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Over expression of Jatropha's dehydrin *jcdhn-2* enhances tolerance to water stress in rice plants

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Abstract

Jatropha curcas's dehydrin (*JC-DHN2*) has been previously shown to play a role during natural dehydration process associated with maturation of *Jatropha curcas* seed. In this study, we generated transformed rice plant (tp) overexpressing Jc-DHN2 gene and examined the role of over expressed gene in improving the drought tolerance. The tp plants showed a stronger growth under water stress condition induced by addition 20% of PEG 6000. It also showed an enhanced water stress tolerance as indicated by growth parameters included, fresh and dry weight, chlorophyll content, maximum quantum yield, actual quantum yield of photosystem II and non-photochemical quenching. Also, tp plants showed higher membrane stability under drought comparing with non-transformed plant (wt) as indicated through determination of membrane electrolyte leakage, the values of malondialdehyde and, hydrogen peroxide content as indicator for oxidation level. The tp plant had higher content of osmoregulators substances such as proline, free amino acids and total soluble sugar. The tp plant showed higher values for enzyme activity such as superoxide dismutase, catalase and ascorbate peroxidase compared with wt. Our results clearly showed that tp rice plant with *JcDHN-2* better coped with drought stress due increasing photosynthetic efficiency and antioxidant enzymes activities.

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Introduction

It's well documented that drought is one of the greatest severe environmental stresses limiting the growth and yield of plants In Egypt, rice is cultivated as a summer crop in about one million feddan (1 feddan is equal to 0.42 hectares) and is one of the main water consuming crops. The continuous flooding is the only method for irrigation and it consumes about 20% of the total water resources (Aboulila, 2015). By the year 2025, with increasing the Egyptian population, it will be required to produce about 60% more rice than what is currently produced to meet the food needs of a growing population (Fageria, 2007). Few decades later, water availability will be the limit factor of rice production in Egypt. Also, the regular global climate changes lead to the challenge of the world to produce enough food required to the increased population (Shao *et al.,* 2005).Improving genotypes productivity under water deficit condition is a great challenge for rice breeders to face water limitation problem**.** Drought tolerant plants have adopted various strategies to cope with environmental fluctuations through number of physiological, biochemical and molecular modifications and produced an array of proteins as a part of a global stress response to protect the cell metabolism (Allagulova *et al.,* 2003; Ingram and Bartels, 1996, Omar *et al.,* 2011; Omar *et al.,* 2013). Late embryogenesis abundant (LEA) encoding genes have been identified in numerous plant species in response to cellular dehydration and are, therefore, suggested to play a role in abiotic stress tolerance (Koag *et al.,* 2003; Saavedra *et al.,* 2006; Omar *et al.,* 2013). Dehydrins (DHNs) are classified as group 2 of LEA proteins (Close, 1997; Saavedra *et al.,* 2006) produced during late embryogenesis or drought, low-temperature, salinity, and ABA (Close, 1996; Allagulova *et al.,* 2003; Omar *et al.,* 2013). Several studies found that accumulation of dehydrin transcripts or proteins is associated with tolerance to freezing, drought, low temperature and salinity (Choi and Close, 2000; Park *et al.,* 2006; Walia *et al.,* 2006; Rorat, 2006; Tommasini *et al.,* 2008; Omar *et al.,* 2013). Recently, there are many direct experimental evidences correlating higher expression of DHNs and protection from osmotic stress. Arabidopsis plants overexpressed a dehydrin fusion protein were found to have better survival to low temperature (Puhakainen *et al.,* 2004). Also, transgenic tobacco expressing citrus dehydrin protein has been shown to give increased tolerance to low temperatures (Hara *et al.,* 2003). Over expression of wheat dehydrin *DHN-5* improves tolerance to salt and osmotic stress in *Arabidopsis thaliana* (Brini *et al*., 2007). It has been suggested that the short amphipathic K segments of dehydrin polypeptides interact with solvent exposed hydrophobic patches on proteins undergoing partial denaturation and thus inhibit protein aggregate (Close, 1996). *Jatropha curcas*'s dehydrin (*JC-DHN2*) has been identified and classified as typeY_2SK_2 according to the YSK shorthand for structural classification of DHNs (Omar *et al.,* 2013). *Jc-DHN2* is 441bp long open reading frame and 156 amino acid residues with a predicted PI of 7.09 and special activity of β-N-acetylhexosaminidases of glycoside hydrolase super family. Our previous data showed that *JcDHN-2* transcript level showed a sharp increase in expression during the dehydration process occurred during seed maturation which supports the protection role of this kind of proteins against the harmful effects of dehydration (Omar *et al.,* 2013). The aim of our study is to generate transgenic rice plants expressing JcDHN-2 protein to investigate the contribution of this gene to improve the ability of plant to resist against drought stress. This investigation included the evaluation of some physiological changes accompanied such as growth parameters, photosynthetic activity, membrane stability measurements, osmolytes content and antioxidant enzymes activities for both transgenic (tp) and non-transgenic (wt) plants under both control and drought induced with PEG (20%) conditions.

