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# Tolerance to soil water stress by *Oryza sativa* cv. IR20 was improved by expression of *Wsi18* gene locus from *Oryza nivara*

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## Abstract

Wild rice genotypes are rich in genetic diversity. This has potential to improve agronomic rice by allele mining for superior traits. Late embryogenesis abundant (LEA) proteins are often associated with desiccation tolerance and stress signalling. In the present study, a group 3 *LEA* gene, *Wsi18* from the wild rice *Oryza nivara* was expressed under its own inducible promoter element in stress susceptible cultivated *indica* rice (cv. IR20). The resulting transgenic plants cultivated in a greenhouse showed enhanced tolerance to soil water deficit. Transgenic plants had higher grain yield, plant survival rate, and shoot relative water content compared to wild type (WT) IR20. Cell membrane stability index, proline and soluble sugar content were also greater in transgenic than WT plants under water stress. These results demonstrate the potential for improving SWS tolerance in agronomically important rice cultivar by incorporating *Wsi18* gene from a wild rice *O. nivara*.

*Additional key words*: inducible expression, electrolyte leakage, LEA proteins, proline, relative water content, transgenic plants.

## Introduction

Abiotic stresses, such as drought, result in huge losses to crop (including rice) production worldwide (Mittler and Blumwald 2010, Maclean *et al*. 2013). An ectopic expression of stress related osmolytes and transcription factors has been a popular approach for producing plants with enhanced tolerance to adverse environmental conditions (Mickelbart *et al*. 2015, Kaur *et al.* 2016). Among the essential gene products accumulating during the dehydration-induced stress in plants, nearly 4 % of the total cellular proteins are late embryogenesis abundant (LEA) proteins that play a major role as cellular protectants (Gao and Lan 2016). LEA proteins have been reported to enhance membrane stabilization, sequestrate ions, act as antioxidants, buffers, and chaperones (Khurana *et al*. 2008, Gao and Lan 2016). Nevertheless, of the many LEA proteins identified in plants, only a small minority have been functionally and structurally characterized (Hincha and Thalhammer 2012). Group 3 LEA proteins are more diverse than other LEA groups. They are highly hydrophilic in nature, lack proper 3D structures, and have a repeat region containing 11 amino acids (TAQAAKEKAGE) with a potential to form α-helical structures upon desiccation, thereby imparting cell membrane integrity (Gao and Lan 2016). It is known that tolerance to drought can be significantly improved by introducing genes belonging to a 3 *LEA* group from different sources, *e.g.*, barley *HVA1* (Checker *et al*. 2012), wheat *TaLEA2* and *TaLEA3* (Yu *et al*. 2005) and rice *OsLEA3*, *OsLEA3-1*, and *OsLEA3-2* (Moons *et al.* 1997, Xiao *et al.* 2007, Duan and Cai 2012) into a variety of plants. Thus, group 3 LEA proteins play a major role in genetic engineering approaches to enhance drought tolerance. Accordingly, there is a need to characterize and test candidate *LEA* gene(s) from diverse sources.

Judicious choice of promoters allows desirable spatio-temporal expression of transgene(s) for improving crop species (Wang and Oard 2003, Potenza *et al*. 2004, Bhatnagar-Mathur *et al*. 2008, Bhunia *et al*. 2014). This

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*Abbreviations*: CMS - cell membrane stability; qPCR - quantitative polymerase chain reaction; REL - relative electrolyte leakage; RWC - relative water content; SWC - soil water content; SWS - soil water stress; WT - wild type.
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is especially applicable to the expression of abiotic stress responsive gene(s), because constitutive expression of such factors may have adverse effects (Rohila *et al.* 2002, Kasuga *et al.*2004). For instance, use of a stress-inducible *RD29A* promoter instead of the constitutive *Actin* promoter for the overexpression of *HvHVA1* in mulberry (Checker *et al.* 2012) minimized the deleterious effects of constitutive transgene expression on growth. Use of an inducible promoter also helps to evade unnecessary utilization of the transcription machinery for the synthesis of products that are redundant under normal climatic conditions. A majority of *LEA* promoters possess abscisic acid response elements (ABREs) and/or low temperature response elements (LTREs) (Hundertmark and Hincha, 2008) that are inducible under abscisic acid (ABA), drought, salinity, or cold stresses in various species (Xiao and Xue 2001, Yi *et al*. 2011). Promoters of the rice *LEA3* gene, *Wsi18*, when fused to the reporter genes, have shown a high degree of inducible expression under water stress (Yi *et al*. 2011, Nakashima *et al*. 2014, Kaur *et al*. 2017). Stress-inducible expression of barley *HVA1* under the control of a synthetic 3XABRC promoter has enabled transgenic rice plants to tolerate drought, salinity, and cold stresses more successfully (Chen *et al*. 2015) than respective wild type plants. Thus, regulated expression of *LEA* genes under the control of stress inducible endogenous promoters can lead to positive effects in managing stress tolerance.

