Plant Omics Journal POJ

POJ 6(1):79-85 (2013) ISSN:1836-3644

Water relation and aquaporin genes (*PIP1;2* and *PIP2;1*) expression at the reproductive stage of rice (*Oryza sativa* L. spp. *indica*) mutant subjected to water deficit stress

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Abstract

Rice (*Oryza sativa* L. spp. *indica*) is a carbohydrate crop that grows well in the aquatic habitat. In the drought prone areas, adaptive growth ability and plant defence mechanisms coping with less water are challenging tasks for rice breeder to maintain rice productivity. Rice plants, including Thai jasmine rice (cv. KDML105; wild type), M401 mutant line derived from KDML105 (upon treatment with EMS and γ-irradiation, and Pathumthani 1 (PT1, a negative check -drought susceptible), were grown in the pot culture until booting stage (inflorescence formation) and then subjected to water withholding for 14 days (water deficit stress) represented by 5.28% soil water content (SWC) and well watering (control; 31.9% SWC). Water use efficiency (WUE), net photosynthetic rate (P_n), transpiration rate (E), stomatal conductance (g_s) and expression of *PIP1;2* and *PIP2;1* were examined in flag leaf tissues of rice grown under control and water deficit stress. WUE in MT401 mutant plant subjected to water deficit stress was increased, whereas P_n in both MT401 and KDML105 was maintained. Transcriptional levels of *OsPIP1;2* and *OsPIP2;1* in the MT401 grown under water deficit stress were up-regulated by 2.0–2.5 folds higher than those in KDML105 and PT1 genotype. The expression of *OsPIP2;1* in MT401 mutant plant was maintained when plants were exposed to water deficit condition, resulting in stabilization of WUE at the cellular levels. In addition, panicle length and number of spikelets per panicle in MT401 mutant were retained well under water deficit, suggesting MT401 as water deficit tolerant type.

Keywords: Aquaporin gene expression, Reproductive stage, Rice, Water relation, Water stress.
Abbreviations: E_Transpiration rate; g_s_Stomatal conductance; P_n_Net photosynthetic rate; PIP_Plasma membrane intrinsic protein; WUE_Water use efficiency.

Introduction

Water deficit is one of the most important abiotic stresses reducing crop production, especially in rain-fed agricultural areas (Chaves and Oliveira, 2004; Kijne, 2006; Passioura, 2007). Rain-fed lowland rice is practically cultivated worldwide; however, significant amount of water supply is essential for proper growth and development of the crop (Biswas and Choudhuri, 1984). Insufficient water supply to the rain-fed lowland rice during growth and development negatively affects crop produce (Mall and Agarrwal, 2002; Mostajeran and Rahimi-Eichi, 2009). The reproductive developmental stage, i.e., peduncle elongation, ovule abortion, sterilized pollen, and spikelet sterility, in rice crop is very sensitive to water deficit (Nguyen et al., 2009). , Water deficit during the initiation and development of inflorescence results in low grain yield (Nguyen et al., 2009; He and Serraj, 2012). Therefore, the development and adoption of high-yield, drought-tolerant rice varieties is one of the best solutions for improving crop productivity in dry-land environments (Guan et al., 2010). Water use efficiency (WUE), examined as the ratio of CO_2 assimilation rate and transpiration rate, has been found to be positively correlated to grain yield in rice in Indian Central Himalaya (Agnihotri et al., 2009). Improved WUE and drought resistance has been opined to maintain crop yield in a water-limiting environment for sustainable agricultural production (Karaba et al., 2007). Plant WUE and drought tolerance show a positive relationship with the amount of plasma membrane intrinsic proteins, i.e., PIP1 and PIP2 (Tsuchihira et al., 2010). Aquaporins are intrinsic proteins that facilitates transmembrane water movement (Johansson et al., 2000).Until recently, plant aquaporins have been found to perform water transport activities in supporting plant growth and development, and maintain cell water potential and development under different environmental conditions such as chilling and water deficit stress (Gomes et al., 2009; Lian et al., 2006; Sakurai et al., 2008). In addition, aquaporins have been reported to transport other substances such as glycerol, urea, ammonia, CO_2, silicon, and boron (Tsuchihira et al., 2010).Plant aquaporins are divided into five subfamilies: plasma membrane intrinsic protein (PIP), tonoplast intrinsic protein (TIP), nodulin 26-like intrinsic protein (NIP), small basic intrinsic protein (SIP), and uncategorized X intrinsic protein (XIP) as reviewed by Kaldenhoff and Fischer (2006) and Sakurai-Ishikawa et al. (2011). There exists a very high diversity of aquaporin genes in plant species, with 35 aquaporins in *Arabidopsis*, 33 in rice, 36 in maize, 23 in moss, 37 in tomato, and 55 in poplar (Hassain et al. 2011). PIP subfamily is divided into two groups, PIP1 and PIP2. Among the aquaporin subfamilies, the PIP members, particularly PIP2, predominantly function