Materials and methods

Construction of a binary vector with JC-DHN2

The full length *JcDhn-2* open reading frame (ORF) (Acsession No. KF113882) previously characterized by (Omar *et al.,* 2013) was amplified from *Jatropha*

curcas seedling using specific forward primer (ATGCTCACTTTCAAAACCAAT) and reverse primer (AAGTAGAGTATTAAAGAACAC). KpnI restriction site (GGTAC/C) were added to the JcDhn2 ORF and cloned into the KpnI site of the binary vector pCambia1301-35SN (kindly supported by –Prof Huang Jirong, shanghai institute of plant physiology and Ecology, shanghai, China) downstream the 35S promoter and upstream of poly adenenylation signal 3'NOS. The vector contains the hygromycin resistance gene *hpt* as a selectable marker between the 35S promoter and terminator. This vector was called pCam-Dhn2 (Fig. 1). pCam-Dhn2 was mobilized into the *Agrobacterium tumefaciens* strains EHA105.

Rice calli production

Rice calli were generated from mature seed scutella of japonica rice cultivar (Oriza sativa L. Giza177) on a callus-inducting medium (NB-AS medium containing 2,4-D-Dichlorophenoxyacetic acid (100mg/ml), casein enzymatic hydrolysate (1mg/ml), M-inositol (0.1mg/ml) and sucrose (30mg/ml). Sub culturing in same medium was repeated until obtaining light-yellow calli. Healthy growing light-yellow fragile calli were used for transformation and growing wt plants.

Transformation of Rice

Rice transformation was achieved by co-cultivation of prepared calli with *Agrobacterium tumefaciens* EHA105 containing pCam-Dhn2 at 25-28 ˚C in dark for 3 days. The infected calli were selected using callus selection medium NB-THA containing: Timentin (100 mglml), ampicillin (50 mg/ml) and Hygromycin (50 mg/ml) for 2 weeks at 25-28˚C in dark. Calli were transferred to NB-TH medium containing Timentin (100 mg/ml) and Hygromycin B (50 mg/ml) at 25-28 ˚C in dark for 10 days. Hygromycin resistant callus were transferred to the pre-regeneration medium containing benzylaminopurine BAP (1 mg/ml), NAA (1mg/ml), ABA (12-15 mg/ml) and Hygromycin (50 mg/ml) for 2 weeks Timentin (100mglml), ampicilin (50 mg/ml) and Hygromycin (50 mg/ml) for 2 weeks in dark at 25-28˚C. the resistant calli were transferred to re generation medium containing 6-BAP(1mg/ml), NAA(1 mg/ml), IAA(1 mg/ml), KT(2 mg/ml)and Hygromycin (50mg/ml) Timentin (100 mg/ml), ampicilin (50 mg/ml) and Hygromycin (50 mg/ml) for 2 weeks at 25-28˚C for 2-3 weeks. The regenerates were transferred to rooting- medium and incubated at 25-28˚C under light. Plants of 3-4 inches long are used for examining water stress tolerance.

