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Research article

Functional analysis of the durum wheat gene *Td*PIP2;1 and its promoter region in response to abiotic stress in rice

Malika Ayadi [a], Delphine Mieulet [b], Denis Fabre [b], Jean-Luc Verdeil [b], Aurore Vernet [b], Emmanuel Guiderdoni [b], Khaled Masmoudi [a,c,*,1]

[a] Plant Protection and Improvement Laboratory, Centre of Biotechnology of Sfax (CBS), University of Sfax, Route Sidi Mansour Km 6, B.P "1177", 3018 Sfax, Tunisia
[b] CIRAD, UMR AGAP, F-34398 Montpellier, France
[c] ICBA, P.O. Box 14660, Dubai, United Arab Emirates

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ABSTRACT

In a previous work, we demonstrated that expression of *Td*PIP2;1 in Xenopus oocytes resulted in an increase in P_f compared to water injected oocytes. Phenotypic analyses of transgenic tobacco plants expressing *Td*PIP2;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 *Td*PIP2;1 gene. A 1060-bp genomic fragment upstream of the *Td*PIP2;1 translated sequence has been isolated, cloned, and designated as the pro*Td*PIP2;1 promoter. Sequence analysis of pro*Td*PIP2;1 revealed the presence of cis regulatory elements which could be required for abiotic stress responsiveness, for tissue-specific and vascular expression. The pro*Td*PIP2;1 promoter was fused to the β-glucuronidase (gusA) gene and the resulting construct was transferred into rice (cv. Nipponbare). Histochemical analysis of pro*Td*PIP2;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 pro*Td*PIP2;1 in fusion with *Td*PIP2;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 *Td*PIP2;1 gene regulated by its own promoter achieves enhanced drought tolerance in rice.

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 *At*PIP1;2, *At*PIP2;1, *At*PIP2;6, and *At*PIP2;7 have been established providing important indications about their function. Transgenic *Arabidopsis* plants carrying a fusion of *At*PIP1;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

* Corresponding author. Plant Protection and Improvement Laboratory, Centre of Biotechnology of Sfax (CBS), Route Sidi Mansour Km 6, B.P "1177", 3018 Sfax, Tunisia. Tel./fax: +216 74 872 091.
E-mail addresses: k.masmoudi@biosaline.org.ae, khaled.masmoudi@cbs.rnrt.tn (K. Masmoudi).
[1] International Center for Biosaline Agriculture (ICBA), P.O.Box 14660, Dubai, United Arab Emirates. Tel.: +971 4 336 1100x260; fax: +971 4 336 1155.

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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 *At*PIP2;2 and *At*PIP2;3, and an increased expression of *At*PIP2;5 (Alexandersson et al., 2005). In rice, osmotic stress resulted in an increase of *Os*PIP2 transcripts in roots (Guo et al., 2006). In barley, drought stress weakened expression of *Hv*PIP2;1 in roots and enhanced the expression in shoots (Katsuhara et al., 2003). In maize, cold stress decreased the level of all PIP2s transcripts (Aroca et al., 2006). These observations indicated that the members of the PIP genes 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 *Mc*MIPB gene (Yamada et al., 1997). *Mc*MIPB 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 Rapeseed (*Brassica napus* var. napus), the *Bn*PIP1 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 *Nt*AQP1, *At*PIP1;2, and *Hv*PIP2;1 reduce the diffusion resistance of CO_2 in leaves. Uehlein et al. (2008) showed that tobacco (*Nicotiana tabacum* L.) aquaporin *Nt*AQP1 functions as a CO_2 transporter, whereas tobacco aquaporin *Nt*PIP2;1 was shown to have no CO_2 permeability (Otto et al., 2010), suggesting that CO_2 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., 2007), groundnut (Bhatnagar-Mathur et al., 2007), rice (Serraj et al., 2009) and pearl millet (Kholová et al., 2010). This also supported the robustness of using the fraction of transpirable soil water (FTSW) as a stress covariable in drought studies (Serraj et al., 2009). Therefore, the soil moisture threshold, the factor of FTSW where transpiration declines is extremely useful to understand and forecast the genotypic behavior in face of water deficit (Ray and Sinclair, 1997; Sadras and Milroy, 1996).