as water channels (Tsuchihira et al., 2010),. In rice crop, there are ten PIP proteins encoded by *OsPIP* genes (Hadiarto and Tran, 2011). Some members of *OsPIP1* and *OsPIP2* subfamilies have been reported as drought and salt responsive. For example, *OsPIP1–1*, *OsPIP2–5* and *OsPIP2–7* in the root tissue and *OsPIP2–3* in leaves were up-regulated by PEG 6000 induced-water deficit stress, while *OsPIP2–1*, *OsPIP2–5* and *OsPIP2–6* in leaves were down-regulated (Hadiarto and Tran, 2011). In addition, *AtPIP1;2*, the most abundantly expressed isoform of PIP family, has been found to account for a significant portion of aquaporin-mediated leaf water transport in *Arabidopsis thaliana* and represent a key component of whole-plant hydraulics (Postaire et al., 2010). Lian et al. (2004) reported that overexpression of RWC3, a member of rice PIP1 subfamily, increased drought tolerance in rice. Previous studies have concluded that*PIP1;2* and *PIP2;1* play a significant role in regulating water transport in relation to plant physiological adaptation under drought stress (Aroca et al., 2006). Also, Aroca et al. (2006) found a correlation between increased expression of *PvPIP2;1* and the changes in plant-physiological water transport in *Phaseolus vulgaris* under water deficit. Recently, enhanced expression of *PIP1;2* and *PIP2;1* has been linked to regulate growth and development in roots of japonica rice (Sakurai-Ishikawa et al., 2011). It is hypothesized that these two genes may play a key role in water dynamic under water deficit stress in *indica* rice genotypes. Recently, we found that the MT401 or DD14 mutant line derived from cv. KDML105 (*Oryza sativa* L. spp. *indica*) was resistance to water deficit and MT401 possess physiological and morphological adaptive processes under water deficit condition (Cha-um et al., 2012). With this background, we investigated the water relation and expression / regulation of *PIP1;2* and *PIP2;1* aquaporin genes during reproductive stage of indica rice under water deficit stress. We examined the transpiration rate (E), water use efficiency (WUE) and the alterations in expression *PIP1;2* and *PIP2;1* gene under water stress in the booting stage of MT401 mutant, KDML105 wild type and PT1 water deficit susceptible genotypes of indica rice.

Results

Plant morphological, growth and yield traits

Overall growth performances of MT401 and KDML105 grown under water deficit stress (5.28% SWC) or water withholding for 14 days were better than those in PT1, water deficit susceptible genotype. Leaf rolling, chlorosis and leaf burn toxicity were evident or greater (?) in water-deficit stressed PT1 plants compared to others (Fig. 1). The percent biomass in all rice lines, especially PT1 (water deficit sensitive) declined significantly under water deficit stress (Fig. 2). Under well watered condition, biomass percentage the maximum in MT401 mutant compared to other cultivars. Likewise, the panicle length in MT401 was maintained under water deficit stress, whereas it was slightly declined in KDML105. The number of spikelets per panicle in MT401 genotype increased when subjected to water deficit stress, whereas the spikelet number in KDML105 grown under water deficit condition declined significantly (Fig. 3).