PCR screening of transgenic plants

Non-transformed plants (wt) and transgenic plants (tp) were screened for the presence of JcDhn-2 using primers corresponding to the 5'and 3' ends of JcDhn-2 with Kpn1 restriction sites added. These primers were 5'-GGTACCATGCTCACTTTCAAAAC-3' and 5'-GGTACCAAGTAGAGTATTAAAG-3'. The PCR was carried out in 25ul reaction volume according the instruction supporting with GoTaq®Green master Mix, (Promega) with implication condition as follows: 94˚Cfor 30sec, 54˚C for 1min followed by 72˚C for 1min. this was repeated for 30 cycles. The PCR product was examined on 1.5 % agarose gel stained with Ethidium bromide.

Growth condition and stress treatment

Rice plants of wt and tp at 3-4 inches long were cultivated in 15cm pots. The experiment was carried out in the incubator at 28-29˚C and 14h light. Nutrient solution and nutrient solution containing 20% PEG-6000 was used for irrigation daily for both control and stress treatment, respectively. The concentration of PEG was maintained daily by changing the nutrient solution.

Determination of fresh weight, dry weight and water content

Fresh weight (FW) of whole plant was scored for individual plants. Dry weight (DW) of individual plants was determined after oven drying at 105˚C for 3h. Water content (wc) of individual plants was calculated as the difference between fresh and dry weight on base of FW and expressed as gH_2O/g FW.

Measurements of photosynthetic parameters

Chlorophyll was extracted from leaf fragment (4cm long) using 2ml of 80% acetone. After incubation at

4˚C for 48h, the absorbance at 470, 649, and 665 nm was measured using a spectrophotometer (Lichtenthaler and Wellburn, 1983). Chlorophyll fluorescence was measured in 30 min dark adapted leaves using hand held leaf fluorometer (FluorPen FP 100, Photon System Instruments, Czech Republic). The following fluorescence parameters: maximum quantum yield of PSII in dark-adapted state (Fv/Fm), effective quantum yield of PSII, and non-photochemical quenching (NPQ) were measured according to (Strasser *et al.,* 2000; Kalaji *et al.,* 2014).

Determination of Osmolytes

Proline content was measured according to (Bates *et al.,* 1973). Soluble sugars were extracted and assessed as described in (Dubois *et al.,* 1956). Total free amino content was determined as described earlier by (Rosed, 1957).

Determination of H_2O_2

Hydrogen peroxide was extracted with cold acetone according to the procedure described in (Patterson *et al.,* 1984).

The extract was reacted quantitatively with titanium tetrachloride and ammonia to produce a peroxide-Ti complex. The complex was collected through centrifugation and then dissolved in 2M sulfuric acid. The absorbance of the solution was measured at 415 nm and H_2O_2 content was calculated according to a standard curve.

Evaluation of Lipid Peroxidation Product

Lipid peroxidation was evaluated by determining malondialdehyde (MDA) content from 0.5 g of plant tissue as originally described by (Heath and Packer, 1968), with slight modifications by (Hendry and Grime, 1993).

Determination of antioxidant enzymes activities

Superoxide dismutase (SOD; EC1.15.1.1) activity was measured according to (Beauchamp and Fridovich, 1971) as described in (Donahue *et al.,* 1997). Catalase (CAT; EC1.11.3.6) activity was assayed according to the method of (Aebi, 1983). Ascorbate peroxidase (APX; EC 1.11.1.11) activity was assayed according to (Nakano and Asada 1981).

RNA extraction and semi-quantitative PCR

Total RNA was extracted from wt and tp using InviTrap® Spin plant RNA kit. DNase-treated RNA (5µg) samples were used to synthesis of first strand cDNA according to the protocol supported by Go Script™ reverse transcription Kit using oligo $(dT)^{15}$ primer. Two microliters of the first strand cDNA were used as a template for PCR amplification with *JcDhn-2* specific primers.

