

# Improving rice (*Oryza sativa* L.) drought tolerance by suppressing a NF-YA transcription factor

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## Abstract

The response to drought stress is a complicated process involving stress sensing, intracellular signal transduction, and the execution of a cellular response. Transcription factors play important roles in the signaling pathways including abiotic stress. In the present study a rice NF-YA transcription factor gene was partially characterized following dehydration. Disrupting the gene via a T-DNA insertion resulted in drought tolerant plants and a high rate of recovery after water re-supply. It was demonstrated that the improved drought tolerance of the mutant is primarily due to non-stomatal mechanisms such as free radical scavenging, which might be related to changes in metabolism of carbohydrates.

**Keywords:** Drought stress; Nuclear Factor Y; Rice (*Oryza sativa* L.)

## INTRODUCTION

Drought is one of the major environmental factors affecting crop growth and productivity in the semi-arid tropics. At critical stages of plant growth, crop yields are greatly reduced by even short-term, 15- to 30-day drought stress. The limitation of water availability may result up to 15% loss in rice production during some years (Dey and Upadhaya, 1996). Estimated average world-wide loss of annual rice production due to drought condition is 18 million tons, or 3.6 billion US dollars (O'Toole, 2004).

Unlike animals which upon facing suboptimal

environmental conditions move to find more favorite conditions, plants are sessile and have to make adaptation to the environmental changes in order to survive and produce seeds for the next generation. When plants experience drought stress, a number of genes belonging to different functional categories are activated (some others are repressed). While a vast majority of the drought-responsive genes are directly or indirectly involved in adaptation responses, their activation is tightly controlled at the expression level of regulatory genes, mainly with transcription factors (TF) (Rabbani *et al.*, 2003; Seki *et al.*, 2002).

TF proteins bind to the promoter region of DNA molecules in a sequence-specific manner to control gene expression and subsequent proper responses of the cell to the endogenous and exogenous signals (Thomas and Chiang, 2006). Very soon after proposing the first drought responsive TF in the late 1990's (Stockinger *et al.*, 1997), several further stress-responsive TFs were identified (Choi *et al.*, 2000; Sakamoto *et al.*, 2000; Shinozaki and Yamaguchi-Shinozaki, 2000; Hobo *et al.*, 1999; Liu *et al.*, 1998). Identifying more TFs differentially expressed under specific environmental constraints led to the construction of regulatory models and networks responses of plants to the stress situations (Yun *et al.*, 2010; Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2005; Shinozaki *et al.*, 2003). Most of the TFs presented in the models belong to the Ap2/ERBP, bZIP, ZFHD, MYB, and NAC families of TFs. TFs can play roles in cross talking of different regulatory pathways. In Arabidopsis, it has been demonstrated that a MYB TF, e.g. MYB96, functions as a molecular link that interconnects ABA and auxin signals in lateral root devel-

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opment (Seo *et al.*, 2009). Most of the discoveries about the role of the TFs come from the studies concerning over-expression of genes and loss-of-function mutants. For instance over-expression of a AP2 rice transcription factor (AP37) resulted in yield improvement under drought stress in rice (Kim and Kim, 2009; Oh *et al.*, 2009).

