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Relationship of root aquaporin genes, *OsPIP1;3*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP2;1* and *OsNIP2;1* expression with drought tolerance in rice

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Abstract

Aquaporins are channel proteins that facilitate the passive uptake and transport of water, ions, molecules and regulate the water balance at the plant level. Genes for aquaporins are important since they play a major role in water uptake. The present study was therefore conducted to assess the relationship of aquaporins predominantly expressed in roots in response to drought tolerance. Transcript abundance of five rice aquaporin genes (PIP2;5, PIP1;3, PIP2;4, TIP2;1 and NIP2;1) in five rice genotypes (Banglami, ARC 10372, Inglongkiri, Ranjit and IR 64) under well-watered and drought stress conditions were measured using qRT-PCR. Significant variation in PIP relative transcript abundance in roots was observed between varieties under both well-watered and drought stress conditions. In roots, the expression levels of most OsPIPs were found to be increased in drought stress conditions. *OsPIP1;3*, *OsPIP2;5*, *OsTIP2;1* and *OsNIP2;1* were upregulated more in upland (drought tolerant) rice and Ranjit lowland (moderately drought tolerant) genotypes than drought susceptible lowland IR-64 rice in drought stress conditions and downregulated/unchanged in well-irrigated conditions. The OsPIP genes were identified as candidate genes based on their high expression under drought stress situations and played a significant role in water uptake and root development under water stress conditions. A positive correlation was observed between fold increase in aquaporin expression and degree of drought tolerance which suggest that these aquaporins genes might play an important role in root development under water stress situation to overcome stress.

Key words: Aquaporins, gene expression, root traits, drought tolerance and rice

Introduction

A large part of agriculture in the north-eastern region (NE) of India is still rainfed and therefore, a slight shift in rainfall pattern would severely affect agriculture. Rice, being the major crop in the NE region suffers due to the erratic rainfall and intermittent drought conditions. Moisture stress is the foremost abiotic stress that constrains the rice production and rice productivity in rainfed ecology (Lanceras et al. 2004). In Asia 10 million ha of rainfed upland rice and 13 million ha of rainfed lowland rice is affected by drought (Pandey et al. 2007). Traits such as deep root length, high root volume and fibrous root are important phenotypic characters for increased drought tolerance. Drought stress causes various changes at the molecular and physiological levels. Drought stress leads to over expression and repression of various genes that cause accumulation of various useful osmolytes and detoxifying action, slow down in transpiration rate and reduced foliage growth (Pareek et al. 2010).

In rice, stress-inducible genes which protect the cells under water stress are OsLEA3-2 proteins, LIP9 and LIP19 (Duan and Cai, 2012); dehydrins, OsDhn1 (Lee et al. 2004) osmotin, enzymatic antioxidants which help in detoxification are peroxidase, superoxide dismutase and catalase, guaiacol peroxidase (Lum et al. 2014), ascorbate peroxidase enzymes (Caverzan et al. 2012) and potent non-enzymatic antioxidants includes ascorbate (AsA) and glutathione (GSH) within the cell (Pandey et al. 2015) polyamines such as putrescine, spermidine and spermine (Yang et al. 2007) and m-RNA binding proteins, key enzymes for osmolyte biosynthesis.

Various regulatory proteins play a directive role

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in signal transduction and expression of stress-related genes in rice. These are a variety of transcription factors such as Myb transcription factor, RAB-16C, WSI76, WSI724, NAC6 (Rabbani et al. 2003), mitogen-activated protein kinases (MAPKs) for eg. OsMPK3 (Xie et al. 2012), OsMAPK33 (Lee et al. 2011) DSM1 (Ning et al. 2010) calcium-dependent protein kinases (CDPKs) for eg. OsCPK10 (Bundo and Coca, 2017) OsCPK9 (Wei et al. 2014) and SOS kinase eg., SOS1, SOS2 and SOS3 (Ji et al. 2013), phospholipases (Frank et al. 2000) and enzymes for phospholoipids' metabolism.