PIP1;2 and PIP2;1 mRNA expression

To examine the changes in *m*RNA expression, the levels of *OsPIP1;2* and *OsPIP2;1* in the flag leaf of rice cultivars, viz., MT401, KDML105, and PT1, grown under well watering and water deficit stress were investigated using *q*-RT-PCR. Under well watering, the expression level of *OsPIP1;2* in MT401 and KDML105 was higher than in PT1. Likewise, *OsPIP2;1* expression was the greatest in the flag leaf of KDML105 compared to in the MT401 and PT1 cultivars grown under well watering condition (Fig. 4A). Under water stress, the expression levels of *OsPIP1;2* and *OsPIP2;1* declined in the leaves of all rice genotypes, , especially in KDML105 and PT1. Interestingly, expression of *OsPIP2;1* in the flag leaf of the MT401 was maintained and higher than that in the KDML105 and PT1 plants subjected to water deficit stress (Fig. 4B). It suggested that the expression of *OsPIP2;1* gene may play an important role in water relation in MT401 mutant line of indica rice exposed to water deficit stress.

Transpiration rate, stomatal conductance, net photosynthetic rate and water use efficiency

Transpiration rate (E) in MT401 was the highest when compared with other two cultivars (KDML105 and PT1) under the well watering condition. Under water deficit stress, transpiration rate of MT401 dropped sharply, whereas it was unchanged in KDML105 (Fig. 5A). In contrast, the flag leaf organ of PT1 was absolutely damaged in the extreme water deficit stress (Fig. 5A; so data not collected). Similarly, stomatal conductance (g_s) was the lowest in KDML105 growing in the well watered (Fig. 5B). Under water deficit, the g_s in MT401 declined slightly (but insignificant) when compared with well watering conditions. Net photosynthetic rate (P_n) in MT401 and KDML105 was unchanged in both well watering and water deficit conditions. In contrast, the P_n in the water deficit stressed PT1 was not measured due to the death of flag leaf (Fig. 5C). The minimal water loss and highest CO_2 assimilation *via* stomatal conductance or stomata closure in MT401 grown under water deficit stress were represented as water use efficiency (WUE), which was the key result of this investigation (Fig. 5D). A There was a positive relation between *OsPIP2;1* mRNA expression and WUE in MT401 mutant line. The WUE in KDML105 grown under water deficit stress declined by 36.26%, whereas it increased significantly in MT401 mutant. The adjustment of E and P_n in the MT401 mutants under water deficit stress suggests their role in providing high WUE , which may be linked to the enhanced expression of *OsPIP2;1* aquaporin protein.

Discussion

Under water deficit, the stomatal conductance was adjusted in stressed rice plants to maintain the transpiration rate rather than CO_2 assimilation as net photosynthetic rate (P_n) as reported by Cabuslay et al. (2002). However, significant decrease in transpiration rate (E) and P_n in water deficit stressed MT401 mutant was observed, leading to enhance water use efficiency (WUE). Under mild drought, the WUE may play as a key role in plant growth and development, especially in reproductive developmental stage (Davies et al., 2002). WUE can be enhanced to achieve better crop performance. Improved WUE can be achieved by a decrease in g_s, and an increase in P_n (Araus et al., 2002). However, how rice cultivars maintain the balance between transpiration and photosynthesis under water deficit conditions needs to be explored (Cabuslay et al., 2002). These physiological relationships in MT401 mutant might be implied to associate with plant habituation linked to genotypic adaptation for WUE in plant tissues under water deficit stress. The MT401

