Functional analysis of the durum wheat gene TdPIP2;1 and its promoter region in response to abiotic stress in rice

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ABSTRACT

In a previous work, we demonstrated that expression of TdPIP2;1 in Xenopus oocytes resulted in an increase in Pf compared to water injected oocytes. Phenotypic analyses of transgenic tobacco plants expressing TdPIP2;1 generated a tolerance phenotype towards drought and salinity stresses. To elucidate its stress tolerance mechanism at the transcriptional level, we isolated and characterized the promoter region of the TdPIP2;1 gene. A 1060-bp genomic fragment upstream of the TdPIP2;1 translated sequence has been isolated, cloned, and designated as the proTdPIP2;1 promoter. Sequence analysis of proTdPIP2;1 revealed the presence of cis regulatory elements which could be required for abiotic stress responsiveness, for tissue-specific and vascular expression. The proTdPIP2;1 promoter was fused to the β-glucuronidase (gusA) gene and the resulting construct was transferred into rice (cv. Nipponbare). Histochromatic analysis of proTdPIP2;1::Gus in rice plants revealed that the GUS activity was observed in leaves, stems and roots of stably transformed rice T3 plants. Histological sections prepared revealed accumulation of GUS products in phloem, xylem and in some cells adjacent to xylem. The transcripts were up-regulated by dehydration. Transgenic rice plants overexpressing proTdPIP2;1 in fusion with TdPIP2;1, showed enhanced drought tolerance, while wild type plants were more sensitive and exhibited symptoms of wilting and chlorosis. These findings suggest that expression of the TdPIP2;1 gene regulated by its own promoter achieves enhanced drought tolerance in rice.

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1. Introduction

Constitutive promoters have been widely used as experimental tools to assess the effects of transgene expression in many plant species. However, in some cases, constitutive expression may be harmful to the host plant, causing sterility, delayed development, abnormal morphology, yield penalty, altered grain composition or transgene silencing (Gago et al., 2011). In order to avoid such problems, the use of tissue-specific promoters is an alternative since gene expression is restricted to tissues of interest and at given developmental stages. Promoters induced in response to environmental stimuli have also an interest in avoiding accumulation of transgene products during the whole life cycle. Aquaporins are encoded by a gene family with members exhibiting both tissue specific and inducible regulation. In Arabidopsis, the expression patterns of AtPIP1;2, AtPIP2;1, AtPIP2;6, and AtPIP2;7 have been established providing important indications about their function. Transgenic Arabidopsis plants carrying a fusion of AtPIP1;2 promoter with GUS gene exhibited strong staining in essentially all leaf cell types upon histochemical assay (Postaire et al., 2010). On the other hand ProPIP2;7::GUS showed patchy expression, including expression in mesophyll cells. The proPIP2;1::GUS and ProPIP2;6::GUS showed, by contrast expression in the veins (Prado et al., 2013). A number of studies indicate the differential expression of aquaporin transcripts or proteins in response to stresses or other physiological or developmental changes. It has been reported...
that in *Arabidopsis* roots, salt stress decreased the expression of all PIP2 genes (Boursiac et al., 2005), but drought stress resulted in a different regulation pattern of the genes, namely a decreased expression of AtPIP2;2 and AtPIP2;3, and an increased expression of AtPIP2;5 (Alexandersson et al., 2005). In rice, osmotic stress resulted in an increase of OsPIP2 transcripts in roots (Guo et al., 2006). In barley, drought stress weakened expression of *HvPIP2;1* in roots and enhanced the expression in shoots (Katsuhara et al., 2003). In maize, cold stress decreased the level of all PIP2 transcripts (Aroca et al., 2006). These observations indicated that the members of the PIP gene families have their own characteristic response to hormones and abiotic stresses and have tissue-specific expression patterns. Most aquaporin genes are repressed by auxin during lateral root formation. Treatment of whole roots with the auxin indole-3-acetic acid (IAA) induced an overall inhibition of aquaporin gene expression. Auxin reduces root hydraulic conductivity both at the cell and whole-organ levels (Péret et al., 2012). Auxin response factor (ARF) proteins function as transcription factors controlling auxin-responsive genes (Calderon-Villalobos et al., 2010). Moreover, auxin alters aquaporin spatial expression during lateral root development. In fact, expression studies using transcriptional and translational fusions revealed that *Arabidopsis* PIP2;1 is highly expressed in the stele and less in outer root layers (Péret et al., 2012). Previous studies have identified elements upstream of tobacco NtAQP1 that regulate expression during development or in response to phytohormones such as gibberellic acid and abscisic acid (ABA) (Siefritz et al., 2002). In ice plants (*Mesembryanthemum crystallinum* L.), an ABA-responsive element (ABRE) has been identified in the promoter region of aquaporin McMIPB gene (Yamada et al., 1997). McMIPB has been identified as a PIP1 type aquaporin and is mainly located at the xylem parenchyma in the ice plants (Kirch et al., 2000). In *Rapsedose* (*Brassica napus* var. *napus*), the *BnPPIP1* promoter activity was detected in the apical meristem and the adjacent tissues, where cells were undergoing rapid expansion (Yu et al., 2005). The analysis of the upstream sequences of rice, maize and *Arabidopsis thaliana* has shown the presence of putative regulatory elements such as DREs (drought responsive elements), LTREs (low temperature responsive elements) and ABREs (ABA responsive elements), upstream of a variety of PIP and TIP genes, indicative of regulation of these genes at a transcriptional level in response to abiotic stresses (Forrest and Bhave, 2008).