The wild progenitors of cultivated rice, belonging to the AA genome, are a useful genetic reservoir for allele mining of known stress responsive genes (Lu and Yang 2009, Leung *et al.* 2015). The potential to confer SWS tolerance has recently been identified for *OsDREB1F* following allele mining of various accessions of *O. nivara* and *O. rufipogon* (Singh *et al.* 2015). If variation in the existing germplasms of a crop is low, then wild relatives may serve as a rich source of novel genetic material to improve crop performance (Ashraf 2010), *e.g*., brown planthopper resistance gene from *Oryza officinalis* has been successfully transferred into cultivated rice (He 2003). Similarly, introgression of a chromosome segment (7DL) from wild wheat relative, *Agropyron elongatum,* has resulted in higher root and shoot biomass and improved drought tolerance in cultivated wheat (Placido *et al*. 2013). However, despite these successful reports, little progress has been made in transferring genes/genetic elements/genetic loci from wild *Oryza* species into elite rice cultivars to improve tolerance to abiotic stresses (Sanchez *et al*. 2014). Although there has been progress in deciphering the role of *OsWsi18*, a group 3 *LEA* gene, in drought stress response of *japonica* rice cv. Somewake (Joshee *et al*. 1998) and cv. Nakdong (Yi *et al*. 2011), there is no report of its characterization through "allele mining" in wild rice genotypes. The aim of the present study is to fill this knowledge gap. *Oryza nivara* is a rich source of potentially useful genetic material (Singh *et al*. 2015) and our group has recently characterized an inducible promoter of *Wsi18* from this species (Kaur *et al*. 2017). In the present study, the DNA coding sequence (DCS) of *Wsi18* gene from *O. nivara* was cloned and then expressed under the control of its own endogenous SWS-inducible promoter (Kaur *et al*. 2017) in the stress sensitive but widely-grown rice cultivar, *IR20*. The results show improved tolerance towards SWS in transgenic *IR20* plants with increased survival rates, relative water content (RWC), cell membrane stability (CMS), and grain yield along with enhanced proline and sugar content. This study demonstrates how the deployment of a complete genome locus (*i.e.*, DCS along with its own inducible promoter element) from *O. nivara* can improve survival and productivity of an elite cultivar of cultivated rice under SWS.

## Materials and methods

**Plants and treatments:** Seed of *Oryza nivara* S.D. Sharma & Shastry and *O. sativa* L. type *indica* cv. *IR20* were obtained from the Central Rice Research Institute, Cuttack, Odisha, India. After germination, the plants were grown in a greenhouse at day/night temperatures of 28/25 °C, a relative humidity of 80 ± 5 %, a 16-h photo-period, and an irradiance of 450 - 600 μmol $m^{-2}$ $s^{-1}$. They were grown under well-watered (WW) conditions (providing approximately 1.2 $dm^3$ of water in each pot per day) and soil water stress (SWS) started at the grain-filling stage by withholding water for a period of 8 d followed by recommencing normal watering until maturity and grain harvest (Gaudin *et al*. 2012). For isolation of *Wsi18* gene coding sequence, leaf samples were collected from the *O. nivara* and *IR20* wild type (WT) plants after 8 d of SWS. For transcript profiling in $T_1$ transgenic and WT plant lines, leaf samples were collected before stress and after 8 d of SWS. Soil water content (was measured before and after SWS treatment by the gravimetric method of Black (1965) and expressed as a percent ratio of mass of water per mass of dry soil.

**Isolation of total cellular RNA and synthesis of 1st strand cDNA:** Total RNA was isolated from leaf samples using *RNeasy*$^R$ mini kit (*Qiagen*, Valencia, CA, USA) followed by on-column DNase treatment to remove any residual DNA. The quantity of RNA was determined using a *Nanodrop ND-1000* (*NanoDrop Technologies*, USA) spectrophotometer and the RNA integrity was evaluated in an *Agilent Bioanalyzer 2100* (*Agilent Technologies*, SA) using a RNA *Lab-On-A-Chip* (*Caliper Technologies*, USA). First strand cDNA was synthesized from 2 μg of total RNA with respective gene-specific 3′ primers (WSI18G RP for isolation of *Wsi18* DCS and WSI18 RP for quantitative real time PCR) using *Transcriptor v. 6.0* 1st strand cDNA synthesis kit (*Roche*, Switzerland) following manufacturer's protocol.

**Isolation, cloning, and sequence analysis of *Wsi18* cDNA coding sequence (DCS):** The DCS of the *Wsi18* gene was isolated from *O. nivara* and *IR20* by reverse transcription (RT)-PCR using gene-specific primers (WSI18G FP and WSI18G RP, Table 1 Suppl.) based on the sequence of the *Wsi18* gene from *O. sativa* var. *japonica* available at NCBI (*GenBank* accession No. D26536). RT-PCR was performed using 2 $mm^3$ of the 1st strand cDNA and *PRIME Star* high-fidelity DNA polymerase (*Takara Bio*, USA) in a thermocycler (*Applied Biosystems,* USA) under the following conditions: initial denaturation at 98 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 15 s, annealing at 58 °C for 15 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min . The amplified products were sequenced and the obtained sequences were submitted to NCBI *GenBank* under the accession numbers: KX219624 (*O. nivara*) and KX219625 (*IR20*). Both the sequences were analyzed for the presence of LEA 3 motifs as previously described (Battaglia *et al*. 2008).

**Construction of plant expression vector, rice transformation, and progeny analysis:** The *OnWsi18* DCS was cloned in *Bam*HI and *Kpn*I sites in the multiple cloning site (MCS) of pCAMBIA1300 vector under the control of its endogenous inducible promoter (cloned in *Hin*dIII and *Bam*HI sites) (Kaur *et al*. 2017) to generate the plant transformation construct, *OnWsi18*-TR (TR stands for "transgenic"). The expression construct was transformed into the *Agrobacterium tumefaciens* L. strain EHA105 followed by subsequent transformation into *O. sativa* var. *indica* cv. *IR20* according to protocol reported by Bhattacharyya *et al*. (2012). Putative transformants were selected on MS medium containing hygromycin B (50 mg $dm^{-3}$), transferred to soil after root development, and after proper acclimatization grown to maturity under greenhouse conditions described above. The $T_0$ plants were self-pollinated and $T_1$ seeds were harvested. The $T_1$ seeds were again screened on hygromycin containing MS medium. Finally, the surviving plantlets were established in a greenhouse to obtain mature $T_1$ plants. Segregation analysis for integrated transgene in $T_1$ progeny plants was carried out using the $\chi^2$ test in which observed values were compared to the corresponding theoretical values. The goodness of fit of the observed segregation ratio of the transgene was tested against the Mendelian segregation ratio (3:1) using the formula: $\chi^2 = \Sigma$ [(observed frequency - expected frequency)$^2$ / expected frequency].