Previously, we isolated a durum wheat PIP2 gene, *Td*PIP2;1 and found that its expression in Xenopus oocytes resulted in an increase in P_f compared to water injected oocytes. Phenotypic analyses of transgenic tobacco plants expressing *Td*PIP2;1 generated a tolerance phenotype towards drought and salinity stress (Ayadi et al., 2011). To address the regulatory mechanism of *Td*PIP2;1 expression, we studied the role of plant hormones and abiotic stresses on the activity of the *Td*PIP2;1 promoter during different stages of development in rice. Our results strongly suggest that the ProTdPIP2;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*Td*PIP2;1 promoter in fusion with *Td*PIP2;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 *Td*PIP2;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 Rabia3, 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 proTdPIP2;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 *Td*PIP2;1 is available in the database (GenBank ID: EU182655). The 5′-flanking region of *Td*PIP2;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 *Td*PIP2;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 1
The primers used in the HE-TAIL PCR, PCR and RT-PCR.

Primers	Sequence
Prom-BamHI	5′-CCCAAGCTTGGGTCACTAGTGATTGTGCGAGT-3′
Prom-HindIII	5′-CGGGATCCCGGCTGCTGCGGAGTGTGGACGC -3′
AQ1	5′CAC GAT GCC GCC CGC GCA CTG CGC CAC GAT-3′
AQ2	5′-AGC AGC GAC ACC TTG CGC GCC AGC AGC AGC-3′
AQ3	5′-GTG CAG TAG ACG AGG ACG AAG ATC ATG CCG-3′
AQ4	5′-GTC GGC CGC GGA CTG CAC CTT G TA GCC-3′
R1	5′-NGTCGASWGANAWGAA-3′
R2	5′-GTNCGASWCANAWGTT-3′
R3	5′-WGTGNAGWANCANAGA-3′
R4	5′-NCAGCTWSCTNTSCTT-3′
ForTdPIP2;1	5′-ATGGCCAAGGAGGTGAGCGA-3′
Rev TdPIP2;1	5′-ATGTCGAACAGCGGCGCCG-3′
GusAF	5′-CTCCTACCGTACCTCGCATTAC-3′
GusAR	5′- ACGCGCTATCAGCTCTTTAATC-3′
OsExpF	5′-CGGTTAGCTAGAGTTCATGTGAGA-3′
OsExpR	5′-ATTGGAGTAGTGGAGTGCCAAA-3′

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/EtBr 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 reactions 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 T-easy vector (Promega). The corresponding promoter "Pro*Td*PIP2;1;1 " of 1060 bp was cloned into pGEM-T Easy vector to generate pGEM-P_r *Td*PIP2;1 and sequencing was performed on an automated four-capillary ABI Prism 3100 genetic analyzer (Amersham). The search for putative cis-elements in the Pro*Td*PIP2;1;1 promoter sequence was carried out using the databases Plant care (HUhttp://bioinformatics.psb.ugent.be/webtools/plantcare/htmlUH) 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 *Td*PIP2;1::GUS and CaMV35S::GUS as positive control) and translational (pro*Td*PIP2;1::*Td*PIP2;1-GUS and CaMV35S::*Td*PIP2;1-GUS as positive control) fusions. The promoter pro *Td*PIP2;1 fragment was released by BamHI/HindIII from pGEM-P_r *Td*PIP2;1 and then cloned upstream of the gusA gene into the pCAMBIA1309Z vector (Cambia, 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 (Sallaud 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::*Td*PIP2;1::GUS and pro *Td*PIP2;1:: *Td*PIP2;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°C/25 °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/iTdesc.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 $M_r \geq 6000$ 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-dT_{18} 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 Pro*Td*PIP2;1;1 promoter was amplified using two primers (GusAF and GusAR) amplifying a fragment of 300 pb 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://rsbweb.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).

$$\mathrm{FTSW} = \mathrm{ATSW/TTSW} = \left(W_t - W_f\right) \Big/ \left(W_i - W_f\right)$$

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 (TR*j*) 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 unstressed 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
= (Gs/Gsmax)

Porometry was also used to determine the last day of measurements (when *gs*(stressed)/*gs*(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*t*) indicating the FTSW threshold below which conductance starts decreasing. The parameter FTSW*t* was thus estimated by fitting a two-slope linear model to the experimental data (Eq. (1):

$$\begin{aligned} &\text{if } \mathrm{FTSW} \geq \mathrm{FTSW}t && y = 1 \\ &\text{else} && y = 1/\mathrm{FTSWt} \times \mathrm{FTSW} \qquad (1) \\ &y \text{ being NTR.} \end{aligned}$$