Recent studies suggest that some plant aquaporins including *NtAQP1*, *AtPIP1;2*, and *HvPIP2;1* reduce the diffusion resistance of CO2 in leaves. Uehlein et al. (2008) showed that tobacco (*Nicotiana tabacum* L.) aquaporin *NtAQP1* functions as a CO2 transporter, whereas tobacco aquaporin *NtPIP2;1* was shown to have no CO2 permeability (Otto et al., 2010), suggesting that CO2 permeability differs largely between aquaporins. On the other hand, some aquaporins play a role in water transport in plants, which affects plant responses to drought.

Several crops show genotypic differences in how leaf gas exchange responds to water stress, with certain genotypes being capable of sustaining plant transpiration until the soil becomes fairly dry, whereas others react with a decline in transpiration when the soil is still relatively wet (Heinemann et al., 2011). This has been observed over a wide range of crops, such as maize (Ray and Sinclair, 1997), soybean (Vadez and Sinclair, 2001; Hufstetler et al., 2011), groundnut (Bhatnagar-Mathur et al., 2007), rice (Serraj et al., 2007) and pearl millet (Kholová et al., 2010). This also has been observed over a wide range of crops, such as maize (Ray and Sinclair, 1997; Sadras and Milroy, 1996).

Previously, we isolated a durum wheat *PIP2* gene, *TdPIP2;1* and found that its expression in *Xenopus* oocytes resulted in an increase in *P* compared to water injected oocytes. Phenotypic analyses of transgenic tobacco plants expressing *TdPIP2;1* generated a tolerance phenotype towards drought and salinity stress (Ayadi et al., 2011). To address the regulatory mechanism of *TdPIP2;1* expression, we studied the role of plant hormones and abiotic stresses on the activity of the *TdPIP2;1* promoter during different stages of development in rice. Our results strongly suggest that the Pro* *PIP2;1* promoter could be used to drive expression of genes (i.e. abiotic stress responsiveness, defense genes, tissue-specific and vascular expression) preferentially in vascular tissues of rice. Minimization of water loss in response to water deficit is a major aspect of drought tolerance and can be achieved through the lowering of transpiration per unit leaf area (stomatal conductance). The transpiration rate (TR) of the generated transgenic rice plants overexpressing pro* *TdPIP2;1* promoter in fusion with *TdPIP2;1* gene was monitored as the soil dried progressively for about 15 days. These lines exhibited high level of tolerance to drought stress. Overexpression of the isolated *TdPIP2;1* gene in rice plants is worthwhile to elucidate the contribution of this protein in the tolerance mechanism to salt and drought stresses.

### 2. Materials and methods

#### 2.1. Plant materials and DNA extraction

Durum wheat (*Triticum turgidum* L. subsp. durum) cultivar *Om Rabi3*, was supplied by INRAT Laboratoire de Physiologie Végétale (Tunis, Tunisia). Genomic DNA was extracted from leaves of young seedlings using the CTAB method (Michiels et al., 2003a).

#### 2.2. Cloning of pro* *TdPIP2;1* by HE-TAIL-PCR method

The *TdPIP2;1* gene from durum wheat (*Triticum turgidum* L. subsp. durum) was previously isolated and functionally characterized (Ayadi et al., 2011). The sequences of *TdPIP2;1* is available in the database (GenBank ID: EU182655). The 5′-flanking region of *TdPIP2;1* was isolated by the use of the high-efficiency thermal asymmetric interlaced (HE-TAIL) PCR method as described by Michiels et al. (2003b). PCR reactions were carried out with genomic DNA, from durum wheat as template, four gene-specific reverse primers (30 nucleotides) (AQ1, AQ2, AQ3, AQ4) designed close to the *TdPIP2;1* 5′-UTR sequence, and four arbitrary degenerate primers (R1, R2, R3 and R4) (Table 1). Three rounds of PCR were performed on a Perkin–Elmer 9600 thermal cycler using the

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prom-BamH1</td>
<td>5′-CCCAACGGTTCTGGTACTAGGTTGCGACT-3′</td>
</tr>
<tr>
<td>Prom-HindIII</td>
<td>5′-CGGGATCCGGGCTGGCGACTGAGGACC-3′</td>
</tr>
<tr>
<td>AQ1</td>
<td>5′-CAACGTCCCACGCGCAGCCTGACCATTGAT-3′</td>
</tr>
<tr>
<td>AQ2</td>
<td>5′-GCAAGCACTGGCCGACGGCCATTGACGAT-3′</td>
</tr>
<tr>
<td>AQ3</td>
<td>5′-GTCTGACATAGCCAGGAGAAATTGCTG-3′</td>
</tr>
<tr>
<td>AQ4</td>
<td>5′-GTCGCTCTGATTGCTG-3′</td>
</tr>
<tr>
<td>R1</td>
<td>5′-GTCGAGGGAGGAGGACGGAGAAGAAC-3′</td>
</tr>
<tr>
<td>R2</td>
<td>5′-GTACGAGGGAGGAGGACGGAGAAGAAC-3′</td>
</tr>
<tr>
<td>R3</td>
<td>5′-GCCGCTCTGATTGCTG-3′</td>
</tr>
<tr>
<td>R4</td>
<td>5′-GTCGAGGGAGGAGGACGGAGAAGAAC-3′</td>
</tr>
<tr>
<td>ForTdPIP2;1</td>
<td>5′-ATGGCCAAAGGAGGCTGCACG-3′</td>
</tr>
<tr>
<td>RevTdPIP2;1</td>
<td>5′-ATGTCGAGGGAGGAGGACGGAGAAGAAC-3′</td>
</tr>
<tr>
<td>GusAF</td>
<td>5′-CTCATGCGTCTGATGCTG-3′</td>
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<td>GusAR</td>
<td>5′-AGCGGGTATGCTGATGCTG-3′</td>
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<td>OsExpR</td>
<td>5′-ATGGCCAAAGGAGGCTGCACG-3′</td>
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product of the previous PCR as template for the next reaction. Thermal conditions and reaction mixture were used as described for the HE-TAIL-PCR method by Michiels et al. (2003b). Products of the tertiary PCR, with the control (Rn–Rn), were loaded on a 1% agarose/EBTBr gel to determine appropriate product lengths. Target products were defined as fragments that are absent in the control reaction, (Rn–Rn) and with similar amplification lengths in re-actions AQ3/R2 and AQ4/R2 (taking the small length difference caused by the specific primers AQ3 and AQ4 into consideration). Target bands were cut from the agarose gel, purified and ligated into the pGEM TEasy vector (Promega). The corresponding promoter “ProTdPIP2;1:1” of 1060 bp was cloned into pGEM-T Easy vector to generate pGEM-P, TdPIP2;1 and sequencing was performed on an automated four-capillary ABL Prism 3100 genetic analyzer (Amersham). The search for putative cis-elements in the ProTdPIP2;1:1 promoter sequence was carried out using the databases Plant care (HUhttp://bioinformatics.psb.ugent.be/webtools/plantcare/html/UIH) and PLACE (http://www.dna.affrc.