**PCR analysis of transgenic plants and Southern hybridization:** PCR screening of the transgenic rice lines was carried out using specific primers COC FP and COC RP (Table 1 Suppl.) encompassing a portion of the transgene and the hygromycin selection marker. This oligonucleotide design helped to confirm the integration of the intact expression cassette with a selection marker and also ensured specific identification of the transgenic lines (because using *Wsi18* gene-specific oligos is thought to provide false positive results due to amplification of the endogenous gene). The thermal cycling parameters were: initial denaturation at 94 °C for 4 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s followed by final extension of 72 °C for 10 min. For Southern hybridization, 12 µg of genomic DNA was digested with *Hin*dIII, run on 0.8 % (m/v) agarose gel and then transferred to a nylon membrane (*Hybond* $N^+$, *GE Healthcare*, USA) (Kaur *et al*. 2017). α-[$^{32}$P]-dCTP (3.7 × $10^{13}$ Bq $mmol^{-1}$) radiolabeled *OnWsi18* was used as probe for hybridization. Autoradiographic exposure was performed at room temperature by exposing the membrane to *Multisensitive* (MS) screen (*Perkin Elmer*, USA) inside a *Hyper* cassette (*Amersham*, UK) for 5 to 30 min depending on radioactive count. The MS screen was scanned in *Storage Phosphor* system (*Cyclone Plus*, *Perkin Elmer*) at 300 dpi resolution using a medium carousel type to generate the autoradiographic image.

**Real time quantitative PCR:** Real time qPCR for the detection of *OnWsi18* expression in the control WT and $T_1$ transgenic *IR20* plant lines was carried out in a *Realplex*$^2$ *Master* cycler (*Eppendorf*, Germany) using *SYBR Green 5 prime* kit (*Eppendorf*) and *Wsi18* specific forward and reverse primers WSI18 FP and WSI18 RP (Table 1 Suppl.). Thermal cycling conditions were: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 15 s, and extension at 68 °C for 30 s. Melting curve analysis in each case confirmed the amplification of specific product. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to calculate relative expression of various transcripts in terms of fold change. The primer pair (OsUbi1) designed from rice *polyubiquitin1* gene (*GenBank* accession No. AF184279, Table 1 Suppl.) was used as internal control for normalization.

**Antiserum production and Western blot analysis:** The DCS of *OnWsi18* was cloned in frame in the pRSETA bacterial expression vector (*Invitrogen*, USA) and then transformed into *Escherichia coli* strain BL21 pLysE cells. The induction was carried out with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 28 ºC for 6 h. The purification of the N-terminal 6× His-tagged Wsi18-recombinant protein was carried out according to the *QIAexpressionist*$^{TM}$ protocol (*Qiagen*) using *Ni-NTA* column chromatography. The purified Wsi18 protein (2.5 mg) was submitted to *Imgenex* (Bhubaneswar, India) for production of affinity purified polyclonal rabbit antiserum. For Western blot analysis, total protein was isolated from the leaf tissue of the transgenic as well as control WT *IR20* plants and 50 µg of total protein from each sample was run on gel as per protocol reported by Bhunia *et al*. 2014; 2016). Anti-Wsi18 polyclonal antibody at 1:1 000 dilution was used as primary antibody and anti-isotype IgG-HRP (horseradish

peroxidase) conjugate was used as the secondary antibody. Monoclonal plant actin antibody (*Sigma*, St. Louis, USA) (1:500 dilution) was used to detect equal loading of protein. Immunodetection was carried out with the *Lumi-Light*$^{PLUS}$ Western blotting kit (Roche), according to the manufacturer's instructions.

**Survival rate and yield measurements:** Plants with visibly recovered leaves (after re-watering) were considered as survived (Joo *et al*. 2014). Survival rates were calculated as number of surviving transgenic lines divided by the total number of lines exposed to stress (Checker *et al*. 2012, Joo *et al*. 2014). Three independent experiments were conducted comprising of 18 plants per experiment to calculate the survival rates. Fully-filled, oven-dried (37 ºC for 48 h), de-hulled grains were collected at maturity and used to measure the rice yield in terms of filled grain percentage and grain mass. Filled grain percentage was calculated as the number of filled grains per panicle divided by the sum of filled and unfilled grains per panicle (Uga *et al*. 2013). For grain mass determinations, 100-grain sub-samples were taken to estimate the average mass of one grain (Jeng *et al*. 2006). Each data point represents the mean of three biological replicates.

**Physiological and biochemical assays:** All the physiological assays were performed using the $T_1$ transgenic and control *IR20* plants before and after SWS treatment. For relative water content (RWC) measurement, samples were harvested from the middle section of the blades of fully expanded green leaves and RWC was determined following protocols of Lal *et al*. (2008) and Degenkolbe *et al*. (2009). Six plants were used per experiment and three independent experiments were conducted. For the measurement of free proline, 500 mg of fresh leaves were homogenized in 10 $cm^3$ of 3 % (v/v) sulphosalicylic acid according to Bates *et al*. (1973) and Lal *et al*. (2008) followed by measuring the absorbance at 520 nm. Total soluble sugar content was analyzed from 100 mg of leaf tissue by the anthrone method (Zhang and Huang 2013). Relative electrolyte leakage (REL) was measured according to Dansana *et al*. (2014). In brief, 0.1 g of fully expanded young leaf was cut into 5 pieces of equal size, washed and submerged into 10 $cm^3$ of deionized water in a test tube for 3 h at room temperature with shaking followed by autoclaving for 15 min. Electrical conductivity was measured by a conductivity meter (*Mettler and Toledo Five Easy Plus FEP30*, Switzerland) before (C1) and after (C2) autoclaving the leaf samples. REL [%] was determined using the equation [(C1/C2) × 100]. Each data point for the biochemical assays represents the mean of three replicate leaf samples.