mutant may obtain the benefit of the water facilitation in cell communication with high WUE under water deficit stress. In fact, a precise involvement of aquaporins in controlling WUE in plants under water deficit stress has been debated (Bacon, 2004). However, overexpression of *NtAQP1* has been shown to improve the water use efficiency and yield in tobacco under salt stress (Sade et al., 2010). Likewise, the expression of Aquaporin genes increased in the wild type, KDML105, and elevated the cellular water-circulation when E decreased. In contrast, these adaptations in MT401 mutant provide an opportunity to increase the rice production under water deficit stress than in KDML105 (wild type) ().The expression level of *OsPIP2;1* in flag leaf MT401 mutant line was higher than the wild type (KDML105) under water deficit stress. After low volume of water loss, water flow occurs through cell-to-cell by aquaporin transport rather than through xylem vessel (apoplastic flow) (Tyerman et al., 1999; Zimmermann et al., 2000). It has been established that a decrease in transpiration rate caused a rise in cell-to-cell water permeability mediated by an abundance of PIP aquaporins (Aroca et al., 2006; Morillon and Chrispeels, 2001). The low transpiration rate may be reflected to stabilize water status by major aquaporin genes at plasma membrane (such as some of PIP2) to facilitate greater water flow between cells in rice. In rice crop (*Oryza sativa* L. spp. *japonica*; cv. Zhonghan 3, upland rice) mRNA expression levels of *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;1* and *OsPIP2;5* in the root tissues and *OsPIP1;2* and *OsPIP1;3* in the leaf tissues of were up-regulated under 20% polyethylene glycol (PEG) stress for 6 h (Lian et al., 2006). In contrast, gene expression level of eleven PIPs in rice cv. Xiushui 63 was unchanged and some genes were down-regulated (Lian et al., 2006). The expression levels of *OsPIP1;1*, *OsPIP1;2*, *OsPIP2;1* and *OsPIP2;3* in the leaf tissues of japonica rice cv. Zhonghua 11 exposed to 20% PEG 6000 induced water deficit were up-regulated, whereas the expression levels of OsPIP2;1, OsPIP2;4 and OsPIP2;5 were down-regulated (Gao et al., 2006). Also, the *m*RNA expression levels of *OsPIP1;1*, *OsPIP1;2*, *OsPIP2;1*, *OsPIP2;2* and *OsPIP2;5* in the leaf tissues of japonica rice cv. Akitakamachi (chilling tolerant) were up-regulated by low relative humidity (47/69% in the light/dark period) in the plant growth chamber for 45-52 days (Kuwagata et al., 2012). In contrast, PIP genes, viz., *OsPIP1;3* and *OsPIP 2;3*, were unchanged in the low humidity condition (Kuwagata et al., 2012). Different rice subspecies (*indica* and *japonica*), rice cultivars, culture systems (*in vitro*, hydroponic and pot culture) and dark-light cycle have been regarded to play the trigger role on the expression levels of *OsPIP* gene family, relating to the water use efficiency and transpiration rate as a major route of water translocation from root-to-shoot in the water deficit condition (Sakurai-Ishikawa et al., 2011). However, the deep basic research on the functional of PIPs proteins in water absorption, translocation and water relation of rice crop needs further elucidation.

Materials and methods

Plant materials

The MT401 or DD14 mutant variety (Cha-um et al., 2012), cv. KDML105 (wild type) and cv. Pathumthani 1 (PT1; drought sensitive) (Cha-um et al., 2010) of rice (*Oryza sativa* L. ssp. *indica*) were used as plant material. Seeds of MT401 mutant variety were obtained from Department of Biotechnology, Faculty of Science, Mahidol University, Thailand. The MT401 mutant variety was generated from

Fig 1. Growth sensitivity of MT401 rice plant compared with wild type (KDML105) and a drought sensitive cultivar; Pathumtani 1 (PT1) at reproductive stage after grown under water deficit stress condition for 14 days. (Bar = 7.5 cm).

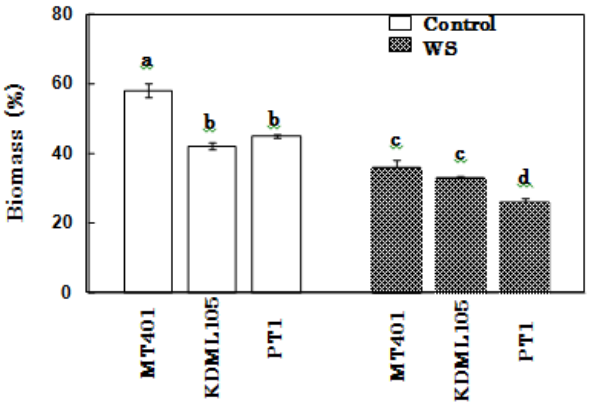

Fig 2. Biomass of leaf blades in MT401, KDML105 and PT1 after grown under control and water stress (WS) conditions for 14 days. Values are mean ± SE (n = 6).

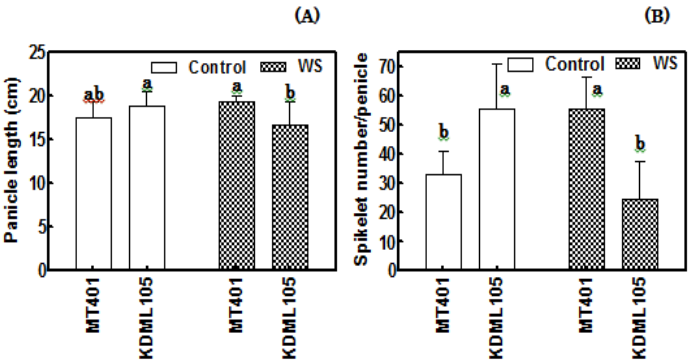

Fig 3. Panicle length (A) and spikelet number per panicle (B) of MT401 and KDML105 after recovery from water deficit stress prior to grain harvesting process. Values are mean ± SE (n = 6).