The cDNA samples were standardized on rice actin gene (RAC1) transcript amount (accession no. X16280) using gene specific primers (F-CATGCTATCCCTCGTCTCGACCT, R-CGCACTTCATGATGGAGTTGTAT). Samples were denatured at 94˚C for 5 min and then run for 28 cycles at 94˚c for 30 s, 54˚C 1 min, 72˚C for 1min and final extension of 3 min at 72˚C. The PCR product was examined on 1.5% agarose gel stained with Ethidium bromide

Results

Generation and identification of Transgenic Rice of JcDHN-2

JcDHN-2 full-length ORF (Fig. 1A) cloned into the KpnI site of the binary vector pCambia1301-35SN (kindly supported by Prof Huang Jirong, shanghai institute of plant physiology and Ecology, shanghai, china) downstream of the 35S promoter and upstream of poly adenenylation signal 3'NOS to produce pCam-Dhn2 vector (Fig. 1B) After agrobacterium-mediated transformation, cullus transformation were confirmed by resistance to hygromycin as selectable marker between the 35S promoter and terminator.

PCR analysis of non-transformed plants (wt) and transgenic plants (tp) using primers corresponding to the 5'and 3' ends of *JcDhn-2* with Kpn1 restriction confirmed the transformation and avoided the amplification of other DHNs from rice. Existing of one specific band in PCR product with size of 490bp

confirmed the transformation in tp comparing with wt plants (Fig.1C).

To examine the expression level of *JcDHN-2* in transgenic plants sq-RT-PCR was performed using gene specific primer to compare wt with tp under control and stress condition. Fig.1D showed that no expression for *JcDHN-2* in wt plants under both control and stress condition.

Expression level of *JcDHN-2* increased in tp plants as a result to stress condition comparing with control condition as appear in increasing of band density and brighten on agarose gel (Fig.1D).