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 *Td*PIP2;1 gene, a 1060 bp genomic DNA fragment upstream of the 5′ region of the *Td*PIP2;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 Pro*Td*PIP2;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 (pro*Td*PIP2;1) in plant grown under stress conditions, we generated transgenic rice plants expressing *Td*PIP2;1 gene under the control of the strong 35S promoter (MA4 construct) or the pro*Td*PIP2;1 (MA5 construct), and the GUS gene under the control of the strong 35S promoter (MA2 construct) or the pro*Td*PIP2;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

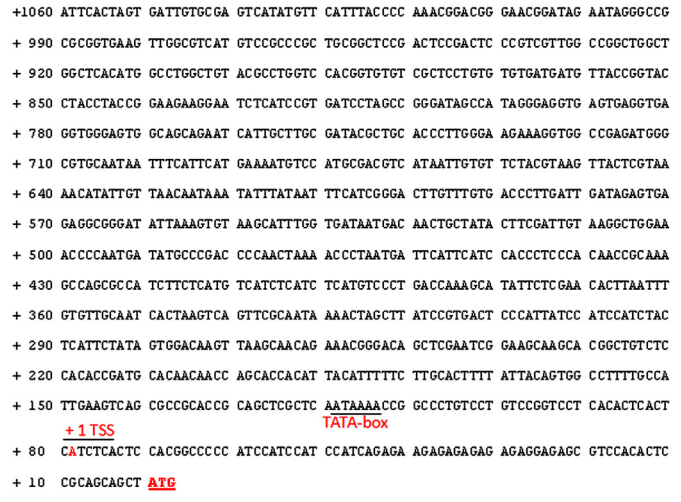

Fig. 1. Nucleotide sequence of the pro*Td*PIP2;1 promoter with the 5′UTR (−80) of the *Td*PIP2;1 gene. The putative transcription start site (TSS) is indicated as +1. The TATA box and putative Cis-acting elements are highlighted. +, positive strand; −, minus strand. Promoter sequence analysis was carried out using software's from the databases: Plant care (HUhttp://bioinformatics.psb.ugent.be/webtools/plantcare/htmlUH) and PLACE (http://www.dna.affrc).

Table 2
Putative cis-acting elements present in the P_rTdPIP2;1 promoter of TdPIP2;1 gene by databases Plant care (http://bioinformatics.psb.ugent.be/webtools/plantcare/htmlUH) and PLACE (http://www.dna.affrc).

Function/responsive	Putative cis-element/consensus	Position[a]	References
Light-responsive elements	Sp1	441(−), 792(+), (445(+) CC(G/A)CCC	Plantcare
	G-box	910(+)CACATGG	PlantCare
	CAATBOX1	148 (+) CAAT	Shirsat et al. (1989)
CT-rich motif, enhance gene expression	CTRMCAMV35S	28 (+) TCTCTCTCT	Pauli et al. (2004)
Anaerobic induced gene elements	ANAERO2CONSENSUS	2(−) AGCAGC	Mohanty et al. (2005)
	ANAERO3CONSENSUS	863 (+)TCATCAC	Mohanty et al. (2005)
High level, light regulated, and tissue specific expression	GATABOX	302 (+) GATA 577 (−)GATA	Lam et al. (1989)
Transcription factor binding sites responsive to water stress and induced by dehydration stress	MYBCORE	261 (+) CNGTTR 435 (+) CNGTTR	Lüscher et al. (1990)
Transcription of CBF/DREB1 gene	MYBCORE	526 (+) CNGTTR	Lüscher et al. (1990)
CBF3/DREB1A and ABF3 increased tolerance to abiotic stress elicitor-responsive transcription of defense genes regulates the transcription of CBF/DREB1 genes in the cold	MYCCONSENSUSAT	526 (+) CANNTG	(Chinnusamy et al., 2004); Lee(2005)
Elicitor-responsive transcription of defense genes	WBOX	340 (+) CTGACY	Yamamoto et al. (2004)
Chloroplast gene expression circadian rhythms light KW regulation chloroplast gene expression	−10PEHVPSBD	997 (+) TATTCT	Thum et al. (2001)
WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling	WRKY71OS	312 (−) TGAC	Xie et al. (2005)

+, Positive strand; −, minus strand.
[a] Position of the cis-elements upstream ATG.

