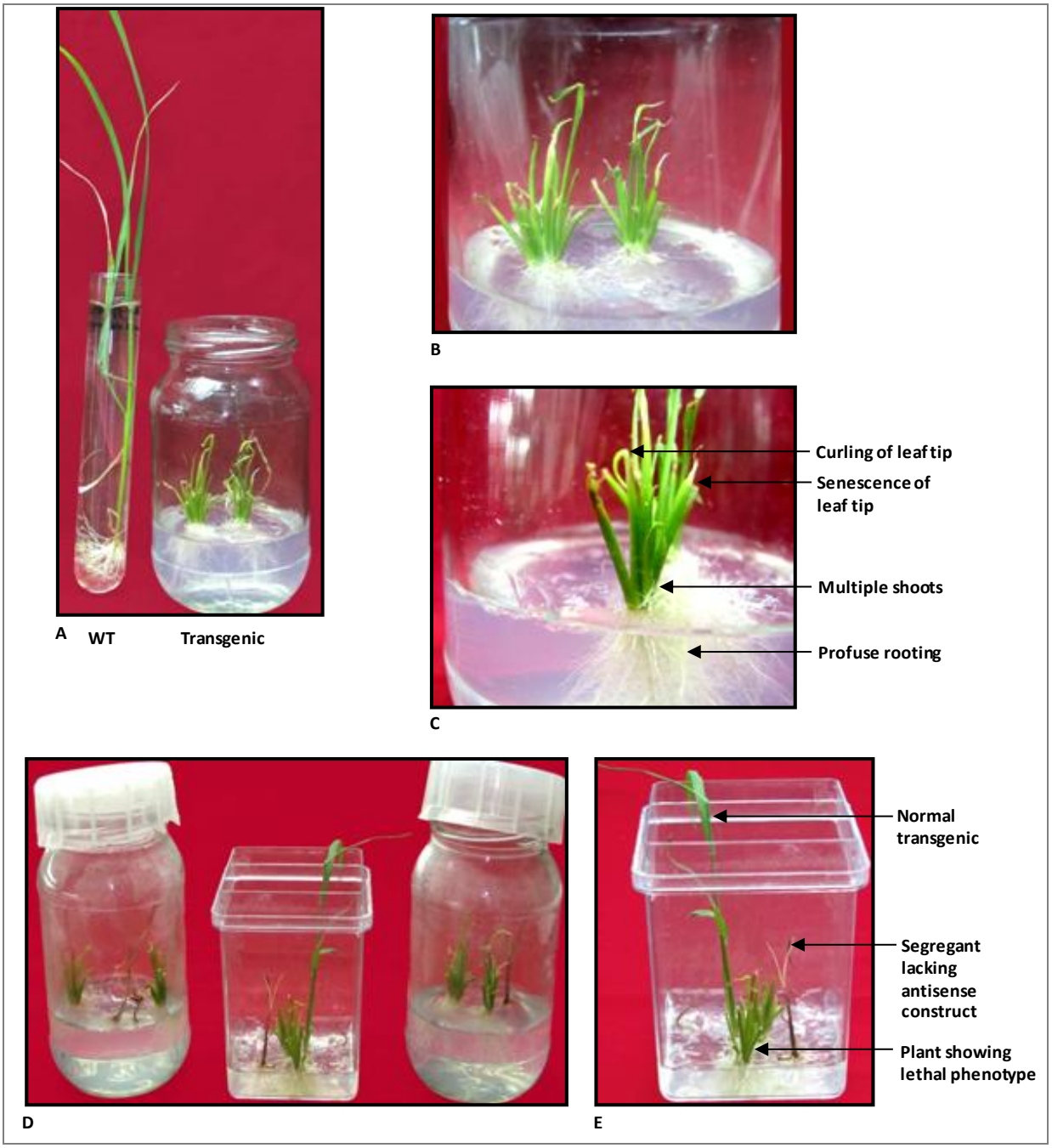


Fig. 7: Appearance of lethal phenotypes in R_2 generation of antisense *OsiEZ1* plants. All the plants have plant D as the parent in generation R_0. In R_1 generation, plants P2 and P3 produced seeds which on germination exhibited the lethal phenotype. A. Comparison of WT with lethal phenotype. B. Magnified view of plants showing lethal phenotype. C. Close-up view of plant showing lethal phenotype with multiple shooting, profuse rooting and leaf senescence progressing from tip to base. D. Transgenic plants with lethal phenotype along with normal ones. E. R_2 segregant lacking the antisense construct dying on selection medium demonstrating complete demarcation between death due to antibiotic selection, and lethality.