Until recently, the role of nuclear factor (NF) transcription factors in stress responses were not clear enough (Li *et al.*, 2008; Nelson *et al.*, 2007). Nuclear factor Y (NF-Y) is a protein complex consisting of three subunits, NF-YA, NF-YC, and NF-YB, all of which are required for DNA binding (Sinha *et al.*, 1995). The NF-YB and NF-YC subunits form a tight dimer, which provides a suitable structure for incorporating of the third subunit, that is NF-YA (Mantovani 1999). The resulting trimer can then bind to DNA with high specificity and affinity (Mantovani, 1999; Maity and de Crombrughe, 1992). The NF-Y transcription factor specifically recognizes the regulatory CCAAT element found in either orientation in proximal or distal enhancer regions of many genes (Mantovani, 1999; Maity and de Crombrughe, 1998). In higher eukaryotes, this element is found in about 30% of all promoters, preferentially in the -60/-100 upstream regions, however distinct flanking sequences are required for efficient binding (Bi *et al.*, 1997). The DNA recognition site of the subunits is called CBF (CCAAT binding factor). The subunits are annotated in two ways: CBF-based and NF-Y-based. The A subunit of CBF corresponds to the B subunit of NF-Y, and the B subunit of CBF corresponds to the A subunit of NF-Y. The subunit C is identical in the nomenclature of CBF and NF-Y (Maity and de Crombrughe, 1998). The assembly of the NF-Y complex and its subsequent binding to the CCAAT box is influenced by cell-internal or external signals (Bezhanian *et al.*, 2001). Unlike the broad information about the biological roles of NF-Y family members in animals, limited number or relatively little is so far known about their functions in plants. The role of the nuclear factor Y in embryo development (Lotan *et al.*, 1998) and seed formation (Gusmaroli *et al.*, 2002) has been investigated. Suppression of OsHAP3A-C gene, a rice NF-YB, via RNAi, led to chloroplast degeneration and down-regulation of photosynthetic genes such as RBCS and CAB, which encode a small subunit of ribulose-1,5 biphosphate carboxylase and the light-harvesting chlorophyll a/b binding protein of photosystem II (Miyoshi *et al.*, 2003). NF-YB has been demonstrated in promoting of flowering in Arabidopsis (Kumimoto

*et al.*, 2008).

The rice genome encodes for 23 NF-YA transcription factors (Riano-Pachon *et al.*, 2007). However, to the best of our knowledge none of these transcription factors have been functionally characterized so far. Here, a nuclear transcription factor with a putative role in drought tolerance is being introduced.

## MATERIALS AND METHODS

**Plant growth and stress treatment:** Seeds of rice varieties DR2 and CR203, belonging to indica rice subspecies, were provided by the Institute of Biotechnology (IBT), Hanoi, Vietnam. Seeds of wild type Nipponbare and homozygous mutant line, *os12g42400.1* was kindly provided by Dr. Caldana, Max-Planck Institute of Molecular Plant Physiology in Golm. The non-dehusked seeds were germinated on wet paper tissue in dark at 28°C for five days. The varieties were appeared as drought tolerant (DR2) and susceptible (CR203) when subjected to a period of 15 days drought stress (Najafabadi, 2008). To accelerate their growth, the germinated seedlings were transferred to a growth chamber set at 12 h/day (600  $\mu$ /Em<sup>8</sup>/s) at 26°C, and 12 h/night at 22°C with 70% relative humidity for subsequent four days. This growing condition was used for all experiments carried out with rice. For expression analysis, the 14 day-old seedlings were grown in hydroponic media by fixing them on polypropylene plates being 10 cm apart and placed in boxes containing macro- and micro-elements (Yang *et al.*, 1994). After 14 days, the seedlings were exposed for 0, 1, and 6 h to drought by air-drying their roots on filter paper in the same growth chamber. Roots were harvested, wrapped in aluminum foil and immediately frozen in liquid nitrogen. Control plants were kept in the media and harvested at the same time as the stressed plants. For physiological studies, the seedlings (14 days old and with a size of 5-6 cm) were transplanted to pots containing 2  $\pm$  0.2 kg well mixed soil and sand in 2:1 ratio (w/w) and fertilizers (3 g PLANTACOTE, DEPOT 4M, 14:9:15 from Spiess-Urania Chemicals, Hamburg, Germany; 0.25 g FETRILON from Compo, Muenster, Germany) with three plants per pot. Pots were put in water for 14 days and then drought stress was applied by removing pots from the water supply, while control plants were kept under full water condition. For the recovery experiments, the stressed plants were re-watered by returning back those into the water supply.