T3 homozygous lines for each construct that harbored one or two T-DNA copies, as determined by Q-PCR assessment 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 pro*Td*PIP2;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 challenged by stress treatments including osmotic stress (20% PEG). To investigate the expression levels of *Td*PIP2;1 under stress conditions gusA specific primers were designed. PCR bands were quantified using Image J processing analysis program. In addition, RT-PCR products were sequenced to confirm the identity of the amplified fragments (data not shown). The *OsExp* gene (Caldana et al., 2007) was used as an internal control not influenced by abiotic stress treatment. Under standard growth conditions (time 0), both gusA and *Td*PIP2;1 genes were expressed at a certain level in roots and leaves (Fig. 4). Leaf tissues accumulated more gusA mRNA than roots, irrespective of plant age (Fig. 4a). After 24 h of stress treatment, the accumulation of gusA transcripts was both detected in leaf and root tissues at the same level (Fig. 4b). Fig. 4b shows the expression patterns of *Td*PIP2;1 gene in rice when subjected to water deficit induced by 20% PEG treatment in MA5 lines along with control line MA4 harboring gusA under the control of the 35S constitutive promoter. The expression pattern of *Td*PIP2;1 gene increases in response to water deficit. The transcript level of *Td*PIP2;1 was up-regulated in roots and leaves in MA5 lines and found up-regulated by PEG treatment (Fig. 4b).

3.5. In-vitro seedling growth under abiotic stresses

The three representative transgenic T3 homozygous lines for each construct previously evaluated for gusA transcripts accumulation, were evaluated for growth in MS medium and MS supplemented with 150 mM NaCl or 125 mM mannitol (Fig. 5). Root and leaf length were measured to identify plant characteristics for adaptation to drought and salt stress. Drought and salt tolerant transgenic rice lines generated longer roots and leaves when 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 *Td*PIP2;1 under the control of its own promoter pro*Td*PIP2;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 *Td*PIP2;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 (Fig. 5 b, c) and leaf (Fig. 5d, e) of WT seedlings by more than 80%. It is worth to notice that the transgenic plants overexpressing the *Td*PIP2;1 (MA4 and MA5) showed the highest leaf and root length than the wild-type plants when challenged with salt and osmotic stress (Fig. 5). When challenged with osmotic stress, the wild-type plants exhibited bleached leaves, a significant decrease in root and leaf length ($P < 0.05$) and died (Fig. 5 c, d, e). Interestingly, the

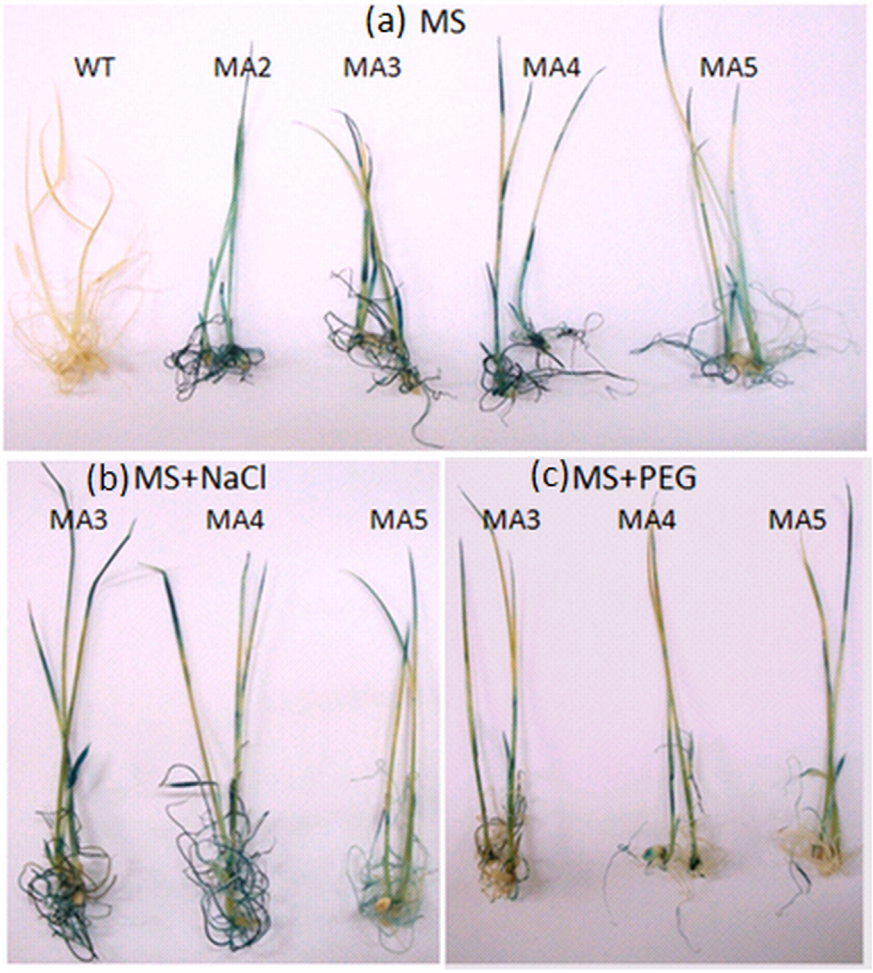

Fig. 2. Histochemical GUS staining of 7-day-old transgenic rice seedlings grown on MS medium and challenged with various abiotic stresses (200 mM NaCl, 20% PEG) for 24 h. MA2: p35S:GUS; MA3: pro*Td*PIP2;1:GUS; MA4: p35S:*Td*PIP2;1:GUS; MA5 : pro*Td*PIP2;1 : *Td*PIP2;1 :GUS.