Various water channel proteins and sugars (various PIP) are engaged in water and ions movement under water stress conditions which help in imparting drought tolerance (Zargar et al. 2017; Wang et al. 2015). Li et al. (2017) reported that GoPIP1 aquaporin is associated with water transport under water stress condition in *Galega orientalis*, a promising perennial forage legume. Recently, Zhou et al. (2016) reported that under moisture stress the induction of OsAHL1 is involved in root development to reduce moisture stress effect. These genes may serve as candidate genes (CGs) for large scale genotyping for association study to reveal a true association between candidate genes and corresponding phenotype to use in marker-assisted selection to improve drought tolerance. (Gudys et al. 2018; Yadav et al. 2019).

Among many candidate genes (CG) so far reported for root traits, genes for aquaporins are very important (Javot and Maurel 2002) since they play a major and critical role in the absorption of water from the soil. Plant aquaporins are involved in the transport of water to support the vital activities of plants for their development. It helps in the maintenance of cell water potential even under moisture and chilling stress for their survival (Gomes et al. 2009; Lian et al. 2006; Sakurai et al. 2008). Studies on plant aquaporins and plant water relations suggest that aquaporins might play a vital and critical role in the uptake of water from the soil (Javot and Mourel 2002). Most importantly, it was found that aquaporin expression is effecting water uptake by rice roots during drought-stress (Lian et al. 2004, 2006). Many studies reported positive and negative expression of genes under moisture stress conditions (Shahebi et al. 2018; Chen et al. 2019). For example, rTip1 aquaporin (Liu et al. 1994) is up-regulated under moisture stress. Chen et al. (2019) reported that overexpression of *OsNRT2.1* in rice improves drought tolerance. Aquaporins are also involved in maintaining high water conductivity and water potential balance at seedlings stage under drought stress (Lian et al. 2004).

Research on aquaporins in rice mostly focused on the major aquaporins subfamily plasma-membrane intrinsic proteins (PIPs). In rice, among 33 aquaporin isoforms have been identified (Sakurai et al. 2005), of which three PIPs and one TIP (OsPIP1;3, OsPIP2;4, OsPIP2;5 and OsTIP2;1) were strongly expressed in the roots and barely expressed in the leaf blades, (Nugyen et al. 2013; Wu and Cheng, 2014). RWC3 aquaporin play a considerable role in moisture stress tolerance (Lian et al. 2004). Therefore we selected OsPIP1;3, OsPIP2;4, OsPIP2;5, OsTIP2;1 and one new NIP (OsNIP2;1) to analyze their differential expression in well-watered and drought stress condition in rice varieties of Assam. Study at genome level using the gene expression knowledge holds great promise in developing functional markers, marker identification for root trait is limited in indica rice concerning drought tolerance. Therefore, the present study aiming at assessing the aquaporins as a candidate gene for their associations with root traits associated with drought tolerance, which may facilitate their use as functional marker in marker-assisted breeding.

Materials and methods

Plant material, growth condition and phenotypic observations

Experiments were conducted using three genotypes of upland rice namely, Banglami, a local drought-tolerant line (http://dbtaau.ac.in/allele.html), ARC10372 and Inglonkiri and two of low land rice genotypes IR-64 (IR 5657-35-2-1/IR 2061465-1-5-5) and Ranjit a mega variety (Pankaj x Mahsuri) obtained from Regional Agricultural Research Station, Titabar, Jorhat (Assam). IR-64 was used as a drought susceptible check and ARC10372 was used as a drought-tolerant check (Mishra et al. 1994). Experiments were performed in a rainout shelter at Assam Agricultural University, Jorhat in pots and PVC pipes (4kg cm3 strength) of 1-meter length and 30 cm diameter arranged in 1meter depth trench in a randomized complete block design with three replications.