2.3. Construction of the binary vector, and rice transformation

To perform expression and histochemical studies, we generated transcriptional (pro TdPIP2;1::GUS and CaMV35S::GUS as positive control) and translational (pro TdPIP2;1::tdPIP2::GUS and CaMV35S::TdPIP2;1::GUS as positive control) fusions. The promoter pro TdPIP2;1 fragment was released by BamHI/HindIII from pGEM-P, TdPIP2;1 and then cloned upstream of the gusA gene into the pCAMBIA1309Z vector (Camiba, Canberra, Australia) digested with the same restriction enzymes.

The resulting constructs were then mobilized into Agrobacterium tumefaciens strain EHA105 to transform japonica rice (Oryza sativa L. cv. Nipponbare) through coculture of seed-embryo callus (Sallaun et al., 2003). Seeds were originally provided by the National Institute of Agrobiological Sciences (NIAS, Tsukuba, Japan). The regenerated, hygromycin-resistant plants were numbered and named as MA1 to MA5. MA1 harbors the empty vector while MA2, MA3, MA4 and MA5 harbors the pCAMBIA1301-CaMV35S::GUS, pCAMBIA1301-proTdPIP2;1::GUS, pCAMBIA1301-CaMV35S::TdPIP2;1::GUS and pro TdPIP2;1::TdPIP2;1::GUS T-DNA constructs, respectively. The WT rice plants were used as controls.

2.4. Histochemical GUS staining

GUS activity was assayed histochemically by incubating tissue sections or seedlings under vacuum infiltration in 50 mM Na2HPO4 buffer (pH 7.0), 0.5 mM K3[Fe(CN)6], 0.5 mM K4[Fe(CN)6], 0.1% Triton X-100, and 1 mg/l X-Gluc (5-bromo-4-chloro-3-indolyl β-d-glucuronide cyclohexyl ammonium salt) for several minutes and then overnight at 37 °C. The pigments and chlorophyll were removed by soaking the tobacco tissues for several hours in 70% ethanol. These stained tissues were photographed or used for histological analysis. Transgenic plants driven by the CaMV35S promoter and wild-type rice were the respective positive and negative controls.

For histological sectioning by vibratome, transverse sections of 35 μm thick were cut after mounting root in a 3% (w/v) agarose blocks. Observation of tissue sections prepared by vibratome was made by use of a Leica DM 4500 fluorescence microscope. Image J tool, a free image processing and analysis program was used to acquire, display, edit and analyze images.

2.5. Abiotic stress experiments in-vitro

Seeds of wild type and transgenic japonica rice (Oryza sativa L. cv. Nipponbare) of homozygous T3 generation were used in the subsequent abiotic stress assays. Thirty seeds of WT and transgenic plants for three experiments repetitions were surface-sterilized by immersion in 70% ethanol for 1 min, rinsed with sterile distilled water and treated with 40% solution of sodium hypochlorite for 30 min. Finally, seeds were rinsed five times with sterile distilled water. Seeds were incubated in sterile distilled water in growth chamber (16 h of light per day, 500 μE m–2 S–1, 28 ± 2°C day/night). After 2 days, to evaluate the growth rate under osmotic and salt stress conditions, young seedlings were transplanted to a new medium supplemented with 150 mM NaCl or 125 mM Mannitol, as optimized by Ben Saad et al. (2012). These dishes were transferred and placed vertically in a growth chamber. After 15 days of culture, root/shoot lengths were determined in transgenic and WT seedlings using UTHSCSA image tool, a free image processing and analysis program was used to acquire, display, edit and analyze images (http://www.ddsdx.uthscsa.edu/dig/iTides.html).