Table 1A. Details of *OsiEZ1* antisense transgenic rice plants: R_0 and R_1 generation.

S. No.	R_0 – Line name	GUS assay	No. of seeds planted	No. of plants obtained in R_1	No. of plants that survived	Phenotype-R_1 generation
1	D	+	10	3	3	Leaf curling at seedling stage
2	E	+	10	7	0	Leaf curling at seedling stage, multiple shooting, lethality
3	F	+	10	3	0	Leaf curling at seedling stage, multiple shooting, lethality
4	G	+	24	16	16	Leaf curling at seedling stage
5	H	_	10	8	4	No phenotypic difference compared to wild type
6	I	_	10	6	6	No phenotypic difference compared to wild type
7	J	_	Not pursued further			_

Table 1B. Details of *OsiEZ1* antisense transgenic rice plants: R_1 and R_2 generation.

Line name in R_0 generation	Plant name in R_1 generation	No. of seeds planted	No. of plants that died on selection	Phenotype-R_2 generation
D	P1	14	2	9 plants did not show any phenotypic difference with wild type, 3 showed minor differences
	P2	12	2	2 exhibited lethal phenotype and 8 did not show any phenotypic difference with wild type
	P3	12	3	4 exhibited lethal phenotype and 5 did not show any phenotypic difference with wild type
H	P2	12	5	3 died and 4 did not show any phenotypic difference with wild type
	P3	18	3	3 died and 12 did not show any phenotypic difference with wild type
	P7	20	4	3 died and 13 did not show any phenotypic difference with wild type
	P8	13	3	1 died and 9 did not show any phenotypic difference with wild type

Antisense transcript production verified by RT-PCR and Real-Time PCR

To check whether more antisense transcripts were being produced in the plants exhibiting lethal phenotype, RT-PCR experiments were performed with R_2 transgenics in the similar fashion as described for the R_1 generation transgenics as explained earlier in the text (Fig. 9). The expression of the antisense construct was, however, found to be similar in the normal plants and the plants showing lethality (Fig. 9). Real-Time PCR experiments were performed with the cDNA obtained from the R_2 lethal and normal transgenic lines, and WT plants. The RNA was extracted from seedlings of WT and transgenics showing normal features, and the whole stunted lethality-exhibiting plants. Real-Time PCR were done with two different sets of primers, one designed from the region present in the inserted cassette, and the other lying outside this region, but specific for *OsiEZ1* gene. The amplification recorded by the primers, of the region present inside the cassette, in case of transgenics, would be a reflection of the production

of antisense transcripts from the ubiquitin promoter, while amplification by primers, of the region present outside the inserted cassette, would give an indication towards the extent of silencing, since this amplification gave an idea of residual sense transcript present in the transgenic plants. The detection of more amplicon in the former case and less amplicon in the latter case would, therefore, mean greater extent of silencing. All the transgenics exhibited high antisense transcript abundance, with four of the plants showing lethality (P3L1, P3L4, P2L1 and P3L2) showed maximum expression (Fig. 10A). WT expression indicated the level of endogenous transcript. The expression of *OsiEZ1* sense transcript was found to be lower in P2L1, P3L2 and P3L4 plants compared to the WT transcript level, but no reductions were observed in the transcript levels of the gene in P2L2 and P3L1 plants (Fig. 10B).