KDML105 seeds using γ-irradiation and then the irradiated seeds were treated with ethyl methane sulfonate (Teerawitaya et al., 2011) and grown until fourth-generation (M_4). A single seed of MT401 mutant was germinated and proliferated on MS medium (Murashige and Skoog, 1962) supplemented with 3 mg L^{-1} benzyl amino purine (BAP), incubated under 25±2°C ambient temperature, 65±5% relative humidity, 60±5 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) provided by fluorescent lamps with 16 h d^{-1} photoperiod.

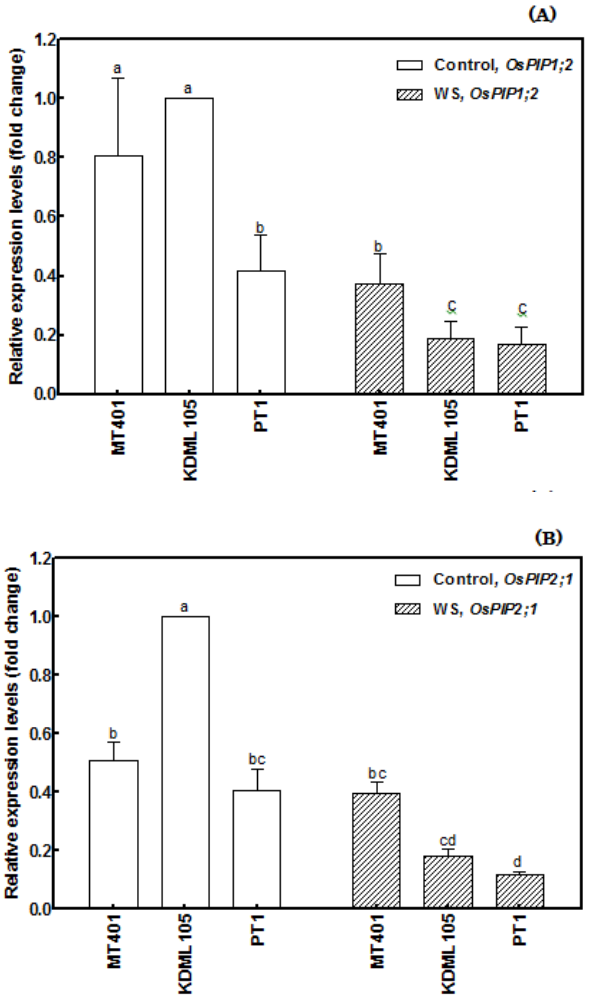

Fig 4. Relative expression levels of *OsPIP1;2* (A) and *OsPIP2;1* mRNAs (B) in flag leaf of MT401, KDML105 and PT1 grown under control and water stress (WS) conditions for 14 days. The fold-change is relative to the expression of ubiquitin as housekeeping gene. Values are mean ± SE (n = 6).

Single plantlets were transferred to root induction medium containing 0.5 mg L^{-1} indole butyric acid (IBA) and then plantlets were directly transferred to pot culture and grown at a greenhouse of the Thailand Science Park, Thailand (Latitude 14°01'12"N Longitude 100°31'12"E) in 15 cm diameter × 30 cm height clay pots filled with clay soil (EC = 2.687 dS m^{-1}; pH = 5.5; organic matter = 10.36%; total nitrogen = 0.17%; total phosphorus = 0.07%; total potassium = 1.19%) and supplemented with chemical fertilizer (15-15-15; N-P-K) for 15 days after planting. Plants were grown at a greenhouse until booting stage under waterlogging (5 to 10 cm from the soil surface).

Soil water content and water deficit treatments

Five-gram of soil sample was collected at a depth of 5 to 10 cm in each pot under well watering and water deficit stress conditions. After keeping in a dry-heat oven at 70°C for 7 days, soil water content was evaluated using the following equations: Soil water content (%) = [(soil fresh weight − soil dry weight)/soil fresh weight] × 100 (Coombs et al., 1987). Soil water contents (SWC) in the pot culture were adjusted to 31.93±3.00% for well watering or 5.28±0.78% for water deficit stress (14 days water withholding). Flag leaf blades of plants grown under each set of conditions were chosen for water relation, transpiration rate and net photosynthetic rate, and biomass and yield traits were evaluated.