2.6. RNA extraction and RT-PCR assay

Total RNA from roots and leaves of 2 weeks-old plants (seeds of WT and transgenic rice plants) under control and drought stress conditions (20% PEG 6000), were extracted using the TRIZOL method (Invitrogen). We used (PEG 6000) solutions to mimic dry soil by lowering the water potential and simulate drought stress in plants, because PEG molecules with a Mr ≥ 6000 cannot penetrate the cell wall pores (Carpita et al., 1979). To remove contaminated DNA, total RNA (10 mg) were treated with RNase-free DNase (Promega). DNase-treated RNA samples (0.5 μg) were reverse-transcribed using 5U M-MLV reverse transcriptase (Invitrogen). The reverse transcription (RT) reaction was performed at 37 °C for 1 h using 2 mM oligo-dT18 as a primer. Two microlitres of the first strand cDNA was used as template for PCR amplification. Primer pairs were designed with Primer 3 software to ensure gene specificity in amplification of the house-keeping OsExp gene (Os06g11070.1) and the gusA gene. The gusA gene under the control of the ProTdPIP2;1:1 promoter was amplified using two primers (GusA Forward and GusA Reverse) at 55°C for 30 cycles from gusA gene (Table 1). The synthesized cDNAs from control and stressed plants (20% PEG) were used as template to amplify the gusA gene. Samples were denatured for 5 min at 94 °C and then run for 35 cycles of 30 s at 94 °C, 45 s at 58 °C and 1 min at 72 °C followed by 5 min at 72 °C as a final extension. The PCR products were separated by agarose gel electrophoresis. Image J, a free image processing and analysis program was used to quantify PCR bands (http://rsweb.nih.gov/ij/). To ensure reproducibility, the experiment was repeated three times.

2.7. Water treatments

The soil water status was monitored using the fraction of transpirable soil water (FTSW). According to Sinclair et al. (2008), plants respond to the progressive drying of soil in a similar manner when water stress is expressed as FTSW. In order to estimate the FTSW value of each pot, soil moisture was kept high by daily full watering of all pots and the day before the start of measurements was followed by one night of drainage. On the next morning, the initial pot water capacity was determined by weighing all the pots. Total transpirable soil water (TTSW) was calculated as the difference between initial pot capacities (Wi) and final pot weight after soil desiccation (Wf). FTSW was estimated as the ratio of actual transpirable soil water (ATSW) to TTSW. ATSW being the mass difference between daily (Wt) and final pot weight. When the plants had 8 on the main stem leaves, a single drought cycle started in half of the pots (four pots for each genotype).
FTSW = ATSW/TTSW = \left( \frac{W_f - W_j}{W_i - W_f} \right)

Four control pots were watered in late afternoon to replace the daily water loss. Water deficit in the stressed pots progressively occurred as the plants were not re-watered and covered with a plastic bag to prevent soil evaporation. Initial values of FTSW may be regarded equal to 1. The experiment ended when the transpiration rate of each stressed pot was less than 10% of that of the fully watered pots (Sinclair and Ludlow, 1986).

2.8. Plant transpiration rate

Each day, plant transpiration (TRj) per unit leaf area (mmol m\(^{-2}\) s\(^{-1}\)) was calculated for each pot as the mass difference between weightings every 24 h (including the watering for un-stressed pots) were divided by plant total leaf area on the previous day. At the same time, stomatal conductance (mmol m\(^{-2}\) s\(^{-1}\)) was measured each morning between 9:00 a.m. and 12:00 p.m. on the last panicle leaf per plant. The measures on both plants, the control and treatment plants were taken by a porometer (Leaf Porometer Model SC-1 DECAGON). Conductance measurements were performed on leaves and on its abaxial face, where stomatal density is greater.

Normalized transpiration rate (NTR) = transpiration of stressed plants/average transpiration of control plants = \(\frac{G_s}{G_{s\text{max}}}\)

Porometry was also used to determine the last day of measurements (when \(g_s(\text{stressed})/g_s(\text{control})\) was less than 0.1), allowing us to estimate the value of TTSW of each pot. Mean TTSW was remarkably stable.

2.9. Modeling whole plant response to increasing water deficit

To account for the daily fluctuation of transpiration rate resulting from changes in evaporative demand, relative daily values of TR were calculated by dividing the drought treatment values by the corresponding mean values on the well watered (control) treatment. To minimize the effect of variation in initial plant size, daily ratio values of TR for FTSW \(>\) 0.6 were normalized to result in a mean value of 1, when the soil was still moist. The calculation of normalized plant transpiration (NTR) was necessary for the quality of further linear model.

The plant responses to water deficit were approached using a two-slope linear relation with one parameter (FTSW) indicating the FISW threshold below which conductance starts decreasing. The parameter FTSW was thus estimated by fitting a two-slope linear model to the experimental data (Eq. (1));

\[
y = \begin{cases} 
1 & \text{if } \text{FTSW} \geq \text{FTSW}_t \\
1/\text{FTSW}_t \times \text{FTSW} & \text{else}
\end{cases}
\]

2.10. Statistical analysis

All data analysis was performed using R software for statistical computing from the institute for statistics and Mathematics of Wirtschafts University of Vienna (http://www.r-project.org/). Means were compared by computing unpaired Student tests and pair wise multiple parameter comparisons were made using Tukey's honestly significant differences (HSD) test to obtain significance groups.

3. Result

3.1. Isolation and in-silico analysis of the ProTdPIP2;1;1 promoter

To further investigate the regulation of the TdPIP2;1;1 gene, a 1060 bp genomic DNA fragment upstream of the 5' region of the TdPIP2;1;1 translated sequence gene, including 80bp of the 5'UTR, was isolated from Triticum durum by the HE-TAIL-PCR method. During tertiary cycling, two major specific bands were amplified using AQ3/R2 and AQ4/R2 primers but no amplification was detected with R2/R2 primers. The cloned 1060 bp fragment was sequenced and analyzed by software's from the databases. PlantCARE showed the presence of a putative transcription start site (TSS +1) at position –80 pb and a TATA box at –30 upstream of the TSS (Fig. 1). This was consistent with the regular features of eukaryotic promoters (Hachez et al., 2006). BLAST search of ProTdPIP2;1;1 against the plant promoter databases PLACE and PlantCARE predicted the presence of some cis-regulatory elements related to transcription levels (enhance gene expression levels), tissue-specific gene expression (vascular expression in xylem and phloem), abiotic (dehydration, cold, salt, anaerobic circadian rhythms and light induction), biotic (fungal elicitors) and hormone (ABA signaling) stress responses (Table 2). In addition, several potential binding sites for transcription factors such as MYB, MYC and WRKY were also identified. Hence, this analysis suggests that the sequence isolated displays characteristic features of a promoter region and may contain stress responsive cis acting elements.