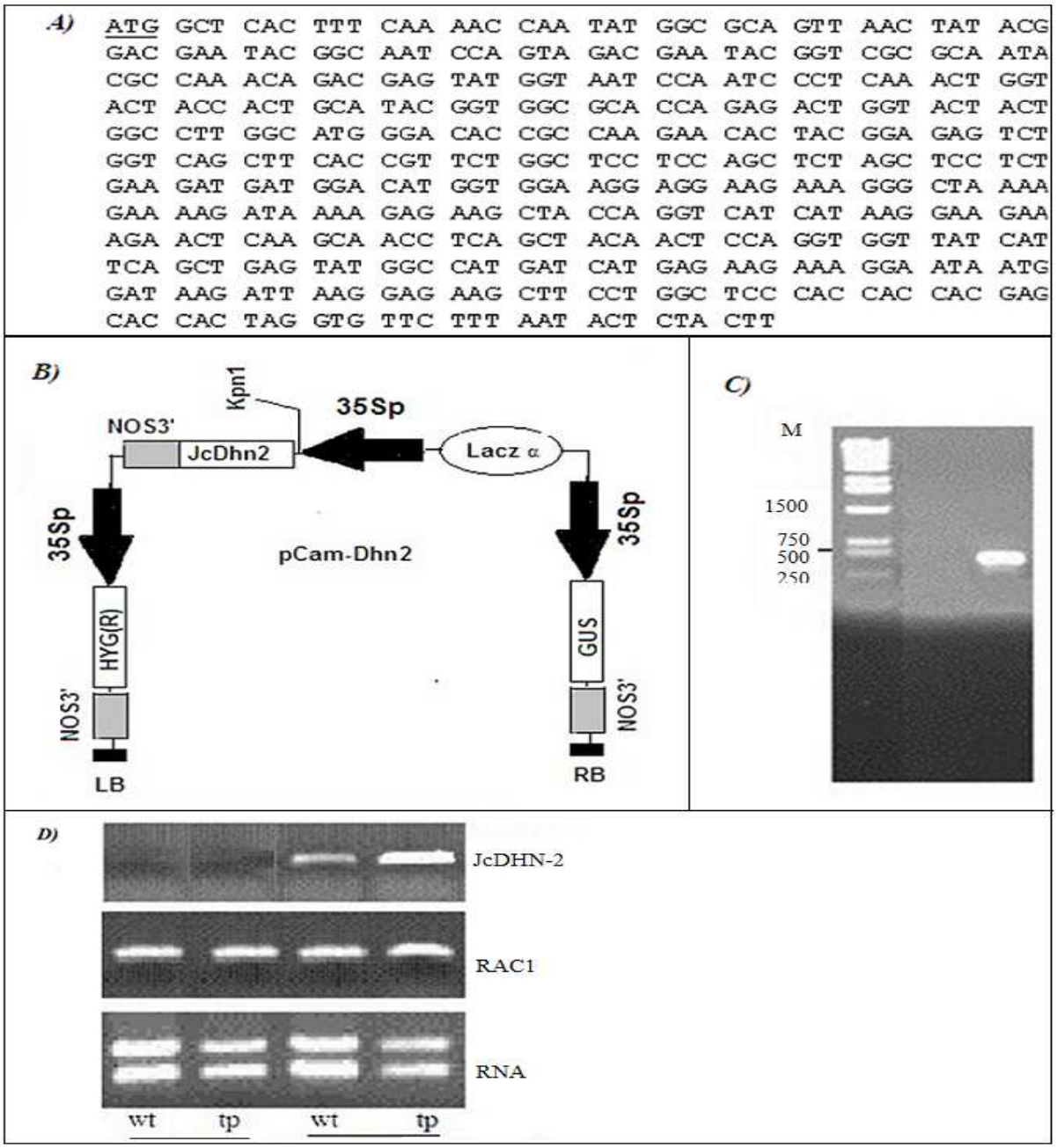

Fig. 1. Creation of pCam-Dhn2: (A)-nucleotide seqJcDhn2 ORF (Accession No. KF113882) isolated from *Jatropha curcas* germinated seeds using JcDhn2 primers. (B)- *JcDhn-2* was inserted between 35Spromotor and polyadenylation signal 3'NOS termination in kpnI site of binary vector Pcambia1301-35SN to create the transformation vector Pcam-Dhn2. **(C)**- PCR screening of transformed plantlet using JcDhn2–ORF- specific primers. A specific PCR product of 490 bp was detected in tp and absent in wt. (D)- Semi quantitative analysis of *JcDhn-2* expression pattern in wt and tp plants under control and PEG (20%) treatments.

Evaluation of drought tolerance in JcDHN-2 transformed plants

To study the role of JcDHN-2 in drought tolerance of tp, we conducted several analyses and investigated stress-tolerance of wt and tp plants.

Fresh and Dry weight

Determination of fresh (FW) and dry (DW) weight indicated to the superiority of tp plants on wt plants under stress condition. Tp plants kept their Fw and Dw values at high level under both conditions, while wt plants affected severely under drought condition (Fig. 2).