Water relation and biomass analysis

Transpiration rate (E), stomatal conductance (g_s), and net photosynthetic rate (P_n) from the flag leaf were measured using an Infra-red Gas Analyser (IRGA) (Model Portable Photosynthesis System LI 6400; LI-COR® Inc., Lincoln, Nebraska, USA). Measurement was done by continuously monitoring H_2O in the air entering and exiting the IRGA headspace chamber. The flow rate of air in the sample line was adjusted to 500 µmol s^{-1}. The micro-chamber temperature was set at 25°C. The light intensity was fluxed by a 6400-02B red-blue LED light source at 1,000 µmol m^{-2} s^{-1} PPFD. E was calculated following Pan et al. (1998). Water use efficiency (WUE; µmol CO_2 mol H_2O) in the leaves was calculated as the ratio of P_n to E, according to Estrada-Luna et al. (2001) Biomass of flag leaf was determined after oven drying at 70°C to a constant mass and calculated by the equation; leaf biomass (%) = [leaf dry weight / leaf fresh weight] ×100.

RNA isolation and quantitative reverse transcriptase-expression analyses

Tissues from the leaf blades were frozen in liquid nitrogen and ground. CTAB-based extraction method was used for the RNA extraction (Chang et al., 1993). One microgram of RNA treated with RQ1 RNase-free DNAse (Promega) was used for the first strand *c*DNA (Super Script II strand synthesis system, Invitrogen Technologies, USA). The quantitative reverse transcription-polymerase chain reaction (*q*RT-PCR) was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA). *OsPp-PIP1;2* (accession no. AK098849) and *OsPp-PIP2;1* (accession no. AF062393) rice aquporin DNA have been previously reported by Sakurai et al. (2005). The specific primers for water channel genes were designed with Primer3 software v. 0.4.0 (Rozen and Skaletsky, 2000), (www. http://frodo.wi.mit.edu/primer3/) as follows. The forward primer for the *OsPp-PIP1;2* class was 5'-AAGTAGAGAGATGGAGGGGAAG-3', and the reverse primer 5'-ACTTGGAGGTGGAGTTGTTG-3'. The forward primer for the *OsPp-PIP2;1* class was 5'-TGATATCAAGGGGTTCGAGA-3', and the reverse primer 5'-ATGAGAACATCCCCTCGATT-3'. The *Oryza sativa* ubiquitin/ribosomal polyprotein (accession no. D12629), the forward primer for which was 5'-TTGTCCTGCGCCTCCGT-3' and the reverse primer 5'-GGCATAGGTATAATGAAGTCCAATGC-3' was used for normalized gene (Lian et al., 2006). The *c*DNAs were amplified with 40 cycles of 94°C for 3 s and 58°C for 30 s, according to the manufacturer's protocol (Kapa SYBR Fast *q*PCR Kit, Kapa Biosystems Technologies) and Yooyongwech et al. (2008) method. The amplification efficiency of reaction was determined from the component data using LinRegPCR (Ramakers et al., 2003). The amount of relative expression was calculated from threshold cycle values using the $2^{-\Delta\Delta CT}$ method (Bulley et al., 2009).