3.2. Characterization of pro TdPIP2;1 in rice plants

In order to investigate the localization and the physiological role of durum wheat PIP aquaporin promoter (proTdPIP2;1;1) in plant grown under stress conditions, we generated transgenic rice plants expressing TdPIP2;1;1 gene under the control of the strong 35S promoter (MA4 construct) or the proTdPIP2;1;1 (MA5 construct), and the GUS gene under the control of the strong 35S promoter (MA2 construct) or the proTdPIP2;1;1 (MA3 construct). Ten transgenic lines of the four different constructs were confirmed by PCR for their transgenic status (data not shown). Three representative transgenic

+1600 ATTACATG CATTTCTCA TCCATTTT TACCATCC ACCAGGAC ACCAGGAT ATAGGGCC
+ 990 CCGGCTCT CTTGCTCT TCCCTGCT TCCCTGCT ACCTCCAAC CCGGCTCT CGGCTCTC
+ 920 CTGCCTCT CAGGCTCT ACCTCCAAC CCGGCTCT CGCTCTCT CTCCCTCT CTCCCTCT
+ 850 TCCCTCTG CAGGCTCT ACCGCTCT CTCCCTCT CCGGCTCT CGCTCTCT CGCTCTCT
+ 780 CGGCCTCT CACGGCAAC CAGGCTCT ACCGCTCT CGCTCTCT CGCTCTCT CGCTCTCT
+ 710 CCTGCCTT CAGGCTCT ACCGCTCT CTCCCTCT CTCCCTCT CCGGCTCT CGCTCTCT
+ 640 CACGGCTT CAGGCTCT ACCGCTCT CTCCCTCT CTCCCTCT CCGGCTCT CGCTCTCT
+ 570 GCCTCTCT ACCGCTCT CTCCCTCT CTCCCTCT CTCCCTCT CCGGCTCT CGCTCTCT
+ 500 ACCGCTCT CTCCCTCT ACCGCTCT CTCCCTCT CTCCCTCT CCGGCTCT CGCTCTCT
+ 430 CCGGCTCT CTCCCTCT CTCCCTCT ACCGCTCT CTCCCTCT CTCCCTCT CTCCCTCT
+ 360 ACCGCTCT CTCCCTCT ACCGCTCT CTCCCTCT CTCCCTCT CTCCCTCT CTCCCTCT
+ 290 TCTCCCTCT CTCCCTCT ACCGCTCT CTCCCTCT CTCCCTCT CTCCCTCT CTCCCTCT
+ 220 ACCGCTCT CTCCCTCT ACCGCTCT CTCCCTCT CTCCCTCT CTCCCTCT CTCCCTCT
+ 150 TCTCCCTCT CTCCCTCT ACCGCTCT CTCCCTCT CTCCCTCT CTCCCTCT CTCCCTCT
+ 100 CCCGCTCT

Fig. 1. Nucleotide sequence of the proTdPIP2;1;1 promoter with the 5'UTR (–80) of the TdPIP2;1;1 gene. The putative transcription start site (TSS) is indicated as +1. The TATA box and putative cis-acting elements are highlighted. +, positive strand; -, minus strand. Promoter sequence analysis was carried out using software’s from the databases: Plant care (HUhttp://bioinformatics.psb.ugent.be/webtools/plantcare/htmlUT) and PLACE (http://www.dna.ufr).
T3 homozygous lines for each construct that harbored one or two T-DNA copies, as determined by Q-PCR assay based on amplification of the hpt gene sequence, were selected and further evaluated for gusA transcript accumulation, phenotypic and stress physiological assays.

3.3. *ProTdPIP2;1* is a organ and tissue-specific promoter

Histochemical staining of whole plants at early developmental stages (i.e. 5 and 15 days-old seedlings), grown under control and stressed conditions enable detection of GUS activity in leaves and in roots (Fig. 2). The intensity of staining was affected when seedlings were challenged especially with PEG. To further investigate the tissue-specific location, histological sections were prepared using a vibratome from histochemically stained leaf and root samples (Fig. 3a). In the root sections of 5 and 15 days old seedlings, GUS precipitates were localized in phloem, xylem, pith and in some cells adjacent to xylem (Fig. 3b). In leaf sections, GUS precipitates were observed in vascular tissues (data not shown) in 5 and 15 days old seedlings. Altogether, these results show that *proTdPIP2;1* activity is not age-dependent but organ and tissue-specific.