**Expression analysis:** Roots from 5 harvested seedlings were pooled for isolation of RNA in each sample. Total RNA was extracted from the samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration and purity was determined by spectrophotometre. Removal of DNA contamination was performed in a two-step way: on-column digestion using DNaseI (Qiagen, Hilden, Germany), and subsequently after RNA extraction by RNase-free DNaseI (Roche, Mannheim, Germany). Integrity of RNA was checked on a 1% (w/v) formaldehyde agarose gel prior to, and after the second DNaseI treatment. Absence of genomic DNA contamination was subsequently confirmed by real-time PCR using three primer pairs which amplify two intergenic regions (AP003727, positions 7366-7426 located at rice chromosome 1 called Intergenic I, and AP006456, positions 27334624-27334684 located at chromosome 7, called Intergenic II), and an intron sequence of gene *Os01g01840* called Intron (Caldana, 2007; Caldana *et al.*, 2007). The total mRNAs (5 µg) were converted into cDNA using SuperScript III reverse transcriptase kit (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions. The efficiency and concentration of the synthesized cDNA was evaluated using real-time PCR amplification of the three rice reference genes encoding Actin (*Os03g50890*), Actin g (*Os05g36290*), and β-Tubulin (*Os01g59150*) (Caldana *et al.*, 2007).

PCR reactions were conducted using an ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Darmstadt, Germany), employing SYBR Green to monitor dsDNA synthesis. Reactions with a final volume of 5 µl contained 0.5 µl of template (cDNA or total RNA), 200 nM of each gene or sequence-specific primer (2 µl of mixed 0.5 mM forward and reverse primers), and 2.5 µl of SYBR Green master mix. The following standard thermal profile, recommended by the manufacturer, was used for all real-time PCR reactions: 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s, and 60°C for 1 min. After 40 cycles, the specificity of the amplifications was checked by heating from 60°C to 95°C with a ramp speed of 1.9°C/1min, resulting in melting curves. Data were analyzed using the SDS 2.2.1 software (Applied Biosystems, Foster City, USA). The threshold cycle,  $C_T$ , which is the cycle number at which SYBR Green fluorescence in a PCR reaction reaches an arbitrary value during the exponential phase of DNA amplification, was set at 0.2 in all experi-

ments. In order to make a foundation for comparing data from different PCR runs or cDNA samples and also gaining an estimation of the cDNA synthesis efficiency, the reference gene Actin g, was included in each PCR run.  $C_T$  values for samples ( $C_{Tg}$ ) were normalized ( $\Delta C_T = C_{Tg} - C_{Tr}$ ) based on the  $C_T$  values of Actin g ( $C_{Tr}$ ). For comparative expression analysis, e. g. comparing situation I with II,  $2^{-\Delta\Delta C_T}$  was used as fold change in gene expression, where  $\Delta C_T = \Delta C_{TI} - \Delta C_{TII}$  and the data were shown as  $\log_2$  of the fold change.

**Gas exchange measurements:** The rate of gas exchange in leaf including net photosynthesis rate, transpiration rate, and stomatal conductivity, was measured on the second-youngest fully expanded leaf using a portable open-flow gas exchange system from LI-COR, model LI-6400 (LI-COR Bioscience, Lincoln, Nebraska, USA). The following conditions were used as measurement parameters: saturating light intensity of  $800 \pm 10$  µmol photons/m<sup>2</sup>/sec over the leaf chamber, CO<sub>2</sub> concentration of 350 µmol/mol, leaf temperature of 28°C, and ambient relative humidity of 60%. Measurements were started one day before stress application four hours after beginning the light period and continued thereafter. For each measurement, three independent seedlings showing similar morphological appearance were used.

## RESULTS

**Structure and features of wild-type *Os12g42400* gene and mutant *os12g42400.1* genes:** The Nuclear factor-Y gene, *Os12g42400*, is 936 bp long and encodes a protein of 311 amino acids. Its DNA binding domain is located between amino acid positions 150 to 206. The gene possesses five exons and the DNA binding domains are encoded by exons 4 and 5.