Biomass, panicle length and number of spikelets per panicle

Biomass of flag leaf was measured based on the ratio of fresh

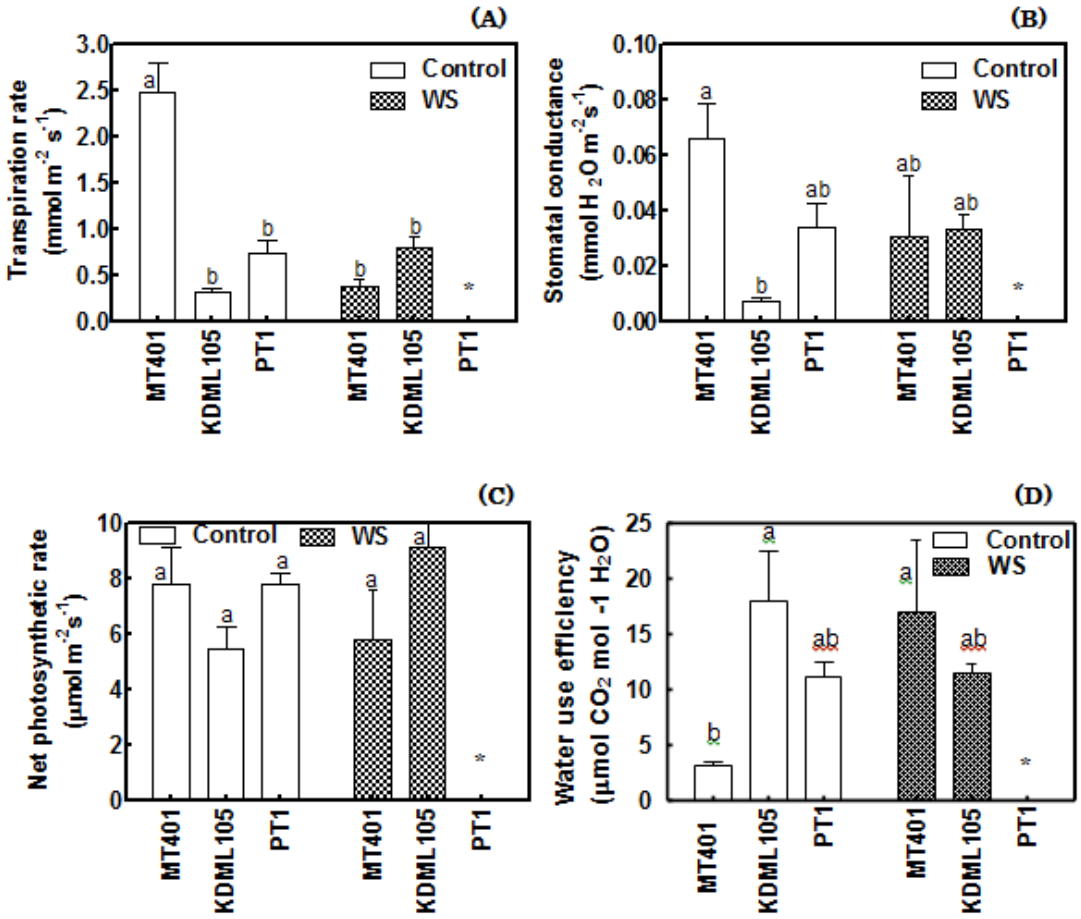

Fig 5. Transpiration rate (A), stomatal conductance (B), net photosynthetic rate (C) and water use efficiency (D) in the flag leaf of MT401, KDML105 and PT1 after grown under control and water stress (WS) conditions for 14 days. *Data could not obtain from PT1 because leaf of PT1 was dried under the water deficit stress. Values are mean ± SE (n = 6).

weight and dry weight. Recovery process of rice plants were done by re-watered after the 14 days of water withholding (5.28±0.78% SWC) prior to grain filling and harvesting process. Finally, the panicle length and number of spikelets per panicle in MT401 and KDML105 cultivars were measured and counted at the harvesting stage in both of the well watering and the water deficit stress.

Statistical analysis

The experiment was arranged as 3 × 2 factorial in Completely Randomized Block Design (CRBD) with six replicates (n = 6). Means and standard errors (±SE) were computed for the measurements. Assessment of the statistical significance of the results was performed using Duncan's multiple range test (DMRT) at the level of $p \leq 0.05$.

Conclusion

In MT401 mutant rice derived from KDML105, decrease in transpiration rate, maintenance of net photosynthetic rate and increase in water use efficiency in plant grown under water deficit stress was observed. It resulted inmaintaining biomass and yield traits, length of panicle and number of spikelets per panicle in MT401. The mRNA expression of *OsPIP2;1* was relatively high in flag leaf tissues of the MT401 mutant grown under water deficit stress. The study concludes that the development of the MT401 plants have possible signs in in their physiology and molecular expression reflecting an improvement of rice yield under water deficit stress.

Acknowledgments

The authors wish to thank Mahidol University (MU) as a funding source (Grant code A04/1/2553), partially supported by the International Atomic Energy Agency (IAEA) (contract no. 12998/R0). We would like to thanks Science Orientation Unit at MU, Kanchanaburi campus for supporting basic experimental tools.

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