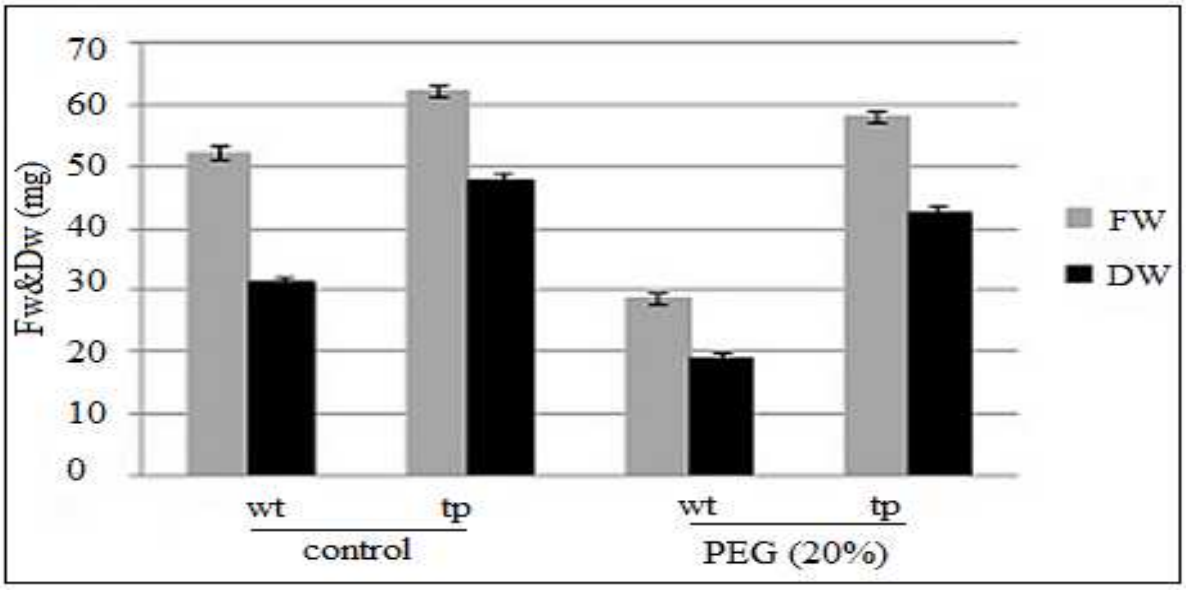

Fig. 2. Fresh and dry weight of plants under control and PEG (20%) treatments.

Monitoring the oxidative state and membrane stability

Tested plants showed that rate of EL, MDA and H_2O_2 content increased under stress conditions (Fig. 3). Drought stress stimulates H_2O_2 production in wt compare with tp plants. The accumulation of H_2O_2 can cause oxidative stress in plant tissues and associated with increasing of MDA content as a direct indicator for membrane's lipid oxidation.

This severs effect of oxidation on membranes induce an increase in EL rate. Lower H_2O_2 content subsequently lower MDA content and EL rate were found in tp plants under drought indicated that overexpression of *JcDHN-2* resulted in less ROS accumulation in tp plants.

These results suggest a lack of capacity for protection from oxidative damage that occurred in wt under drought stress and reveal the great adaptation and membrane stability of tp plants to drought stress.

Analysis of photosynthesis pigments and their activity

Determination of chlorophyll pigments such as a, b, and carotenoids and chlorophyll fluorescence parameters as Fv/Fm, PSII and NPQ showed increasing in their values in tp plants comparing with wt plants under control condition (Fig. 4). Under stress condition, the contents of chlorophyll a, b, and carotenoids were greatly reduced in wt plants, whereas those values in tp plants were slightly reduced. These results clearly indicated that transgenic plants over expressing *JcDHN-2* had a greater tolerance to drought stress compared with wt plants.

Osmolytes compounds and antioxidant enzymes activities

Measurements of proline, FAA and TSS (Fig. 5) showed that values of proline, TSS and FAA were higher in wt plants than tp plants under both conditions. Decreasing the values of proline, TSS and

FAA in tp plants under drought conditions indicating that tp plants was less affected with drought than wt plants, where it reveal that these plants were protected against water loss. Determination of antioxidant enzymes (Fig. 5) showed variation and differentiation between wt and tp plants. SOD enzyme showed similar activity in both wt and tp plants under control and also increased dramatically under drought stress in both of wt and tp. Although, CAT activity showed similar activity in both wt and tp plants under control condition, it showed great decrease in wt plants under drought stress while it increased slightly in tp plants under drought stress. APX activity showed higher values in tp plants comparing with wt plant under both control and drought conditions.