Analysis of the genomic sequence revealed that three alternative splice forms, differing in 5' untranslated regions, exist. The *Os12g42400* gene contains four introns located 237, 1459, 1583 and 2335 nucleotides downstream of the translation initiation codon, respectively (Fig. 1). The mutant gene, *os12g42400.1*, harbors a T-DNA insertion in the fourth exon.

**The *Os12g42200* gene is repressed by drought stress:** Based on the transcription profiling of rice under drought stress using a rice transcription factor

platform representing 2221 gene models of transcription factors (Caldana *et al.*, 2007; Najafabadi, unpublished data), a nuclear factor Y gene, *Os12g42400*, was identified. The gene showed a decreased expression level in two *indica* rice varieties, i.e. DR2 and CR203, after drought stress (Fig. 1). Gene expression was analyzed by quantitative real-time PCR (q-RT-PCR) on the root samples subjected to drought stress for one or six h, respectively. The experiment was repeated three times. The general trend was down-regulation of NF-Y upon drought stress (data not shown).

**Mutation in the gene *Os12g42400* enhances drought tolerance in rice:** For functional characterization of *Os12g42400*, plants containing a T-DNA insertion in the fourth exon of the gene, 2250 bp downstream of the start codon, were analyzed. The tolerance of the *os12g42400-1* mutant to drought stress was compared with that of the Nipponbare wild-type. For this, plants were germinated for 7 days and transplanted to pots, where they were fully watered for further 14 days. We did not detect any obvious differences in morphology between the wild-type and the *os12g42400-1* mutant under control condition. Drought stress was imposed by removing the pots from the boxes containing water. The stress was applied for up to 21 days. Fourteen days after stress-induction a difference between wild-type and *os12g42400-1* mutant plants was found. Leaves of *os12g42400-1* mutant plants appeared erected with slight curling, whereas those of the wild-type fully wilted and had breaks at their leaf-sheath junctions. Prolongation of the stress for additional 7 days following with resupplying of water for 10 days resulted in

recovery of normal growth and development of *os12g42400-1* mutant plants. The wild-type plants did not survive the treatment and were dead within 22 days after start of the drought stress (Fig. 2).

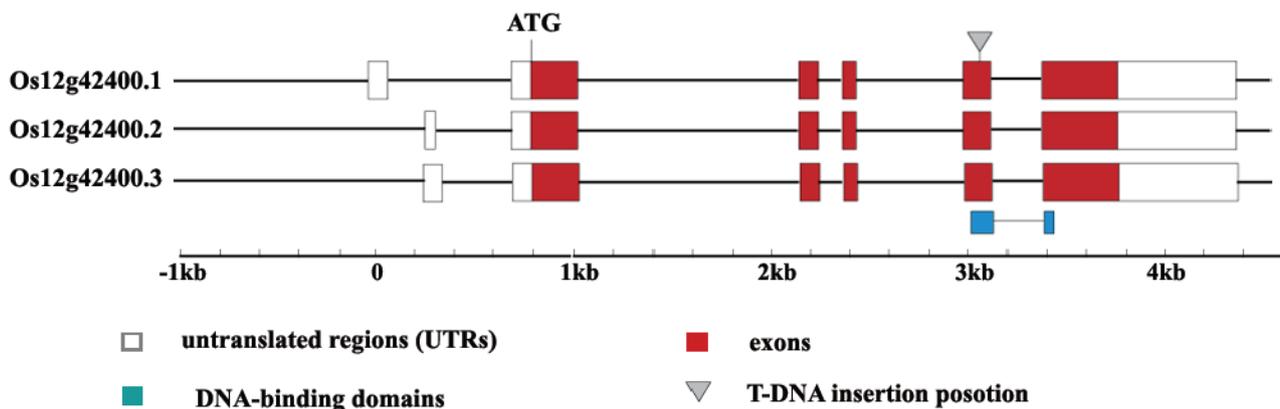
**Physiological responses of the *os12g42400-1* mutant to drought stress:** To investigate the probable physiological basis for the enhanced drought tolerance in the mutant line, physiological traits including gas exchange parameters were measured in wild-type and transgenic plants exposed to drought stress and control conditions.