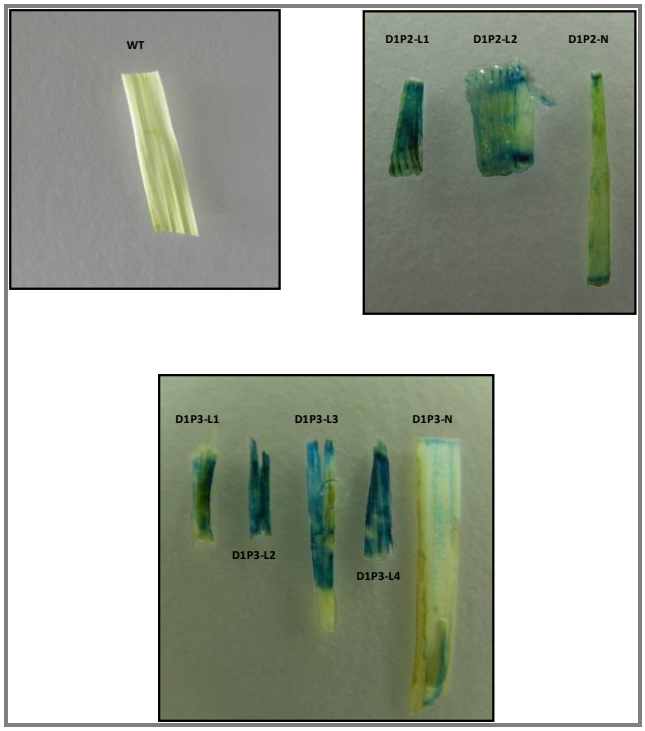


Fig. 8: GUS staining of leaf segments taken from WT and antisense *OsiEZ1* plants of R_2 generation after identical incubation time in GUS buffer. The plants with lethal phenotypes show more GUS expression than normal transgenics. D1P2-L1, D1P2-L2, D1P3-L1, D1P3-L2, D1P3-L3 and D1P3-L4 are the lethal lines while D1P2-N and D1P3-N represent the normal transgenic lines.

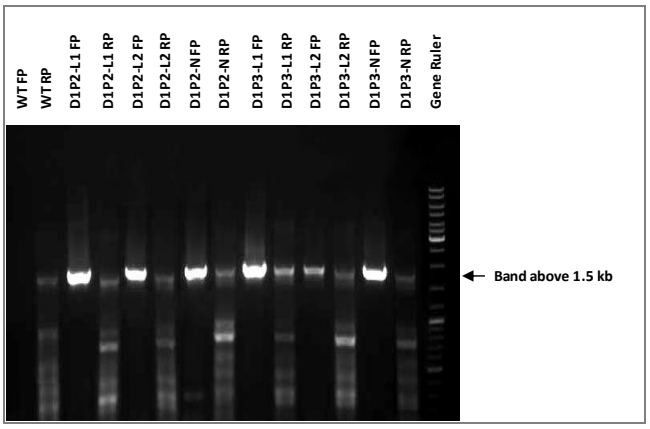


Fig. 9: High expression of antisense transcript from transgenic rice plants harboring antisense construct for *OsiEZ1*. First strand cDNA syntheses were carried out using either forward primer (FP) or reverse primer (RP), designed from the region used for transgenic raising, taking RNA from leaf tissues. Second strand cDNA syntheses were performed using both FP and RP. WT FP shows no product while WT RP shows endogenous *OsiEZ1* transcript level. D1P2-L1, D1P2-L2, D1P3-L1, D1P3-L2 are transgenic plants showing lethal phenotype while D1P2-N and D1P3-N grew normally. D is the R_0 plant and P2 and P3 represent two independent R_1 plants obtained from D. The analysis has been carried out in R_2 generation obtained from P2 and P3 plants.