transgenic plants overexpressing *Td*PIP2;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 *Td*PIP2;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 pro*Td*PIP2;1 promoter in fusion with *Td*PIP2;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 = Gs/Gsmax) 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 ($r2 = 0.67$) 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 *Td*PIP2;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

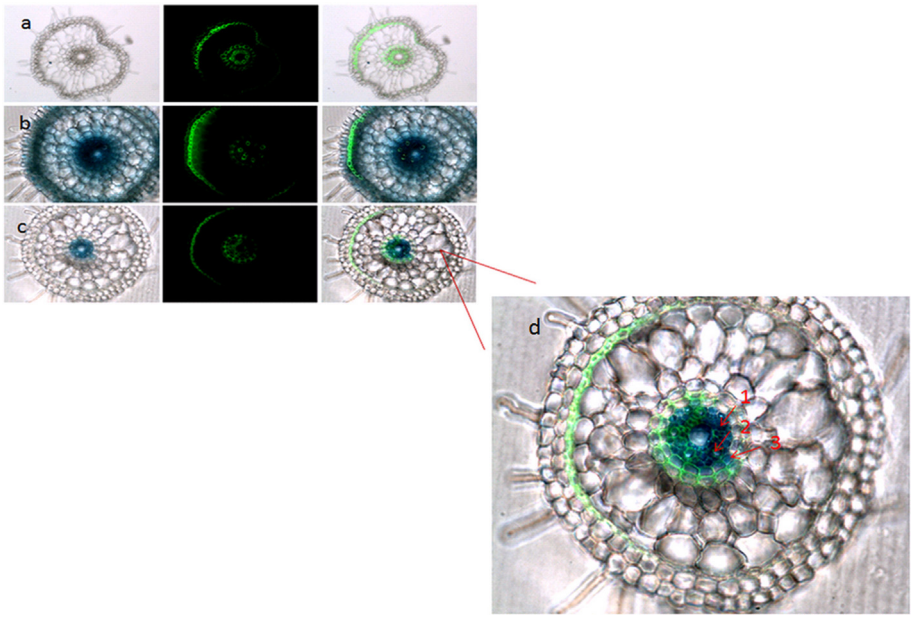

Fig. 3. Spatial GUS staining of root section of T2 transgenic rice plants harboring pro*Td*PIP2;1:GUS and pro35S:GUS fusions. Binocular observation of GUS staining and overlay of two images of root section of WT (a), MA4 (b), MA5 (c). Observation under bright-field illumination microscopy (GUS crystals appear blue) of transverse sections prepared by vibratome (20 μm) after GUS histochemical staining of root from MA5 transgenic rice line (d). *Td*PIP2;1 fused to GUS and driven by its own promoter (pro*Td*PIP2;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.)

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 expected to be a potentially powerful tool for improving plant resistance to abiotic and biotic stresses. The present work, focused on the regulation and function of *Td*PIP2;1, one of the most highly expressed PIPs. We isolated and characterized the promoter pro*Td*PIP2;1 region of the *Td*PIP2;1 gene and determined its activity throughout the life cycle of the plant using GUS and the *Td*PIP2;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. There are very few examples in the literature of monocot gene promoters that are functional in a dicot plant (Karandashov et al., 2004). Therefore, these results suggest that not all promoters will behave the same in dicots and monocots. Interestingly, our isolated promoter can carry out a different regulation in tobacco (data not show) than that discussed here in rice, 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 pro*Td*PIP2;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 dehydration (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 pro*Td*-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