Seeds were soaked for 12 h and kept in a muslin cloth in the dark area for 40 h at 29°C all germinated seeds of upland and lowland rice genotypes were grown in pots in rain out shelter. Pots were filled with 5 parts finely ground silty clay loam soil and 1 part vermicompost. Plants were supplemented with NPK chemical fertilizer @ 40:20:20 kg per hectare based on the available area for sowing in the pots. In the non-stress experiment, all the genotypes were grown in lowland conditions up to the end of the experiment (2-4 cm from the soil surface) and in the stress experiment irrigation was done at every alternate day to keep moisture content above field capacity up to 28 days. The drought was induced by withholding irrigation after the 29th day of sowing in the stress experiment. Manual weeding was done at intervals of seven days. The gravimetric method was used for the determination of soil moisture content in soil (Reynolds et al. 1970). Soil moisture contents in the pots were brought down to 4.8±0.78% for creating water stress condition (14 days water withholding).

Leaf rolling score of nine in IR-64 (drought susceptable) and Ranjit (mnoderately tolerant to drought) according to the IRRI manual suggest a strong drought ("Standard Evaluation System for Rice (Ses)", 2002). The various root traits of 45-day-old plants of stress and non-stress situation were recorded using the standard method (Shashidhar 2012). Randomly selected plants along with roots were dried in the oven at 80°C for 120 h and root dry weight and shoot dry weight were recorded and paired t-test was applied.

Expression analysis of rice aquaporin genes

Roots of 42 days old seedlings stress treatment and non-stress pots (control) were harvested and put in liquid nitrogen and ground in a mortar with a pestle. RNA was extracted from the frozen powder of the tissue with Triazol reagent (Invitrogen, CA, USA). The expression analysis of the five aquaporin genes in roots of five rice varieties both in control and stressed conditions were tested by quantitative real-time PCR. The sequence for the chosen aquaporin genes were retrieved from rap-db database (http://rapdb.dna.affrc.go.jp/). The primers OsPIP2:4, OsPIP2;5, OsNIP2;1, OsPIP1;3, OsTIP2;1 and Actin were designed by Gene Runner v.6.1.23 with GC% of 60-65% and melting temperature ranging from 60-65^{o}C (Table 1). The first-strand cDNA was synthesized by using the PrimeScript TM 1st strand cDNA synthesis kit (Takara, clontech) as per the manufacturer's instruction. The relative expression of MEP pathway genes in the same 3 tissues were checked with quantitative real-time PCR by —"CT method (Livak et al. 2001) on Step One Plus Real-Time PCR System (Applied Biosystem) using the SuperScript III Platinum SYBR Green One-Step qRT-PCR with ROX Kit (Invitrogen, CA, USA) according to manufacturer's instruction. PCR amplification was performed under the following conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute finally melting at 95°C for 15, 60°C for 1 minute. The gene expression was normalized against an internal reference gene, rice actin gene and IR-64 control sample was arbitrarily chosen to be the calibrator of gene expression.

Statistical analyses were performed for root trait data and drought tolerance data in SPSS using ANOVA to identify significant differences between varieties.

Results

Phenotypic observations

In the present study based on leaf rolling score Banglami, ARC10372 and Inglonkiri were found to be drought-tolerant variety while IR-64 behaved like a drought susceptible cultivar and Ranjit showed moderately drought tolerance response under water stress condition (Table 1).

Table 1. Drought sensitivity score as leaf rolling of genotypes in the present study

S.No.	Name of genotypes	Mean	Range	Variance
1.	Banglami	0.66	0-1	0.58
2.	ARC 10372	1.3	0-3	1.53
3.	Inglongkiri	0.66	0-1	0.58
4.	Ranjit	5.66	5-7	1.15
5.	IR-64	8.33	9	0

Root traits

The analysis of variance revealed significant variation among genotypes for root length, fresh and dry root weight studied (Table 2). The highest root length was observed in Inglongkiri (22 cm) followed by ARC 10372 and Banglami with root length of 16 cm and 15 cm, respectively in well irrigated situation. In the water stress situation significant increase in root length of drought tolerant genotypes *viz*., Banglami (34%), ARC 10372 (14%) and Inglongkiri (8%) was observed using student t-test analysis (Table 3). A significant increase in root fresh weight and dry weight of roots was also being observed under drought stress condition.