3.4. Accumulation of GUS transcripts in relation to stress treatment

The gusA mRNA expression levels were estimated by semi-quantitative RT-PCR performed on young leaves and roots from seeds of WT and transgenic rice plants. Two week-old plants were grown in MS medium supplemented with 125 mM mannitol or 150 mM NaCl. Under normal MS growth conditions, the transgenic plants overexpressing either *TdPIP2;1* under the control of its own promoter *proTdPIP2;1* (MA5) or the strong constitutive CaMV-35S promoter (MA4) showed no significant difference in root length compared to wild-type plants (Fig. 5a). Whereas, in the same condition the transgenic plants overexpressing *TdPIP2;1* (MA5) showed a significant enhancement of leaf length compared to the transgenic plants (MA4) and wild-type plants (P < 0.05) (Fig. 5a). Drought and salt stress decreased significantly the plant biomass (data not shown) and this is may be due to the considerable decrease in plant growth, photosynthesis and leaf senescence during the stress. Fifteen day exposure of two-day old seedlings to lower NaCl (150 mM) and mannitol (125 mM) concentrations gave highly significant differences among the genotypes (Fig. 5). Mannitol and NaCl stresses similarly reduced length of both root and leaf (P < 0.05) and died (Fig. 5 c, d, e). Interestingly, the

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**Table 2**

Putative cis-acting elements present in the TdPIP2;1 promoter of TdPIP2;1 gene by databases Plant care (http://bioinformatics.psb.ugent.be/webtools/plantcare/htmlUHJ) and PLACE (http://www.dna.affrc).

<table>
<thead>
<tr>
<th>Function/responsive</th>
<th>Putative cis-element/consensus</th>
<th>Positiona</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light-responsive elements</td>
<td>Sp1 441(−), 792(+), 445(+)</td>
<td>572(+), 577(+)</td>
<td>Plantcare</td>
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<tr>
<td></td>
<td>G-box 910(+)</td>
<td>766 (+), 769(+)</td>
<td>PlantCare</td>
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<tr>
<td>CT-rich motif, enhance gene expression</td>
<td>CAATBOX1 148(+)</td>
<td>150 (+)</td>
<td>Shirsat et al. (1989)</td>
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<td>Anaerobic induced gene elements</td>
<td>ANAERO2CONSEQUENSUS 2(−)</td>
<td>2(−) TACGAG</td>
<td>Mohanty et al. (2005)</td>
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<td>ANAERO3CONSEQUENSUS 863(+)</td>
<td>863(+), TCTATAC</td>
<td>Mohanty et al. (2005)</td>
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<td>High level, light regulated, and tissue specific expression</td>
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<td>302 (+), GATA</td>
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<tr>
<td></td>
<td>GATA BOX 577 (+), 579(−)</td>
<td>577 (+), 579(−)</td>
<td>Lüscher et al. (1990)</td>
</tr>
<tr>
<td>Transcription factor binding sites responsive to water stress and induced by dehydration stress</td>
<td>MYB GATA 536(+)</td>
<td>536 (+), CNGGTR</td>
<td>(Chinnusamy et al., 2004); Lee(2005)</td>
</tr>
<tr>
<td></td>
<td>MYC BOX 261 (+)</td>
<td>261 (+), 264(+)</td>
<td>CNGGTR</td>
</tr>
<tr>
<td></td>
<td>435 (+)</td>
<td>435 (+), 438(+)</td>
<td></td>
</tr>
<tr>
<td>Transcription of CBF/DREB1 gene</td>
<td>MYC CONSENSUS 526 (+)</td>
<td>526 (+), CNGGTR</td>
<td>Lüscher et al. (1990)</td>
</tr>
<tr>
<td></td>
<td>MYC CONSENSUS SAT 526 (+)</td>
<td>526 (+), CNGGTR</td>
<td>(Chinnusamy et al., 2004); Lee(2005)</td>
</tr>
<tr>
<td>Elicitor-responsive transcription of defense genes</td>
<td>WBOX 340 (+)</td>
<td>340 (+), CTGACY</td>
<td>Yamamoto et al. (2004)</td>
</tr>
<tr>
<td></td>
<td>−10PEHPSPBD 997 (+)</td>
<td>997 (+), TATTCT</td>
<td>Thum et al. (2001)</td>
</tr>
<tr>
<td>Chloroplast gene expression circadian rhythms</td>
<td>WRKY71OS 312 (+)</td>
<td>312 (+), TGAC</td>
<td>Xie et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>GATA BOX 302 (+)</td>
<td>302 (+), CTGACY</td>
<td></td>
</tr>
</tbody>
</table>

a. Positive strand; −, minus strand.

Position of the cis-elements upstream ATG.
transgenic plants overexpressing TdPIP2;1 under its own promoter (MA5) showed white roots compared to the other transgenic plants and wild-type plants ($P < 0.05$), which showed brown oxidized roots (Fig. 5). It seems that overexpression of the TdPIP2;1 in rice plants results in better growth, vigor and tolerance to stress conditions than the wild-type plants.

3.6. Fraction of transpirable soil water (FTSW)

The transpiration rate (TR) of the generated transgenic rice plants overexpressing proTdPIP2;1 promoter in fusion with TdPIP2;1 gene was monitored as the soil dried progressively for about 15 days. The three representatives transgenic T3 homozygous lines for each construct that were evaluated for growth in-vitro condition were evaluated for growth in greenhouse (Fig. 6).

In this experiment, NTR (transpiration of stressed plants/average transpiration of control plants = $G_s/G_{smax}$) was calculated to reflect daily transpiration rate and FTSW was calculated to reflect soil-water content. The relationship between NTR and FTSW fitted well to the plateau regression function (Fig. 7). The FTSW thresholds for the NTR decline were similar for both lines of transgenic rice (MA3 and MA4) and wild type. Whereas the thresholds for the NTR decline were higher for the transgenic MA5 line than for wild type. The threshold for the decrease transpiration (NTR) occurred when FTSW values of about 0.363, 0.394 and 0.314 were reached for WT, MA3 and MA4, respectively. Using a plateau regression procedure, the FTSW threshold at which transpiration rates began to decline was determined for each line tested. FTSW threshold values ranged from 0.31 to 0.39 for WT, MA3 and MA4 (Table 3). No significant trend in a change of the FTSW threshold for transpiration rate decrease with MA3 and MA4. However, there was a significant linear ($r^2 = 0.67$) decline of FTSW threshold with MA5. For this line a preventive strategy was observed to avoid excessive water loss by a stomata closure for a high FTSW. In this case, line MA5 was the most tolerant to drought stress, whereas; lines MA3 and MA4 were sensitive to drought stress.