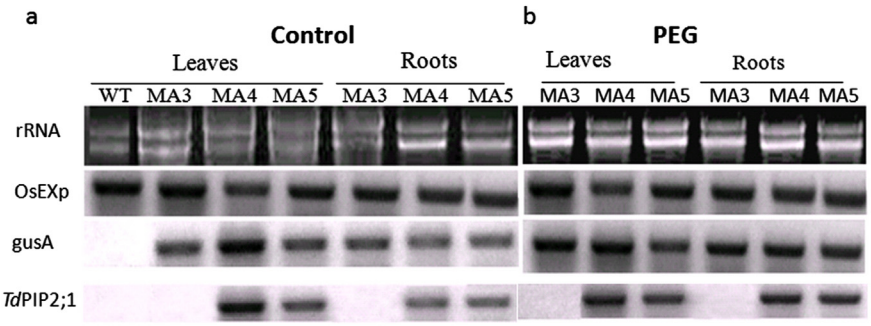

Fig. 4. RT-PCR analysis for monitoring the steady-state level of gusA mRNA in transgenic rice lines cultured in MS medium. Comparative expression levels of gusA mRNA relative to untreated control plants (a) and treated plants with 20% PEG in roots and leaves (b). A specific PCR product of 0.3 Kb corresponding to gusA gene was detected in the transgenic T3 homozygous lines. OsExp gene was used as an internal control.

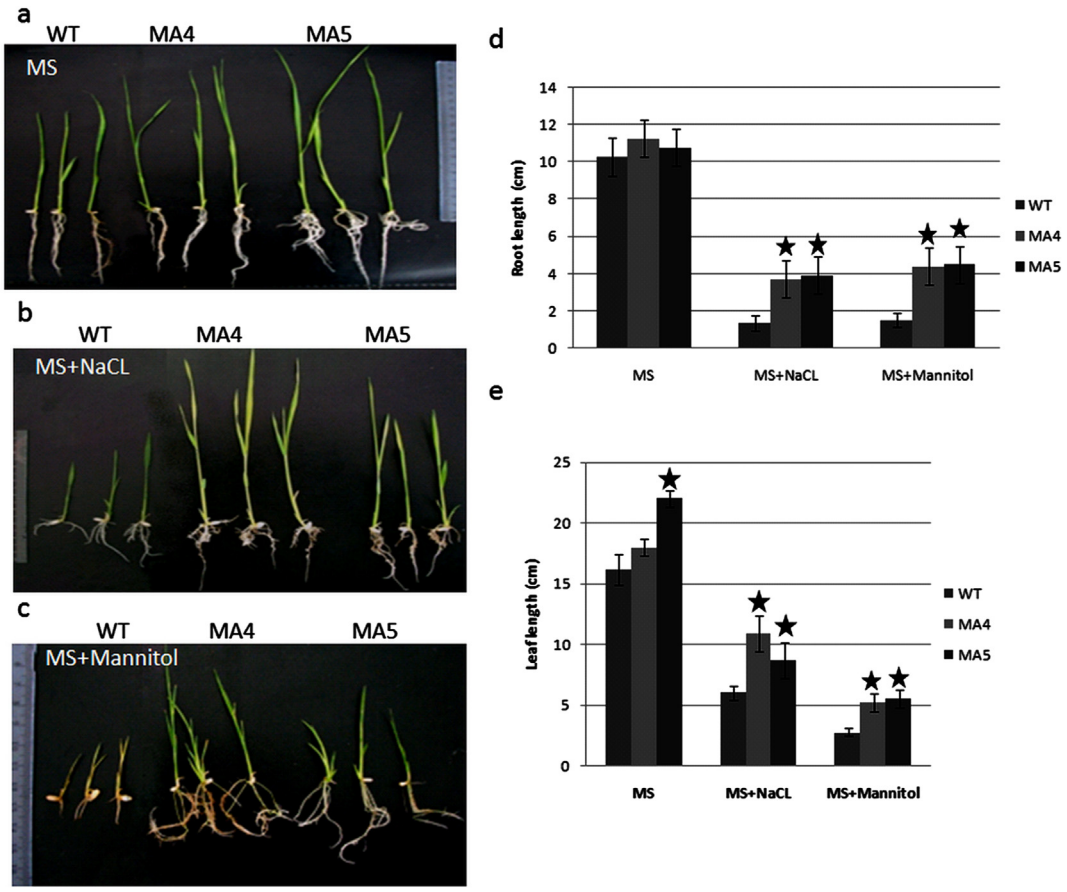

Fig. 5. (a, b, c) Phenotype and growth rate of wild type (WT) and transgenic MA4 and MA5 rice plants cultured in growth chamber on MS medium (a), MS medium supplemented with 150 mM NaCl (b), and MS medium supplemented with 125 mM Mannitol (c), respectively; (d, e) statistic analysis of root and leaf length of WT, MA4 and MA5 transgenic plants cultured on MS medium, MS medium supplemented with 125 mM Mannitol and MS medium supplemented with 150 mM NaCl, respectively. Transgenic and wild-type rice plants were gown on MS medium during 1 week, then transferred to culture plates containing MS medium supplemented with either 125 mM Mannitol or 150 mM NaCl. The photographs were taken 15 days after stress application. The results are expressed as the means SE of measurements from three different experiments. Values are means SD ($n = 5$). Asterisks indicate values significantly different from the control in the same group according to *t*-test ($P = 0.05$).

binding protein, Cab22 gene; conserved in the promoter of all LHCII type I Cab genes.