Expression analysis of rice aquaporin genes

In the present study, the transcript abundance of 5 rice aquaporin genes (PIP2;5, PIP1;3, PIP2;4, TIP2;1

Table 2. ANOVA of genotypes for root traits under irrigated and drought condition

Source of variation	df	Mean square					
		Root length		Fresh root weight		Dry root weight	
		Irrigated	Drought	Irrigated	Drought	Irrigated	Drought
Genotypes	4	53.25*	165.70*	2.13*	6.01*	0.33*	0.54*
Replication	2	0.39	0.62	0.0026	0.0016	1.7E-05	0.0013
Error	8	0.47	0.31	0.0029	0.0016	1.1E-04	0.0003

*Significant level at P < 0.05

and NIP2;1) in five rice genotypes under well-watered and drought stress conditions was measured using qRT-PCR in experiment to assess the association between root aquaporin expression and drought tolerance.

Under both well-watered and drought stress conditions, significant variation in PIP relative transcript abundance in roots was observed among varieties. Under well-watered conditions among the 5 genotypes, ARC 10372 exhibited comparatively low PIP transcript abundance in roots while Banglami exhibited the highest relative transcript abundance of PIP2;5, PIP2;4 and TIP2;1. ARC10372 showed the highest relative transcript abundance of PIP2;5 in drought condition. Ranjit and ARC 10372 both expressed the highest transcript of PIP2;4 under drought stress situation. Differential expression of aquaporins in roots under water stress was examined by calculating the relative fold-level increase in each aquaporin under drought to its expression under irrigated situation for each genotype (Fig. 1).

For instance, *OsPIP2;5* was highly induced by 16 folds in ARC10372, 14 folds in Banglami and 4 folds in Inglongkiri; while downregulated by almost 1 fold in Ranjit and IR-64 in drought stress as compared to well-watered condition. The transcript level of *OsPIP1;3* was highly upregulated by 15 folds in Banglami followed by ARC 10372 with 11 folds, 7 folds in Inglongkiri and 5 folds in Ranjit in drought stress as compared to well-watered situation. Further investigation of differential expression in between upland drought tolerant and lowland susceptible showed that transcript level of *OsPIP1;3* was upregulated by 15, 11 and 7 folds in Banglami, ARC10372 and Inglongkiri upland drought tolerant genotypes respectively, as compared to drought susceptible IR-64 in drought stress condition.

The expression level of *OsTIP2;1*, a root-specific TIP was higher in drought tolerant and moderately drought tolerant genotypes, approximately 11 times higher in Banglami and 7 fold higher in ARC 10372 and 3 fold higher in Inglongkiri than IR-64. Similarly,