**Statistical analysis:** Student's *t*-test was used for analysis of statistical significance following a Gaussian distribution. Significance was evaluated at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

## Results

The DCS of *Wsi18* gene was amplified from the *O. nivara* and *IR20* by RT-PCR (Fig. 1*A*). When aligned with the 645 bp reported sequence of *Wsi18* DCS from the *japonica* rice cv. Somewake (GenBank accession No. D26536; Takahashi *et al.* 1994), the DCS region of *O. nivara* (obtained in this study) was found to be 648 bp and possessed 99 % similarity (Fig. 1 Suppl.). Analyses of the *Wsi18* gene sequence from both the genotypes showed the presence of 5 motifs characteristic of group 3 *LEA* genes (Fig. 1*B*). However, no significant differences, such as addition, deletion, or re-organization of motif sequences, could be detected between *O. nivara* and *IR20*. As both DCSs were found to be nearly identical, *OnWsi18* DCS was expressed under its endogenous promoter (Fig. 2 Suppl.) in anticipation of better regulatory control of gene expression. The plant expression construct *OnWsi18*-TR (Fig. 2*A*) was transformed into the SWS-susceptible cultivar, *IR20*. The number of independent primary ($T_0$) transgenic rice lines obtained after stringent screening was ten. However, sequence-specific PCR screening (Fig. 2*A*) showed amplification of 1455 bp PCR product for only seven plants (Fig. 2*B*). These plants were then subjected to further molecular analysis. Southern blot analysis confirmed the presence of the transgene in the putative $T_0$ transformants (Fig. 3*A*). All the transgenic lines were found to represent independent transgenic events with single, double, and triple integration sites of the *OnWsi18* transgene. The uniform common bands in each case signify the endogenous *Wsi18* gene. Three $T_0$ transgenic plants $T_0$-1, $T_0$-4, and $T_0$-6 showed three integration sites whereas two $T_0$ plants, $T_0$-7 and $T_0$-10, showed two integration sites for the transgene within the rice genome. Only two of the transgenic plants ($T_0$-2 and $T_0$-8) showed single integration sites. Seeds obtained from all the seven independent $T_0$ rice lines were subjected to progeny analyses. It revealed the Mendelian inheritance pattern for a monohybrid cross (3:1) for each independent transgenic event, indicating transgene(s) integration in a single chromosome (Table 2 Suppl.). Randomly chosen $T_1$ transformants ($n$ = 2) were further tested for stable transgene inheritance by Southern blot analysis. All the tested $T_1$ plants showed a similar pattern of *OnWsi18* transgene integration (Fig. 3*B*) as was found in $T_0$ plants. Representative $T_1$ progenies of two independent plants with single integration sites of the transgene ($T_1$-2.1, $T_1$-2.2, $T_1$-8.2 and $T_1$-8.4) were selected for further analyses.

The $T_1$ transgenic plants were analyzed for the *Wsi18* transcription after SWS application in comparison to the

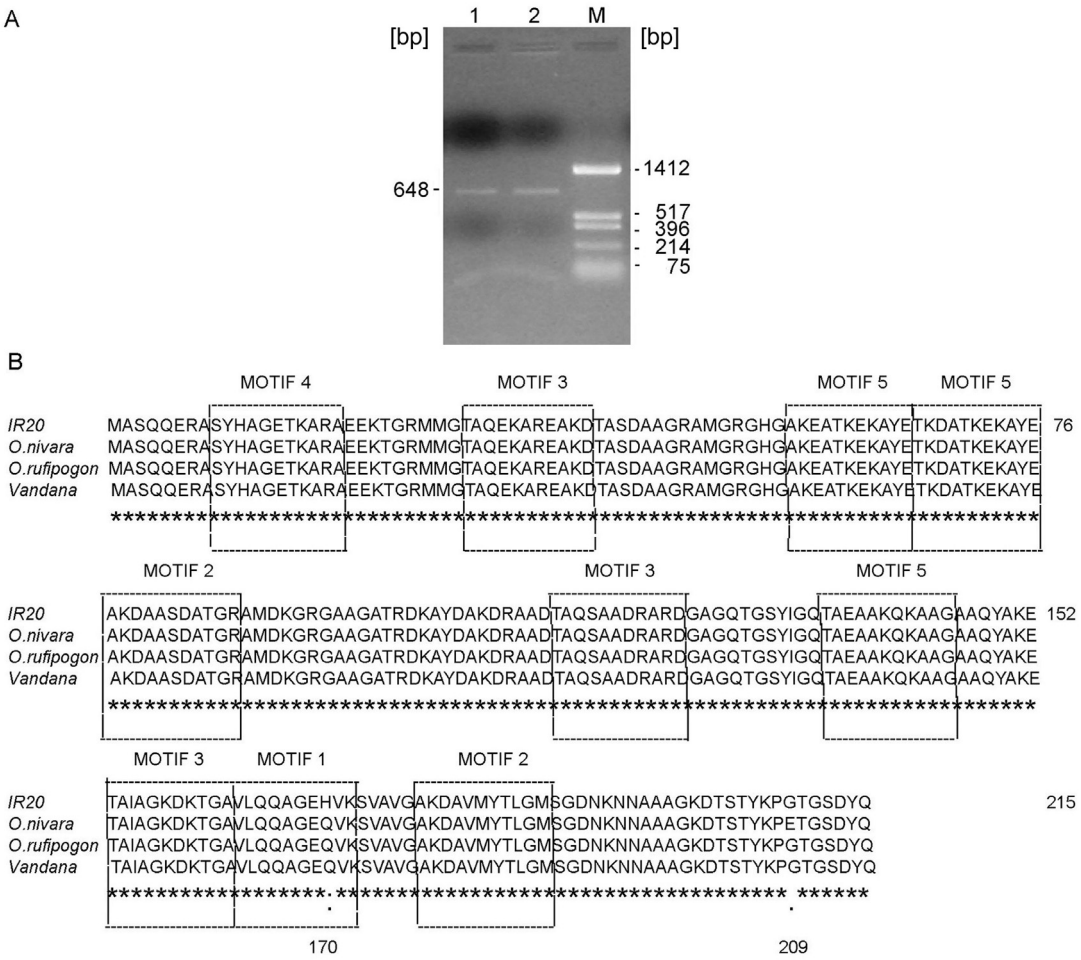


Fig. 1. Cloning and sequence analysis of *Wsi18* from *O. nivara* and *IR20*. *A* - Agarose gel analysis of PCR amplified *Wsi18* gene DCS from *O. nivara* cDNA (*lane* 1) and *IR20* cDNA (*lane* 2); *lane* M represents *Hin*fI digested pUC18 plasmid as DNA molecular marker. *B* - Alignment of Wsi18 amino acid sequence from *O. nivara* and *IR20,* showing the presence of the different motifs characteristic of group 3 LEA proteins along with the changes in the amino acid mark by points.