It is worth to determine whether the pro*Td*PIP2;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 pro*Td*PIP2;1 fused to *Td*PIP2;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 pro*Td*PIP2;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 *Td*PIP2;1 transcripts in response to abiotic stress, suggesting a role of *Td*PIP2;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 *Td*PIP2;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 *Td*PIP2;1 was positively correlated with stress–response pathways in transgenic tobacco plants (Ayadi et al., 2011).

Expression studies using transcriptional (pro*Td*PIP2;1:GUS) and translational (pro*Td*PIP2;1: *Td*PIP2;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 pro*Td*PIP2;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 *Mc*MipA promoter activity appeared at a high level in the vascular bundles, while the *Mc*MipB promoter activity was present at a high level in the rapidly expanding tissues (Yamada and Bohnert, 2000). The *Mc*MipB promoter exhibited the strongest activity in the root meristems which

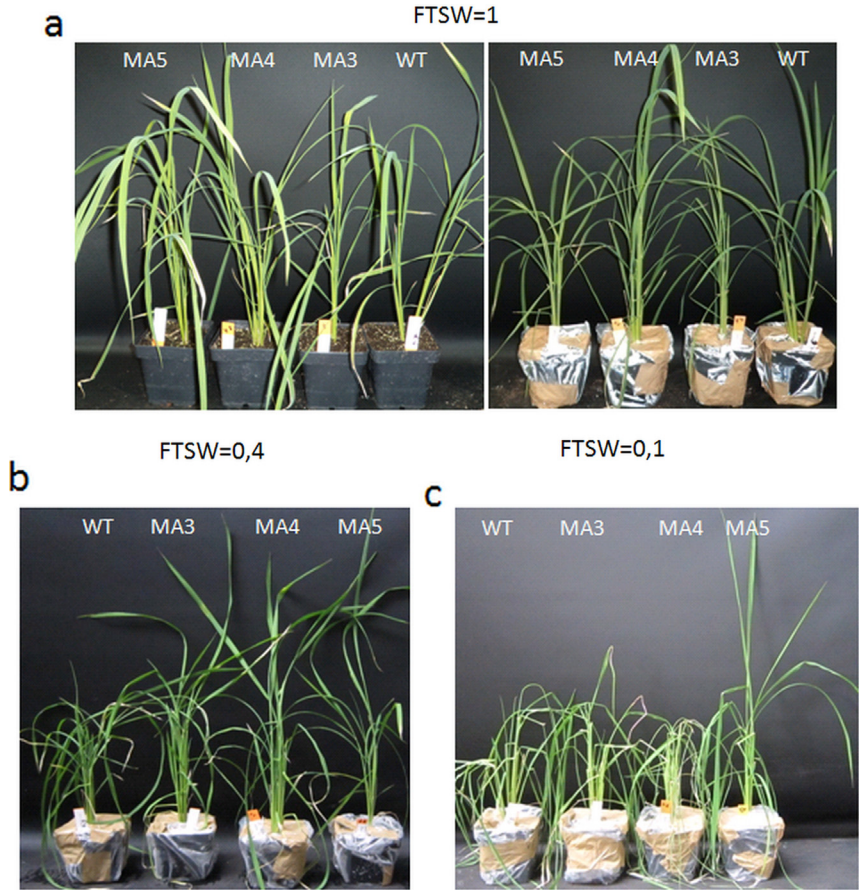

Fig. 6. Photographs of plants of WT cv Nipponbare and pro*Td*PIP2;1 transgenic lines MA3, MA4 and MA5 grown under control conditions (a) or subjected to an average (b, FTSW = 0.4) and severe (c, 15 days of water withholding, FTSW = 0.1).

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 *Bn*PIP1 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 in these studies (Ray and Sinclair, 1997; Rosental et al., 1987). The virtue of these models is that they allow definition of critical soil water contents at which physiological process start to decline (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 *Td*PIP2;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 *Td*PIP2;1, was tightly associated with stress–response pathways in tobacco (Ayadi et al., 2011), and here we found and confirmed that the expression of *Td*PIP2;1, was tightly associated with stress–response pathways in rice, an important agronomic crop, without causing undesirable growth phenotype or any penalty.