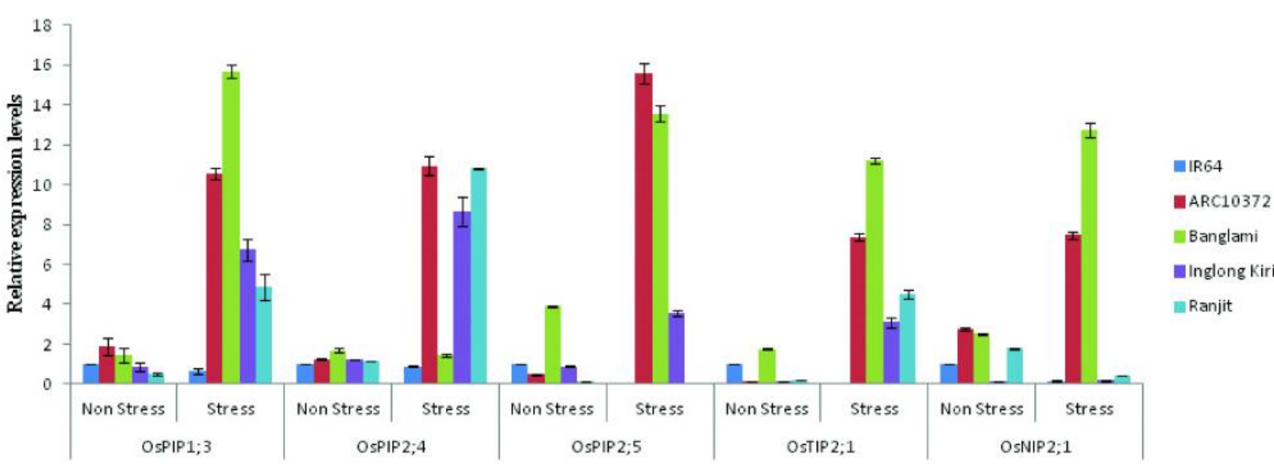

Fig. 1. The relative expression patterns of *OsPIP, OsTIP* and *OsNIP* genes in rice roots in response to well-watered and drought stress condition

In roots, the expression levels of most *OsPIP*s were found to increase in drought stress condition. Ranjit showed a 5 fold increase in relative expression of *OsTIP2;1* over IR-64. The transcript level of

Table 3. T-test value for root length, fresh root weight and root dry weight in irrigated and drought condition among drought tolerant genotypes

Source of variation	Root length		Fresh root weight		Dry root weight	
	Irrigated	Drought	Irrigated	Drought	Irrigated	Drought
Banglami	14.83	19.83	0.77	1.3	0.18	0.28
ARC 10372	16.17	18.5	0.7	1.1	0.25	0.33
Inglongkiri	22.33	24.33	2.77	3.9	0.93	1.14
't' test	0.041*	0.046*	0.042*			

*Significant level at $P < 0.05$

OsNIP2;1 was highly upregulated in drought tolerant and moderately drought tolerant genotypes under drought situation. Banglami showed 13 folds increase and ARC10372 showed 7 folds increase in expression Transcript level of OsPIP2;5, OsPIP1;3 OsTIP2;1 and OsNIP2;1 upregulated in drought tolerant cultivars only under drought stress. However the transcript level *OsPIP2;*4 was unspecific for upland and low land cultivar, it was upregulated in Ranjit, ARC 10372 and Inglonkiri whereas unchanged in Banglami. In this study, we observed that upland rice and lowland rice showed different physiological responses to water deficit condition such as higher expression of aquaporins and increased in root length, root weight in upland rice genotypes as compared to low land rice genotypes. These differences in physiological responses of lowland and upland rice revealed that upland and lowland rice have different mechanisms for drought tolerance.

Discussion

Among various abiotic stresses, drought is the major abiotic factor limiting the rice productivity in the rainfed upland ecosystem. Various studies have been conducted to understand the genetic mechanisms of drought tolerance for developing drought tolerant varieties. Plant breeders are targeting root traits for improving yield under water stress situation for developing drought tolerant rice varieties. The consensus-based on research of last 35 years revealed that deep and thick roots might help in improving drought tolerance (Fukai and Cooper 1995; Govda et al. 2011; Uga et al. 2013; Verma et al. 2019). Aquaporins play a very important role in water uptake and regulate the water balance at the plant level. Aquaporin is identified as one of the key trait differential adaptation of different rice varieties in water limiting environments (Matsunami et al. 2016). For example, MzPIP2;1, an aquaporin involved in radial water movement in both water uptake and transportation and controls water absorption. Expression of MzPIP2;1 increased the drought and salt tolerance in transgenic *Arabidopsis* (Wang et al. 2015). *FaPIP2;1* aquaporin help in maintaining leaf water potential, chlorophyll content, and photosynthetic rate even during moisture stress condition (Zhuang et al. 2015). So an establishment of relationship of aquaporin gene expression with the of root growth and drought tolerance is essential to develop gene based marker for improving root traits for drought tolerance. The present study aims at analysis of response pattern in lowland and upland rice to moisture stress *vis-a-vis* expression of some aquaproin gene to aid in marker assisted breeding.