Transpiration rate and stomatal conductance were analyzed in plants immediately before the stress treatment and 6, 12 and 16 days after start of the drought stress (deprivation from water supply; Fig. 3).

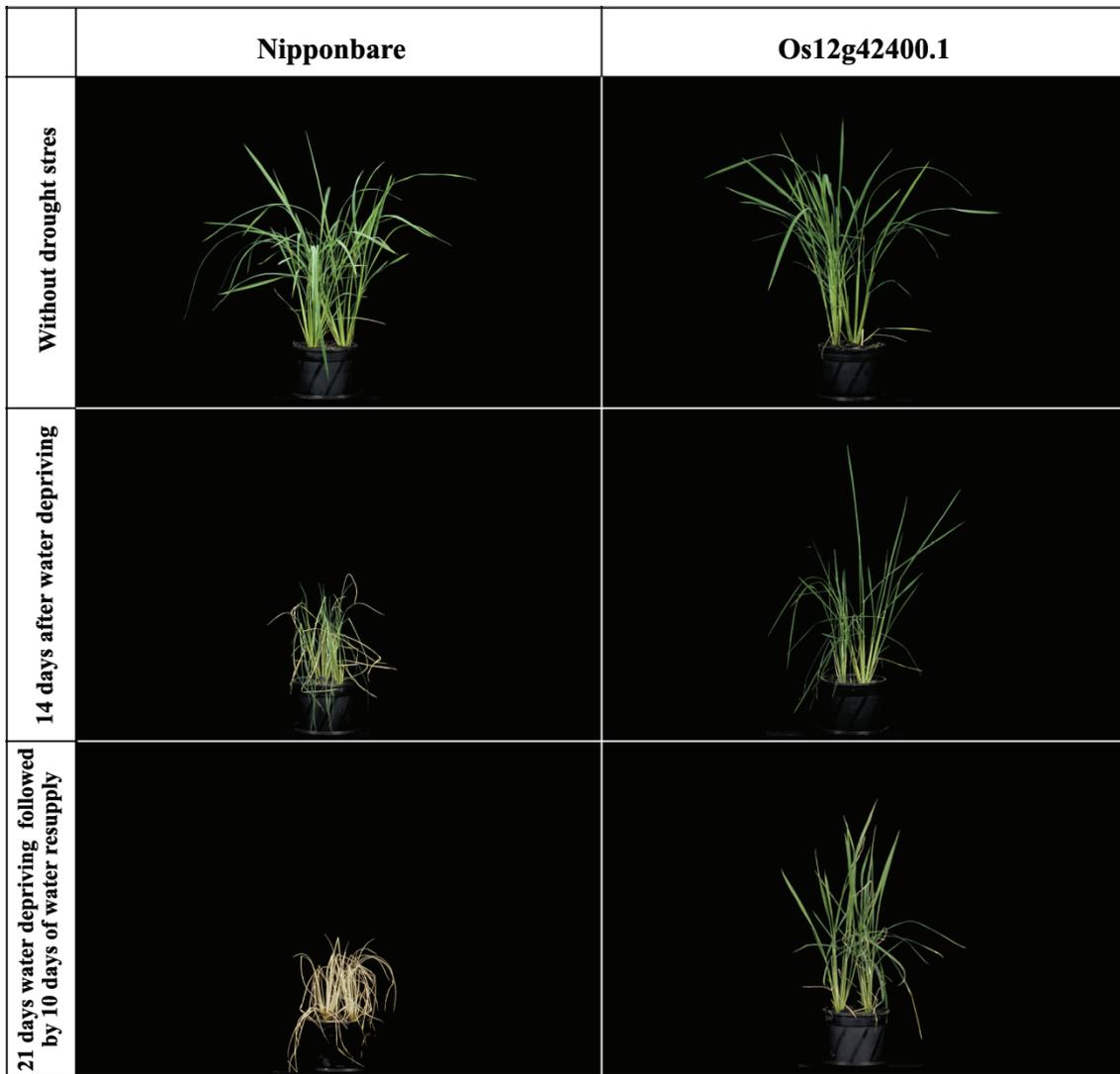
At 12 days, leaves of Nipponbare showed a reduction by 90% of the original transpiration rate, whereas the *os12g42400-1* mutant displayed a decline of only 55%. Stomatal conductance displayed a similar behavior, with a reduction of 95% and 32% of the initial rates in the mutant and the wild-type, respectively. Surprisingly, the *os12g42400-1* mutant showed a photosynthesis rate of about 70% of control plants after 12 days of stress (Fig. 4). Caldana (2007) observed a reduced water usage by the *os12g42400-1* mutant compared to the wild-type, however she did not observe a significant difference in water use efficiency between the mutant and the wild-type.