These lines exhibited high level of tolerance to drought stress. Minimization of water loss in response to water deficit is a major aspect of drought tolerance and can be achieved through the lowering of transpiration per unit leaf area (stomatal conductance). Overexpression of the isolated TdPIP2;1 in rice plants is worthwhile to elucidate the contribution of this protein in the tolerance mechanism to salt and drought.

4. Discussion

PIPs, the plasma membrane associated aquaporins, can control water transport across the plasma membrane, and can be expressed in a temporal-and spatial-specific manner (Vaucheret et al., 1998). Transgenes driven by constitutive promoters may
result in homology-dependent gene silencing (Oettgen, 2001),
while inducible-promoters are highly organized sequences
required for the correct spatial and temporal gene expression (Liu
and Stützel, 2002). The use of stress inducible promoters is ex-
pected to be a potentially powerful tool for improving plant resis-
tance to abiotic and biotic stresses. The present work, focused on
the regulation and function of TdPIP2;1, one of the most highly
expressed PIPs. We isolated and characterized the promoter
region of the TdPIP2;1 gene and determined its activity
throughout the life cycle of the plant using GUS and the
TdPIP2;1 as the reporter in transgenic rice plants, suggesting that it contains
conserved transacting factors and cis-acting elements that enable
this promoter to be regulated in a tissue-specific in both dicots and monocots.

In silico, sequence analysis of proTdPIP2;1 revealed the presence
of cis-regulatory elements which could be required for abiotic
stress tolerance. We identified three highly conserved motifs in
positions (261(+) CNGTTR; 435(+) CNGTTR; and 526(+) CNGTTR or
CANNTG); they are identified at MYBCORE transcription factor
binding sites responsive to water stress and induced by dehydra-
tion (Abe et al., 2003; Oh et al., 2005). In Arabidopsis, the cis-
regulatory elements CBF3/DREB1A and ABF3 in transgenic rice
increased tolerance to abiotic stress without stunting growth
(Monneuse et al., 2011). In addition, sequence analysis of proTd-
PIP2;1 revealed the presence of WRKY transcription factor required
for positive and negative regulatory of abscisic acid signaling in this
position (312(-) TGAC). It also revealed the presence of GATA DNA
motifs which are implicated in light-dependent and nitrate-
dependent control of transcription (Reyes et al., 2004). These
GATA motifs were found in the promoter of Petunia chlorophyll a/b

![Fig. 3. Spatial GUS staining of root section of T2 transgenic rice plants harboring proTdPIP2;1:GUS and pro35S:GUS fusions. Binocular observation of GUS staining and overlay of two images of root section of WT (a), MA4 (b), MA5 (c). Observation under bright-field illumination microscopy (GUS crystals appear blue) of transverse sections prepared by vibratome (20 μm) after GUS histochemical staining of root from MA5 transgenic rice line (d). TdPIP2;1 fused to GUS and driven by its own promoter (proTdPIP2;1) was expressed in the phloem (1), xylem (2), and in some cells adjacent to xylem (3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image1)

![Fig. 4. RT-PCR analysis for monitoring the steady-state level of gusA mRNA in transgenic rice lines cultured in MS medium. Comparative expression levels of gusA mRNA relative to untreated control plants (a) and treated plants with 20% PEG in roots and leaves (b). A specific PCR product of 0.3 Kb corresponding to gusA gene was detected in the transgenic T3 homozygous lines. OsExp gene was used as an internal control.](image2)
binding protein, Cab22 gene; conserved in the promoter of all LHCCI type I Cab genes.

It is worth to determine whether the proTdPIP2;1 and or the transcription factor binding sites responsive to water stress and induced by dehydration, have a physiological significance allowing regulation of water membrane permeability in plant cells. Analysis of transgenic rice plants carrying proTdPIP2;1 fused to TdPIP2;1 gene (MA5) and challenged with either salt or drought stress, showed an enhanced growth and vigor compared to wild-type plants and thus conferring a better tolerance to abiotic stresses. The temporal and spatial expression pattern of proTdPIP2;1 was investigated using a gusA reporter gene system in leaves and roots of transgenic rice plants grown under control or stressed conditions. Transcript profiling shows an increase in the accumulation of TdPIP2;1 transcripts in response to abiotic stress, suggesting a role of TdPIP2;1 in stress acclimation. Based on previous expression surveys, four PIP isoforms (PIP1;2, PIP2;1, PIP2;6 and PIP2;7) were found to be the most highly expressed in Arabidopsis rosette. Arabidopsis PIP2;1 is one of the most highly expressed leaf isoforms and it may contribute to leaf water transport capacity (Prado et al., 2013). Preliminary characterization of transgenic rice plants transformed with TdPIP2;1 gene under the control of the 35S promoter (MA4) has revealed enhanced growth and vigor compared to wild-type plants when cultured under salt and drought stress in vitro, thus conferring a better tolerance to these abiotic stresses. Similar results were reported in our previous work, where the expression of TdPIP2;1 was positively correlated with stress—response pathways in transgenic tobacco plants (Ayadi et al., 2011).