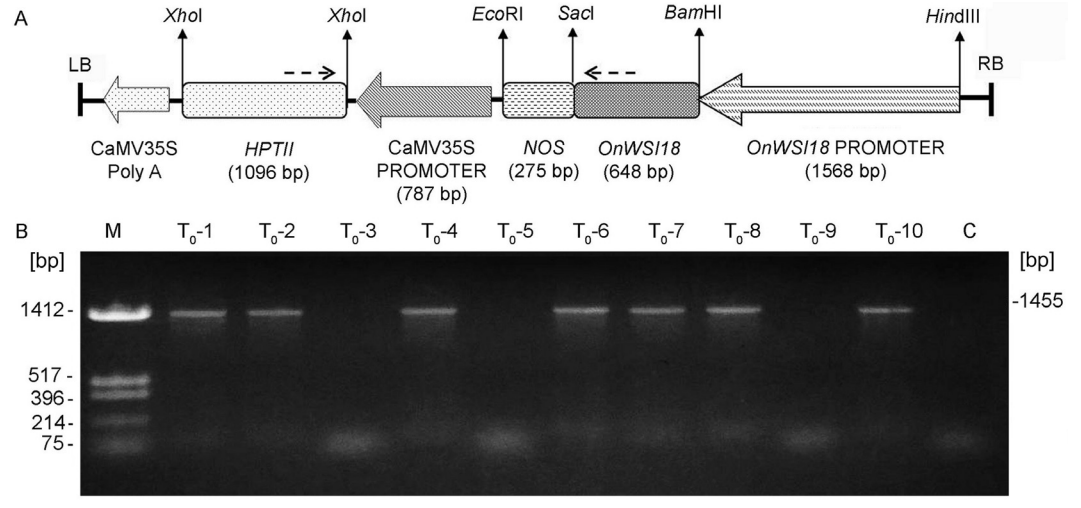


Fig. 2. Molecular analysis of transgenic plants carrying *OnWsi18*-TR casette. *A* - Schematic diagram showing the *OnWsi18*-TR cassette. *Broken arrows* show region (~1 455 bp) for PCR amplification of transgene. *B* - PCR analysis of the $T_0$ rice plants that survived hygromycin selection. *Lane* M - *Hin*fI digested pUC18 plasmid as DNA molecular marker, *lane C* - wild type.

control *IR20*WT plants by means of real-time qPCR (Fig. 4*A*, Table 3 Suppl.). The transgenic plants showed enhanced expression of *Wsi18* gene (7.6 to 9.1-fold) after SWS as compared to WT plants whose expression was normalized using expression of rice *polyubiquitin 1* gene and set as 1. The highest expression was observed in transgenic plant $T_1$-2.2 (Fig. 4*A*). To confirm the SWS-mediated inducible expression of *OnWsi18* at the protein level, the representative transgenic ($T_1$-2.2) plant was subjected to Western blot analysis (Fig. 4*B*) in the backdrop of a WT plant. The transgenic ($T_1$-2.2) plant showed a faint band before application of SWS (Fig. 4*B*) which denoted the basal level of expression of the gene even without stress. However, upon application of SWS, the transgenic plant $T_1$-2.2 showed a more intense band as compared to the WT plant under similar SWS (Fig. 4*B*). This further confirmed the inducible expression of the *OnWsi18* gene under the control of its endogenous promoter in the transgenic plants under SWS. The control *IR20* plant did not show any detectable content of Wsi18 protein without SWS (Fig. 4*B*). This may be due to extremely low basal expression which remained undetectable under the control conditions. The β-actin was used as an internal control and it showed a similar band intensity in the transgenic and WT plants (Fig. 4*B*), thereby confirming the equal loading of protein from each sample.

Physiological traits, such as survival rate and RWC, were studied for transgenic and WT plants before and after SWS application. When compared to WT plants,