## DISCUSSION

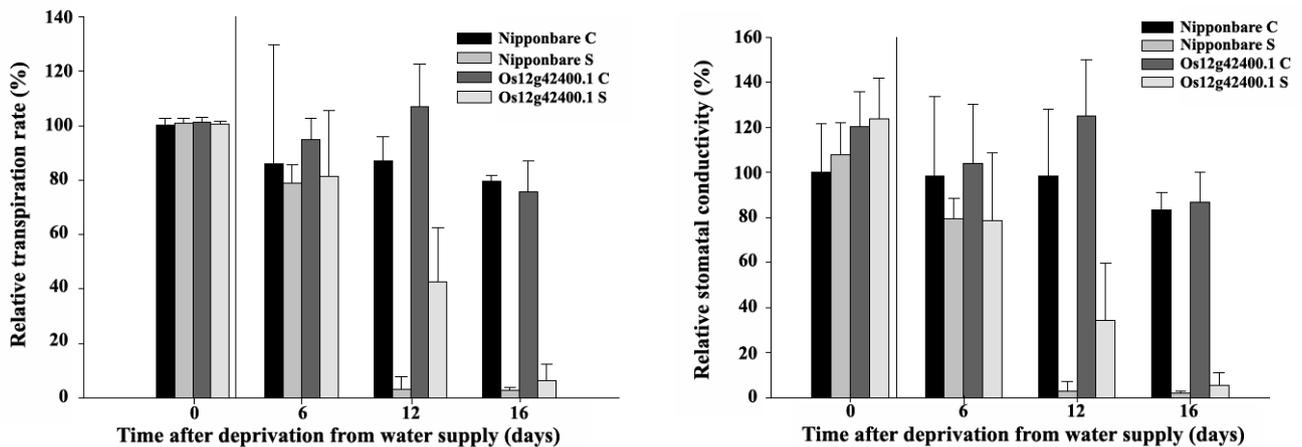
Transcription factors play crucial roles as regulatory