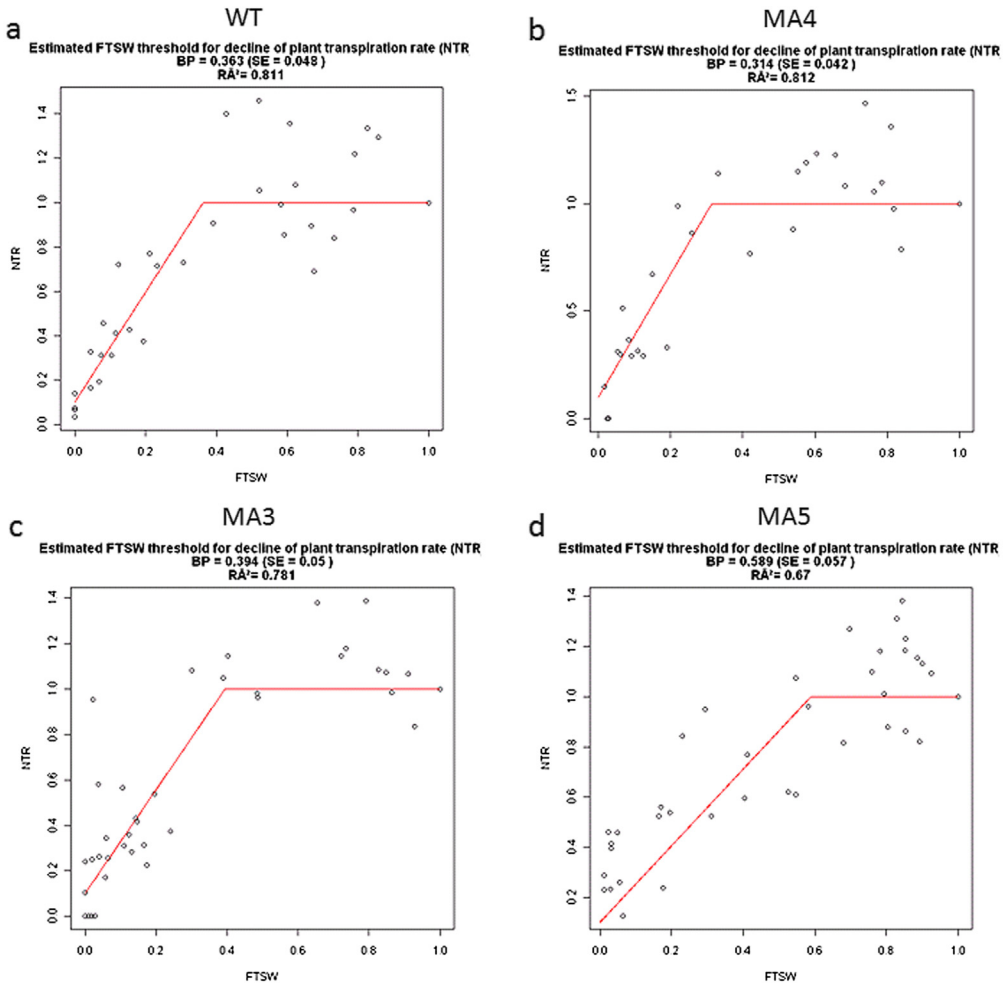

Fig. 7. Variability for normalized transpiration rate (NTR) control under drought stress among the four different lines tested. They are ordered from the least sensitive one WT, MA4 and MA3 to the most sensitive (stomata closure for a high FTSW) (MA5).

On the other hand, we found that mannitol, an osmotic substrate, enhanced the activity of the *Td*PIP2;1 promoter, suggesting that pro*Td*PIP2;1 may be involved in dehydration response. The transpiration rate (TR) of the generated transgenic rice plants overexpressing pro*Td*PIP2;1 promoter in fusion with *Td*PIP2;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 *Td*PIP2;1 gene from durum wheat. Sequence analysis of pro*Td*PIP2;1 revealed the presence of cis-regulatory elements which could be required for abiotic stress responses. Transgenic rice plants overexpressing pro*Td*PIP2;1 in fusion with *Td*PIP2;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 *Td*PIP2;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.

Table 3
FTSW threshold values.

	BP	(R^2)
WT	0.363	0.811
MA3	0.394	0.781
MA4	0.314	0.812
MA5	0.589	0.67

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