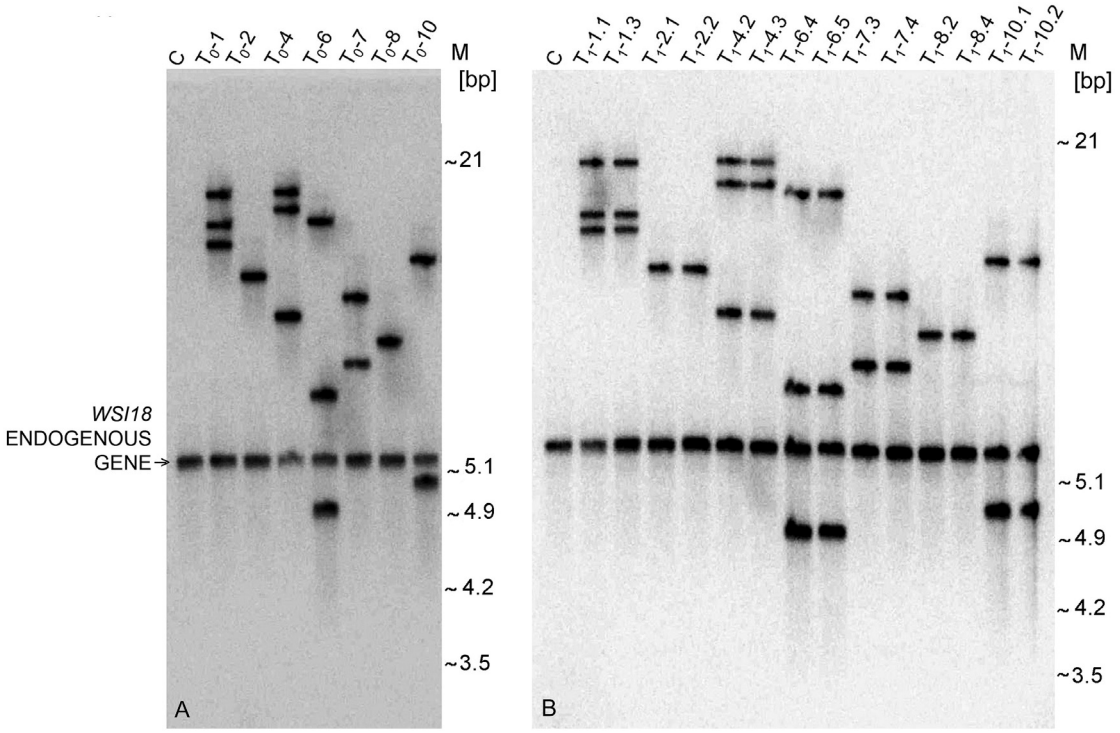


Fig. 3. Southern blot analysis of transgenic plants. *A* - Southern blot analysis of $T_0$ PCR positive plants. *B* - Southern blot analysis of $T_1$ plants ($n$ = 2), showing similar pattern of integration as $T_0$. *Lane* M - *Eco*RI-*Hin*dIII digested λ DNA as molecular mass marker. *Lane* C - wild type.

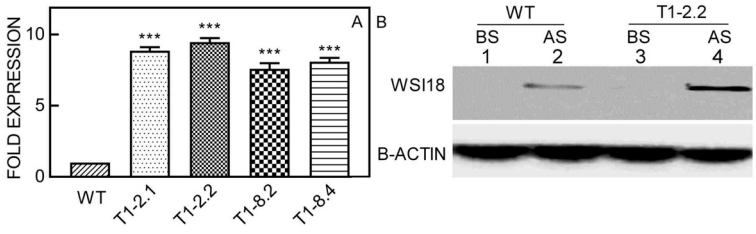


Fig. 4. Expression analysis. *A* - Relative expression of the *OnWsi18* gene after 8-d SWS application in the T1 transgenic plants in comparison to wild type (WT) plants after normalization to unity. Rice *polyubiquitin1* gene was used as the internal control for normalization of data. Means ± SDs of triplicate assays (*** - $P < 0.001$). *B* - Western blot analysis of *On*Wsi18 expression before stress (BS) and after SWS (AS). *Upper panel* - *lanes* 1 and 2 represent WT plants, *lane*s 3 and 4 represent $T_1$ transgenic line ($T_1$-2.2). *Lower panel* shows β-actin as the loading control.

transgenic plants exhibited delayed signs and symptoms of stress in terms of leaf rolling and withering (Fig. 5). Survival rate (SR) of control *IR20* WT plants was ~18.5 % under SWS whereas the SR of transgenic plants ranged from 83 - 89 % (Fig. 6*A*, Table 3 Suppl.). At the end of the SWS period, the soil water content (SWC) of the stressed plants had dropped to ~8 - 10 % compared to ~65 % in the control well-watered plants (Table 3 Suppl.). Plants expressing *OnWsi18* showed greater SWS tolerance than the control *IR20* WT lines. This was confirmed by the higher RWC (68 - 72 %) in transgenic plants compared to the WT plants (~35 %) after 8 d of SWS (Fig. 6*B*, Table 3 Suppl.). The yield measurements were estimated from the filled grain percentage as well as the grain mass of both transgenic and control *IR20* WT plants. Under well-watered conditions, all transgenic and WT plants had similar yield characteristics: 82 - 85 % of filled grains as well as grain mass 27 - 28.4 mg grain$^{-1}$. This indicated that expression of *OnWsi18* had no detrimental effect on the growth and development of *IR20* plants. After being subjected to SWS, WT plants produced fewer filled grains (~28.6 %) whereas the transgenic lines had 63 - 73 % filled grains (Fig. 6*C*, Table 3 Suppl.). Grain mass was also affected in both transgenic and WT plants, but the effect was less in the transgenic plants (20 - 22 mg grain$^{-1}$) in comparison to the WT plants (~13 mg grain$^{-1}$) (Fig. 6*D*, Table 3 Suppl.). These results suggest that *OnWsi18* acts as a

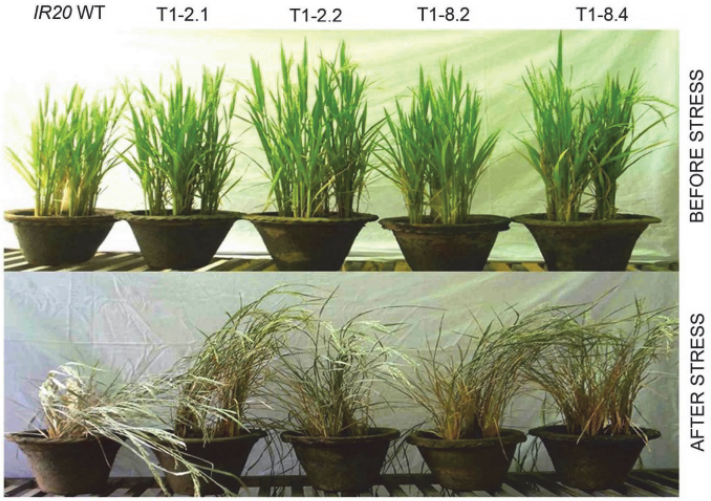


Fig. 5. Representative *IR20* wild type (WT) and selected $T_1$ transgenic plants before stress and after stress (8 d of SWS).