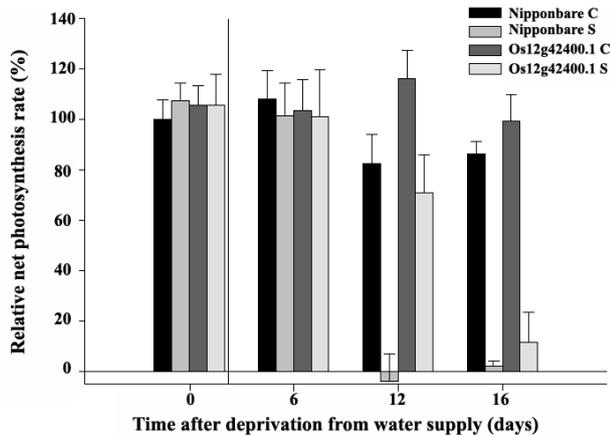
**Figure 1.** Structure of the *Os12g42400* gene. The structure obtained from TIGR v5. The *Os12g42400* locus generates three alternative splice forms (*Os12g42400.1*; *Os12g42400.2* and *Os12g42400.3*). Red boxes represent exons, thin black lines (between two boxes) depict introns, and white boxes represent untranslated regions (UTRs). ATG mark indicates the predicted translation initiation codon. The insertion point of the T-DNA in the *Os12g42400* gene is shown above, close to the stop codon in the fourth exon (gray triangle). The position of the DNA-binding domain is represented by blue boxes under the transcript models.



**Figure 2.** Effect of drought stress on wild-type Nipponbare and *os12g42400-1* mutant plants. Plants were grown on soil and subjected to drought stress by deprivation from water supply. Non-stressed plants served as control. Plants exposed for 14 days to the drought stress as well as plants exposed for 21 days to stress followed by 10 days of water re-supply or control conditions are shown.



**Figure 3.** Effect of drought stress on gas exchange parameters in *os12g42400-1* mutant and Nipponbare wild-type. Response of transpiration (left) and stomatal conductance (right) in Nipponbare (gray bars) and *os12g42400-1* plants treated by drought stress for 16 days (gray bars). C and S indicate control and drought stress conditions, respectively. Data are means  $\pm$  SD of three replicates.



**Figure 4.** Effect of drought stress on net assimilation rate in *os12g42400-1* mutant and Nipponbare wild-type. Response of net photosynthesis in Nipponbare and *os12g42400-1* plants treated by drought stress for 16 days (gray bars). C and S indicate control and drought stress conditions, respectively. Data are means  $\pm$  SD of three replicates.

switching molecules in drought stress signal perception and transduction (Yamaguchi-Shinozaki and Shinozaki, 2005). They either directly bind to the promoter of their downstream targets (*e.g.* C2H2) to regulate their expression, or interact with other proteins/subunits to build a regulatory complex, such as nuclear factor Y proteins (Thomas and Chiang, 2006; Mantovani, 1999). Expression profiling in root of transcription factors allowed us to identify the *Os12g42400* gene as a drought-responsive target in rice roots (Najafabadi, 2008). Twenty five gene models annotated as NF-YA have been identified in rice genome (Riano-Pachon *et al.*, 2007; <http://plntfdb.bio.uni-potsdam.de/v3.0/>) of which, only one (*Os12g42400*) showed differential expression under drought stress (Najafabadi, 2008). Similarly only one drought responding NF-YA has been reported in wheat, and of particular interest, down regulated under drought condition (Stephenson *et al.*, 2007). Moreover, in the NF-Y protein complex, NF-YA has the most affinity and specificity in protein-DNA interaction (Zemzoumi, *et al.*, 1999; Mantovani, 1999) indicating possibility of at least one drought responsive NF-YA with crucial role in drought responses among the all NF-Y genes in cereals.

The use of over-expression techniques in enhancement of tolerance to abiotic stress conditions has been postulated for many gene families. New lines of evidence are arising reporting the effects of NF-Y transcription factors in stress responses. Examples include *AtNF-YB1* (*At2g38880*) (Nelson *et al.*, 2007) and

NFYA5 in *Arabidopsis* (Li *et al.*, 2008 b). By means of large scale transcription factor profiling we found a line of evidence that may indicate the involvement of NF-YA gene in rice drought responses. Phenotype analysis of the T-DNA insertion line of this gene resulted in vigorously drought tolerant plant as compared to wild type. The earlier examples were indicative of drought tolerance improvement upon over-expression of drought-induced NF-Y genes (Nakashima *et al.*, 2007; Nelson *et al.*, 2007; Sakamoto *et al.*, 2004; Hsieh *et al.*, 2002). In contrary, we have noted that the disruption of *Os12g42400* gene, which is down-regulated upon drought stress, led to increasing drought tolerance in rice.