The normally growing transgenic plants, P2Nm and P3Nm, showed relatively less antisense transcript production but more silencing relative to some of their lethal counterparts. Hence, the production of the lethal phenotype could not be directly correlated with the extent of gene silencing, as the normally growing transgenics, P2Nm and P3Nm, showed higher extent of endogenous gene silencing than the plants showing lethality (P2L2 and P3L1). The expression of antisense transcript from the ubiquitin promoter also did not correlate well with the extent of silencing observed in those very plants. From all these experiments, we could conclude that *OsiEZ1* might be having important role in early stages of seedling development in rice. The transcript levels of the gene were earlier shown to be developmentally regulated with expression levels peaking up at 3-4 days after germination followed by a decline in 5-7 days-old seedlings, before it attained stabilization (Thakur et al., 2003). Thus, it could be hypothesized that a minimal transcript level of the gene might be required at very early stages of seed development.

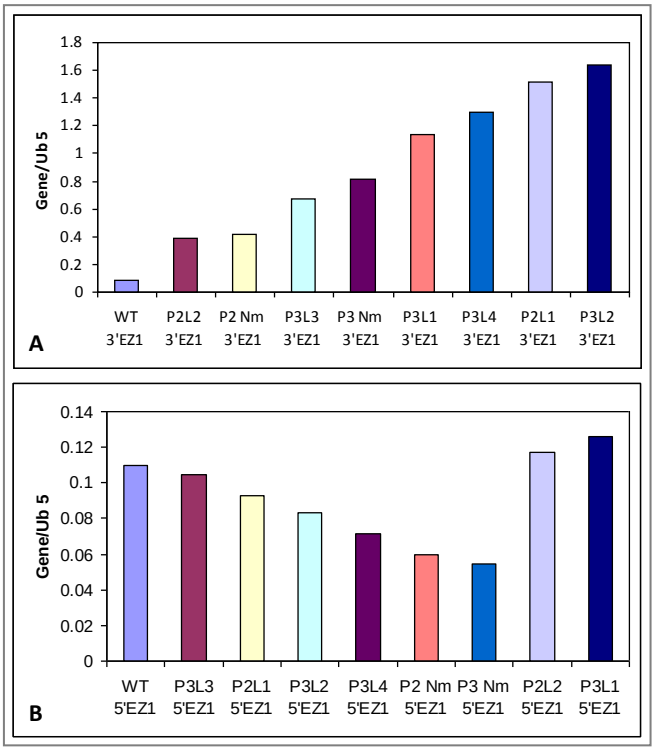


Fig. 10: Real-Time PCR of wild-type and transgenic antisense *OsiEZ1* R_2 plants using primers designed from the region of the gene present in the inserted cassette (A) and region present outside the inserted cassette (B). A) High expression of antisense transcript could be observed in most of the plants with lethal phenotype (P3L1, P3L4, P2L1 and P3L2) than normal plants (P2 Nm and P3 Nm). B) Reduction of endogenous *OsiEZ1* levels is observed in six of the transgenic plants.

The antisense transgenic plants, whose endogenous transcript levels fall below this basal minimal level, at this crucial time period, due to gene silencing, do not show proper vegetative development with abnormal root and shoot growth, and may eventually lead to lethality. The antisense plants, which however, cross this time period, start to behave as normal plants. In many transgenic plants, curling of leaves was observed in young seedling stages but not in the adult ones. Also, both *OsiEZ1* and *OsClf* belong to *E(Z)* class of genes, and *clf* mutants in *Arabidopsis thaliana* showed curled leaf phenotype (Chanvivattana et al., 2004). The *OsiEZ1* homolog in *Arabidopsis*, *Swinger* (*SWN*), acted in redundancy with *CLF* gene, and enhanced the phenotype of *clf* mutant in *clf swn* double mutants (Chanvivattana et al., 2004). Thus, *OsiEZ1* gene might also be essential for normal leaf development in rice, and probably act in redundancy with its homolog, *OsClf*, in controlling leaf, shoot and root development. Additional experiments need to be done for further verifications of these speculations.

Conclusion

This work gives an idea of the role of *OsiEZ1* in rice development. Phenotypic variations, compared to wild type plants, were observed in certain transgenic lines, and these differences were visible in the subsequent generation as well. Further analysis needs to be done to find out the genes and pathways influenced or controlled by this important Polycomb Group member.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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