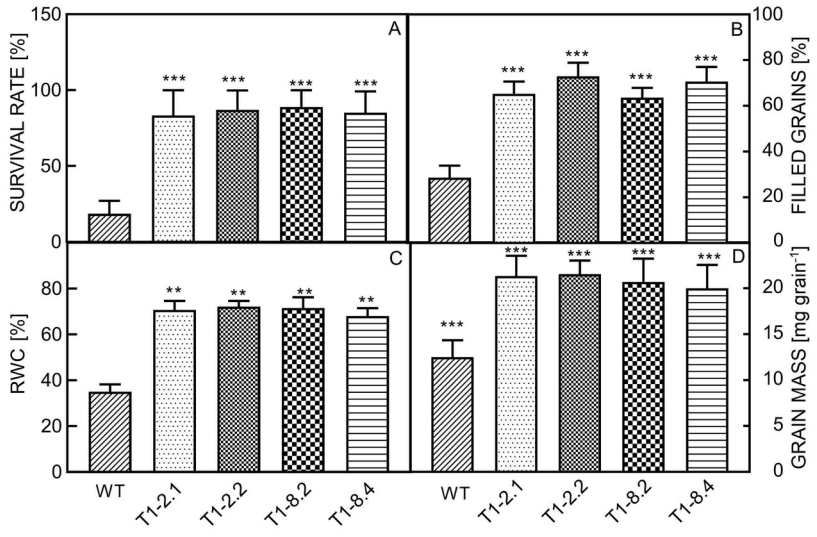


Fig. 6. *A* - Survival rate of *IR20* WT and selected $T_1$ transgenic lines after SWS for 8 d. *B* - Relative water content (RWC) in the leaves of the WT and selected $T_1$ transgenic plants after SWS for 8 d. *C* - Percentage of filled grains in the control WT and $T_1$ transgenic lines at maturity. *D* - Grain mass in the control WT and $T_1$ transgenic plants at maturity. Means ± SDs of triplicate assays (*** - $P < 0.001$, ** - $P < 0.01$).

positive regulator of SWS tolerance in rice and helps to maintain relatively higher yield of otherwise drought susceptible *IR20* plants under SWS.

To elucidate the physiological mechanism by which *OnWsi18* confers tolerance to SWS and improves the ability of plant to retain water, proline and soluble sugars were quantified in the WT and transgenic lines before stress and after SWS. The results showed no significant differences in proline and sugar content of WT and transgenic plants before stress (Table 3 Suppl.). Under SWS, free proline content increased in both the WT and transgenic plants, however, the increase was higher in transgenic plants (Fig. 7*A*, Table 3 Suppl.). Similarly, total soluble sugar content also increased more in all the transgenic plants in comparison with WT plants under AS conditions (Fig. 7*B*, Table 3 Suppl.). Cell membrane stability (CMS) analysis showed that REL was comparable (~2.5 - 4.1 %) in WT and transgenic plants before application of SWS. When SWS was applied, cell membrane injury increased in both WT plants and transgenic plants but the magnitude of increase was greater in WT plants (their REL value was ~29 %; wheares REL of transgenic plants was 10.3 - 15.4 %; Fig. 7*C*, Table 3 Suppl.). We conclude that *OnWsi18* expression improved CMS index for transgenic *IR20* plants.

## Discussion

"Allele mining" has become an attractive tool for geneticists to exploit potentially useful genetic variation and to bring about desirable changes in crop species towards enhancing their stress-tolerance (Kumar *et al*. 2010, Kaur *et al.* 2016). As an important family of highly hydrophilic proteins involved in plant drought tolerance, the *LEA* group of genes constitute a potential target for "allele mining" from wild genotypes (Khurana *et al*. 2008, Gao and Lan 2016). The present study identified and characterized genetic elements for the well-documented SWS responsive group 3 *LEA* gene, *Wsi18*. In our previous study (Kaur *et al*. 2017), we characterized the promoter region of *Wsi18* gene from wild rice *O. nivara*, in comparison to distantly related elite rice genotype *IR20*. Analysis of the *OnWsi18* promoter revealed the presence of various *cis*-regulatory elements commonly associated with SWS inducible promoters (Fig. 2 Suppl., Kaur *et al*. 2017) and showed high inducible expression of the reporter *gusA* gene under SWS (Kaur *et al*. 2017). However, the role of the *OnWsi18* promoter under SWS conditions in an elite *indica* rice cultivar remained unexplored. The present study addresses this issue by employing an SWS inducible promoter from a wild rice genotype *O. nivara* to drive SWS responsive *Wsi18* gene expression in the agronomically important but drought-susceptible rice cultivar *IR20*. A detailed analysis for the *Wsi18* gene sequence from *O. nivara* and *IR20* did not show significant differences of structural motifs and hence both are probable candidates for transgene expression. In order to express an intact genetic locus from a wild rice genotype, *OnWsi18* DCS was preferred over *IR20* DCS for transgene expression in genotype *IR20*.

The rice genome contains 34 *LEA* genes of which six are categorized under group 3, which share conserved regions (Wang *et al*. 2007). Over-expression of different *LEA3* genes in transgenic rice plants has resulted in better survival and improved yield under drought (Babu *et al*. 2004, Xiao *et al*. 2007, Duan and Cai 2012). In our study, transgenic *IR20* plants carrying the expression cassette *OnWsi18*-TR showed enhanced *Wsi18* gene expression at both transcript (Fig. 4*A*) and protein levels (Fig. 4*B*) after application of SWS. The $T_1$ transgenic plants with single integration sites were selected for molecular and physiological analyses as this provided an even premise