Plant responses to water deficit can be viewed in short or long responses. Short term drought responses include but not limited to root water deficit recognition, stomatal closure, and xylem hydraulic changes whereas, examples of long term drought responses include shoot growth inhibition, metabolic acclimation, and osmotic adjustment (Chaves *et al.*, 2003). Stomata function as a gate facilitating CO<sub>2</sub> and H<sub>2</sub>O exchange between leaf and the surrounding atmosphere. Water limitation causes reduction in stomatal aperture to reduce water loss. Both, T-DNA insertion and the wild-type plants have shown reduced transpiration rate and stomatal conductivity (reduction in stomatal aperture) under progressively increasing drought stress (Fig. 3). Reducing stomatal aperture under water deficit, *i.e.*, reduction of CO<sub>2</sub> exchange rate across the stomata, represents an adaptation response to minimize water loss via transpiration (Chaves *et al.*, 2003). The reduced CO<sub>2</sub> exchange as a consequence of water limitation vanishes photosynthesis rate, a phenomenon called as stomatal limitation to photosynthesis (Chaves *et al.*, 2008). On the other hand, changes in metabolism resulting from drought stress can affect photosynthesis. Damaging the photosynthesis machinery along with the production of reactive oxygen species, the causative agents of cell membrane or DNA damage, are amongst the non-stomatal mechanisms that adversely affect plant photosynthesis and growth (Mundree *et al.*, 2002). Stomatal closure is among the short time responses of plants to drought stress and is one of the most prominent factors limiting photosynthesis initially (Babu *et al.*, 2004), however, non-stomatal limitation to photosynthesis becomes more prominent under conditions or prolonged stress (Lawlor, 2002).

Different trends in responses of stomatal conductance and net photosynthesis to drought stress reflect

non-stomatal limitation to photosynthesis (Flexas and Medrano 2002). Non-stomatal limitation to photosynthesis in this study was observed in dried (12 days after the onset of drought stress) *os12g42400-1* T-DNA insertion plants (Figs. 4 and 5). This may include well-developed protective mechanisms such as free radical scavenging and/or changes in metabolism of carbohydrate in the mutant plants as compared to wild-type controls. Six days after deprivation of water supply, the rate of transpiration and stomatal aperture between mutant and wild type plants were similar. Nevertheless, as the stress continued, mutant plants continued to lose significantly higher amounts of water than wild type controls in terms of transpiration. This may be a result of having more water in the mutant plants as compared to the wild type controls. Keeping more water in shoot is a water avoidance component (Levitt, 1980) which reduces free radical generation and protects photosynthetic apparatus (Asada, 1999).

Improving drought tolerance via over-expression of transcription factor genes is a genetic engineering strategy widely used in various plant species (reviewed in Yang *et al.*, 2010). On the contrary, we demonstrated that the degrees of drought tolerance may be gained through disrupting a gene that encodes for a regulatory transcription factor. Here, the drought stress reduced the expression of *Os12g42400* gene suggested that transcription factor might be a negative regulator of drought response, providing an adaptive mechanism for the plant. We further investigated the role of this TF by phenotypic characterization of a T-DNA insertion line where this gene was disrupted. We observed reasonable degrees of drought resistance. Interestingly, this gene disruption was not associated with any significant reduction in the seed yield of the *os12g42400-1* mutant plants when grown in control condition (data not shown).

The research presented here describes the initial stages of functional characterization of a dehydration-responsive NF-YA transcription factor and a regulatory protein with pronounced effect on drought tolerance in rice. Further characterization of this regulatory protein and the downstream cascades are required to better understanding the role of this protein.

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