In the present study the qPCR analysis revealed that OsPIP1;3, OsPIP2;5, OsTIP2;1 and OsNIP2;1 were upregulated in Banglami, ARC10372 and Inglongkiri upland rice and down-regulated in IR-64 lowland rice in drought stress condition compared to well-watered condition. Differential expressions of aquaporins were observed in drought tolerant upland rice and lowland rice in well-watered and drought stress condition. Similarly, Lian et al. (2006) reported that the mRNA expression levels of OsPIP1;3, and OsPIP2;5 in the root tissues were up-regulated in upland rice under water stress whereas the corresponding genes remained unchanged or down-regulated in lowland rice it imply that upland rice and lowland rice follow different adaptive mechanism to avoid water stress. The reduction of aquaporins in lowland rice may reduce membrane water permeability, thereby avoiding excessive loss of cellular water under water deficit. Matsunami et al. (2016) showed the higher expression of OsTIP2;1 in upland rice to lowland rice under osmotic stress condition. Whereas OsPIP 2;4 was downregulated in Banglami and IR-64 and upregulated in ARC 10372, Inglongkiri and Ranjit. Similarly, Grondin et al. (2016) showed downregulation of OsPIP2;4 in upland rice as well as in lowland rice in drought stress. Guo et al. (2006) found that OsPIP1;3, OsPIP2;4, OsPIP2;5 were induced under water stress in lowland rice. Further analysis in between lowland rice, level of aquaporin expression revealed that OsPIP1;3, OsPIP2;4 and OsPIP2;1 were up-regulated in Ranjit lowland rice whereas the same genes remain unchanged or down-regulated in lowland rice IR-64. Although Ranjit variety is popular variety for lowland condition and perform well under irrigated condition was showing moderately drought tolerant behavior during water stress condition in the present study based on up-regulations of OsPIP1;3, OsPIP2;4 and OsPIP2;1 aquaporin genes and phenotypic data for drought tolerance, while some of the genes were unchanged or down-regulated. Upregulation and downregulation/unchanged expression under water stress is considered as a mechanism of internal homeostasis to maintain water balance at the plant level to face water stress (Kapilan et al. 2018). Ranjit despite being a lowland ecology variety, it is mainly grown in the rainfed situation and sometimes experience moisture stress condition due to erratic rainfall pattern prevailing in eastern India. Under such situation, the Ranjit crop does not suffer to a great extent. It might be attributed to upregulation of OsPIP1;3, OsPIP2;4 and OsPIP2;1 aquaporin gens which are not upregulated in lowland cultivars (Lian et al. 2006).

Further insight into correlation between root parameter (root length and root dry weight) and transcript level of aquaporin expression and drought tolerance revealed that the genotypes showing high drought tolerance in the present study have a better root system to explore water from deep soil profile and showed 2-16 fold increase in aquaporin expression under water stress condition. Upregulation of OsPIP1;3, OsPIP2;5, OsTIP2;1 and OsNIP2 aquaporins in upland drought tolerant rice variety and moderately drought tolerant Ranjit lowland rice variety than drought susceptible lowland IR-64 rice in drought stress situation suggests that OsPIP genes can be used as candidate genes based on their high expression under drought stress situation for enhancing water uptake and root development under water stress condition. A positive correlation was observed between fold increase in aqauporin expression and degree of drought tolerance which indicate that these aquaporin genes might play an important role in root development under water stress situation to overcome stress situation. Allelic composition of these aquaporin genes might play an important role in understanding the mechanism of drought tolerance and will be helpful in the manipulation of drought tolerant traits. From these genes, breeders can design functional markers to be used in breeding programmes for improvement of drought tolerance in elite cultivars.

Authors' contribution

Conceptualization of research (RNS); Designing of the experiments (RNS, ARB); Contribution of experimental materials (RNS, ARB); Execution of field/lab experiments and data collection (HV, KD); Analysis of data and interpretation (HV, KD, RNS); Preparation of manuscript (HV, RNS).

Declaration

The authors declare no conflict of interest.

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