Expression studies using transcriptional (proTdPIP2;1:GUS) and translational (proTdPIP2;1: TdPIP2;1:GUS) fusions revealed that TdPIP2;1 is expressed in the heterologous transgenic rice system. Histochemical staining and histological sections revealed the ability of the proTdPIP2;1 promoter to drive gusA expression with inducible and tissue-specific patterns. Indeed, GUS activity was detected in phloem, xylem, pith and in some cells adjacent to xylem. Additionally, the activity was detected at the very early stage of plant development, in 5 and 15 days old seedlings, even in leaf, GUS precipitates were observed in vascular tissues. Similar works concerned with the temporal and spatial specificity of the PIP promoters have been done and the results are consistent with our findings. In transgenic Arabidopsis harboring the Rh-PIP2;1 promoter::GUS fusion, the activity of Rh-PIP2;1 promoter was found to be developmental-dependent in almost all of the tested organs, and was particularly active in organs that were rapidly expanding, and in tissues with high water flux capacity (Li et al., 2008). In ice plants, among all of the tested tissues, the McMipA promoter activity appeared at a high level in the vascular bundles, while the McMipB promoter activity was present at a high level in the rapidly expanding tissues (Yamada and Bohnert, 2000). The McMipB promoter exhibited the strongest activity in the root meristems which...
was different from our observations, although the activity of the Rh-PIP2;1 promoter was also strong in the root meristems (Yamada et al., 1997). It has been reported that GUS expression driven by the BnPIP1 promoter of a Rapeseed PIP gene, was mainly located in the young shoots of 3-day-old seedlings and was obviously weakened when the seedlings grew to 6-day-old in transgenic tobacco (Yu et al., 2005).

A number of studies indicate the differential expression of aquaporin transcripts or proteins in response to stresses or other physiological or developmental changes (reviewed in Forrest and Bhave, 2008). However little is known so far about the transcriptional regulation of expression of these genes. In one study, elements were identified upstream of tobacco NtAQP1 gene that regulate expression in response to development or phytohormones such as gibberellic acid and abscisic acid (ABA) (Siefritz et al., 2002). The analysis of the upstream sequences of rice, maize and A. thaliana has shown the presence of a putative regulatory elements such as DREs (drought responsive elements), LTREs (low temperature responsive elements) and ABREs (ABA responsive elements), upstream of a variety of PIP and TIP genes, indicative of regulation of these genes at a transcriptional level in response to abiotic stresses (Forrest and Bhave, 2008).

Response of leaf expansion, stomatal conductance, and transpiration to soil water deficit could be described successfully by linear plateau models. Linear-plateau models were able to distinguish genotypes with respect to their physiological response to a drying soil (Liu and Stützel, 2002). We have adopted this strategy to distinguish genotypes with respect to their physiological response to a drying soil. Moreover, water deficit is one of the most important abiotic stress limiting upland rice yields. Analysis of transgenic rice plants transformed with TdPIP2;1 driven by its own promoter (MA5) or the constitutive 35S promoter (MA4) under salt and osmotic (PEG) stress in vitro, showed an enhanced growth and vigor compared to wild-type plants and thus conferring a better tolerance to these abiotic stresses. Therefore, it is worth to determine whether this result is confirmed in greenhouse experiments. Three experiments were conducted under greenhouse conditions to investigate drought-stress responses of WT and transgenic rice plants (MA3, MA4 and MA5). Drought affected transpiration rates by closure of stomata and changes in leaf morphology of the rice plant. The first observed response, if drought was initiated at the vegetative phase, was a relatively abrupt decline in leaf expansion. The first signs of declining soil water are leaf rolling and stomata closure. The most sensitive MA5 line to stomata closure for a high FTSW, was the most tolerant line to drought stress. Whereas, the MA3 and MA4 lines were the sensitive ones to drought stress.

To conclude, in our previous work, we found that the expression of TdPIP2;1, was tightly associated with stress—response pathways in tobacco (Ayadi et al., 2011), and here we found and confirmed that the expression of TdPIP2;1, was tightly associated with stress—response pathways in rice, an important agronomic crop, without causing undesirable growth phenotype or any penalty.

Fig. 6. photographs of plants of WT cv Nipponbare and proTdPIP2;1 transgenic lines MA3, MA4 and MA5 grown under control conditions (a) or subjected to an average (b, FTSW = 0.4) and severe (c, 15 days of water withholding, FTSW = 0.1).
On the other hand, we found that mannitol, an osmotic substrate, enhanced the activity of the TdPIP2;1 promoter, suggesting that proTdPIP2;1 may be involved in dehydration response. The transpiration rate (TR) of the generated transgenic rice plants overexpressing proTdPIP2;1 promoter in fusion with TdPIP2;1 gene was monitored as the soil dried progressively for about 15 days. These lines exhibited high level of tolerance to drought stress. Minimization of water loss in response to water deficit is a major aspect of drought tolerance and can be achieved through the lowering of transpiration per unit leaf area (stomatal conductance).

Contributions

In this study, we describe the isolation and characterization of the promoter region of TdPIP2;1 gene from durum wheat. Sequence analysis of proTdPIP2;1 revealed the presence of cis-regulatory elements which could be required for abiotic stress responses. Transgenic rice plants overexpressing proTdPIP2;1 in fusion with TdPIP2;1 and Gus genes revealed accumulation of GUS products in vasculature tissues. These plants showed enhanced drought tolerance when soil was left to dry progressively for 15 days without irrigation, while wild type plants exhibited symptoms of wilting and chlorosis in the same conditions.

This work provides insights for the TdPIP2;1 gene regulated by its own promoter which achieves enhanced drought tolerance in rice. This gene could be used in the breeding program to improve rice tolerance to drought.

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References