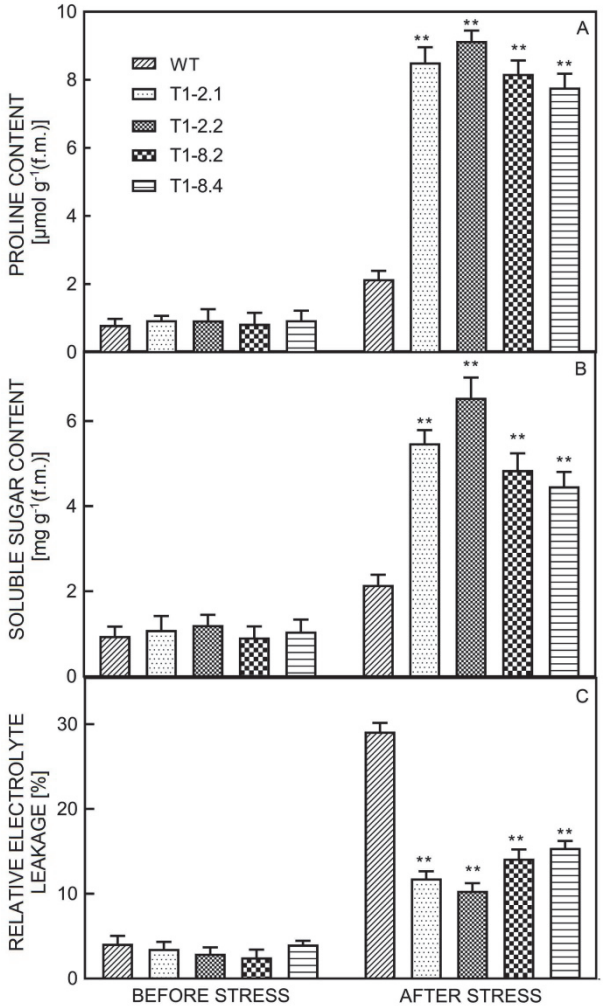


Fig. 7. Free proline content (*A*), soluble sugar content (*B*), and relative electrolyte leakage (*C*) in the leaves of the WT and $T_1$ transgenic plants before stress and after stress (8 d SWS). Means ± SDs of triplicate assays (** - $P < 0.01$).

for comparison and avoided a transgene silencing effect due to multiple integrations (DeBuck *et al*. 2001, 2004). OsWsi18 is expected to contain significant sequence homology (~55 %) with another group 3 LEA proteins, *e.g.*, OsLEA3 (*GenBank* accession No. AAV67829; Moons *et al*. 1997), thereby causing further difficulty in specific detection of expressed Wsi18 in the background of other LEA proteins. Also, there is no significant difference in the size of Wsi18 (215 aa) *vs*. OsLEA3 (200 aa), which remained inseparable on our Western blots. Despite this, the increased band intensity of Wsi18 protein after SWS in the backdrop of equal amount of protein in each lane clearly indicated inducible transgene expression under the regulatory control of the *O. nivara* promoter element. Efficient survival of the transgenic plants with a relatively higher filled grain percentage and grain mass in comparison to WT plants under SWS established that the inducible transgenic expression of *OnWsi18* enhanced drought tolerance and yield characteristics in *IR20*. This is agronomically significant since even a small improvement in yield during environmental stress such as drought can improve future food security (Checker *et al*. 2012, Mickelbart *et al.* 2015). Also, changes in the physiological parameters we measured are comparable with transgenic plants expressing different *LEA* genes from a variety of crops including rice and mulberry (Xiao *et al*. 2007, Lal *et al*. 2008, Checker *et al*. 2012). In our study, the RWC of the transgenic plants remained considerably higher under SWS in comparison with WT which points towards improved internal water status of the transformed plants (Gao *et al*. 2015). Osmolytes, such as free proline and total soluble sugars, increased more in our transgenic plants under water stress (Fig. 7*A,B*) as compared to the WT. Accumulation of these compounds has been positively correlated, by others, to the enhanced osmo-regulation and reduced oxidative damage induced by reactive oxygen species (Liang *et al*. 2013). Although the exact mechanism of action of LEA3 proteins is still unclear, circular dichroism and infra-red spectroscopy of various group 3 LEA proteins indicates that they are mostly devoid of secondary structure, being largely in a random coil conformation in solution (Tompa 2012). However, in the presence of sucrose, glycerol, ethylene glycol, or methanol, or after fast drying, they adopt an α-helical conformation which is fully reversible (Tompa 2012). We therefore presume that the presence of enhanced amounts of soluble sugars after stress-relief in the transgenic plants will aid *OnWsi18* function thereby augmenting SWS tolerance in transgenic plants. Cell membranes are generally disrupted under SWS thereby allowing the release of electrolytes (Bajji *et al*. 2002, Babu *et al*. 2004). In our study, all the plants released electrolytes when subjected to SWS. However, the transgenic plants over-expressing *OnWsi18* were less leaky. This result is consistent with the earlier reports of LEA proteins playing crucial roles in SWS tolerance by maintaining the CMS under dehydration (Babu *et al*. 2004, Tripathi *et al*. 2012). Interestingly, the percentage of REL is highly reduced in the transgenic plants developed in the present study as compared to transgenic rice plants carrying *LEA* genes from different sources, such as *HvHVA1* under drought (Babu *et al*. 2004) and *SiLEA14* under salinity (Wang *et al*. 2014), thereby emphasizing the role of *OnWsi18* as a positive regulator of CMS in rice under SWS. Hence, the use of wild rice accessions for allele mining is expected to provide superior stress tolerance traits compared to genetic elements from other sources.

In summary, we report the successful deployment of a previously characterized SWS inducible promoter element (Kaur *et al.* 2017) from wild genotype *O. nivara* to drive transgenic expression of the group 3 *LEA* gene *Wsi18* DNA coding sequence from the same genotype. This, in turn, provided the opportunity to test the effect of transgenic expression of a complete genetic locus comprising of the allelic variant of the *Wsi18* gene "mined" from a wild rice. Its introduction into a cultivated rice improved grain yield and SWS tolerance. This study extends the boundaries for improving abiotic stress tolerance in rice by allele mining of distantly related wild species.

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