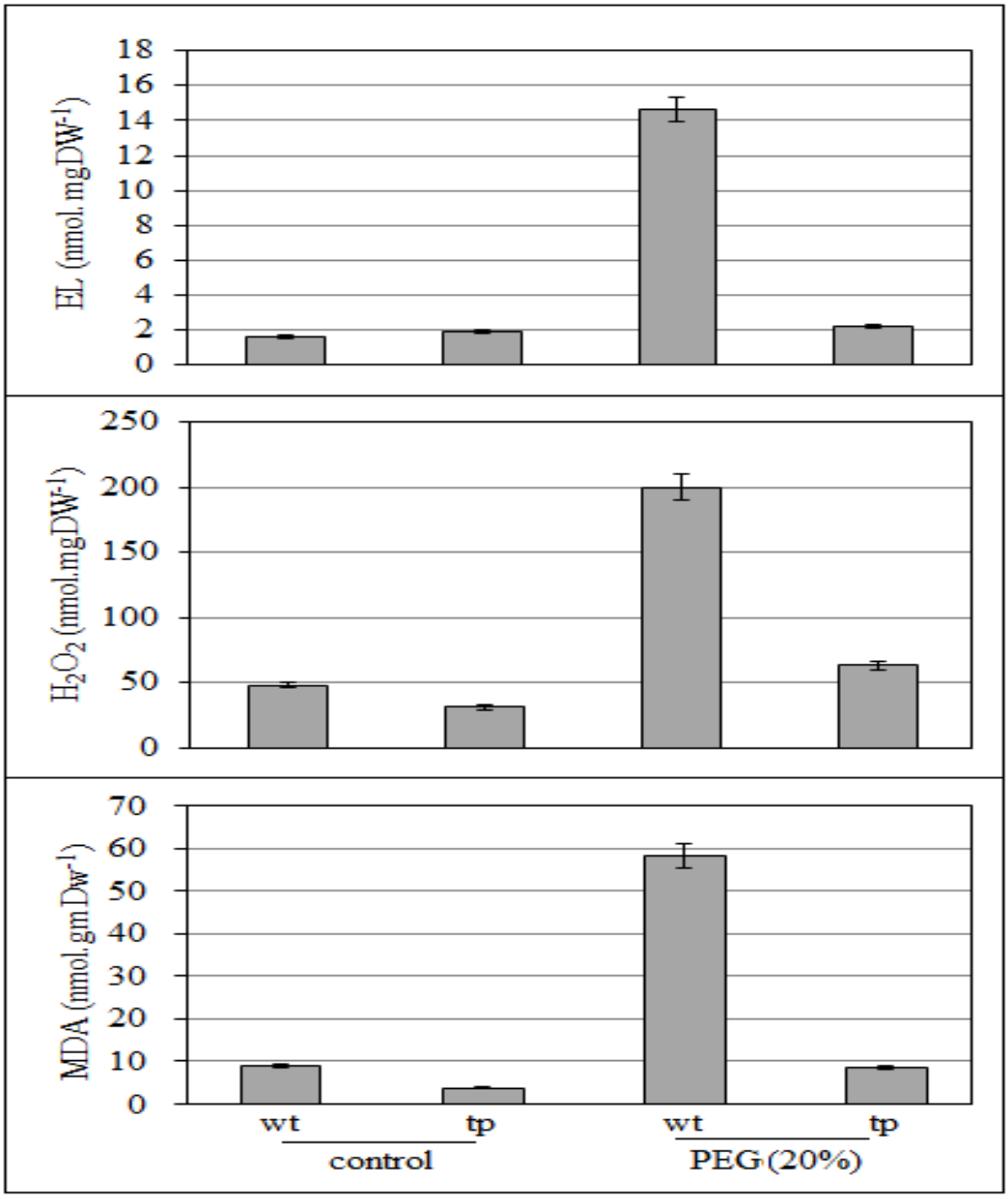

Fig. 3. Stress tolerance monitor: changes in Electrolyte leakage rate of membrane, H_2O_2 content and MDA content of wt and tp plants under control and PEG(20%) treatments.

Discussion

JcDHN-2 full-length ORF used to produce transgenic rice plants to cope with drought stress. In this study, transformed rice plants (tp) showed a great adaptation to drought stress treatment as shown by determination of membrane stability parameters or growth and metabolism parameters.

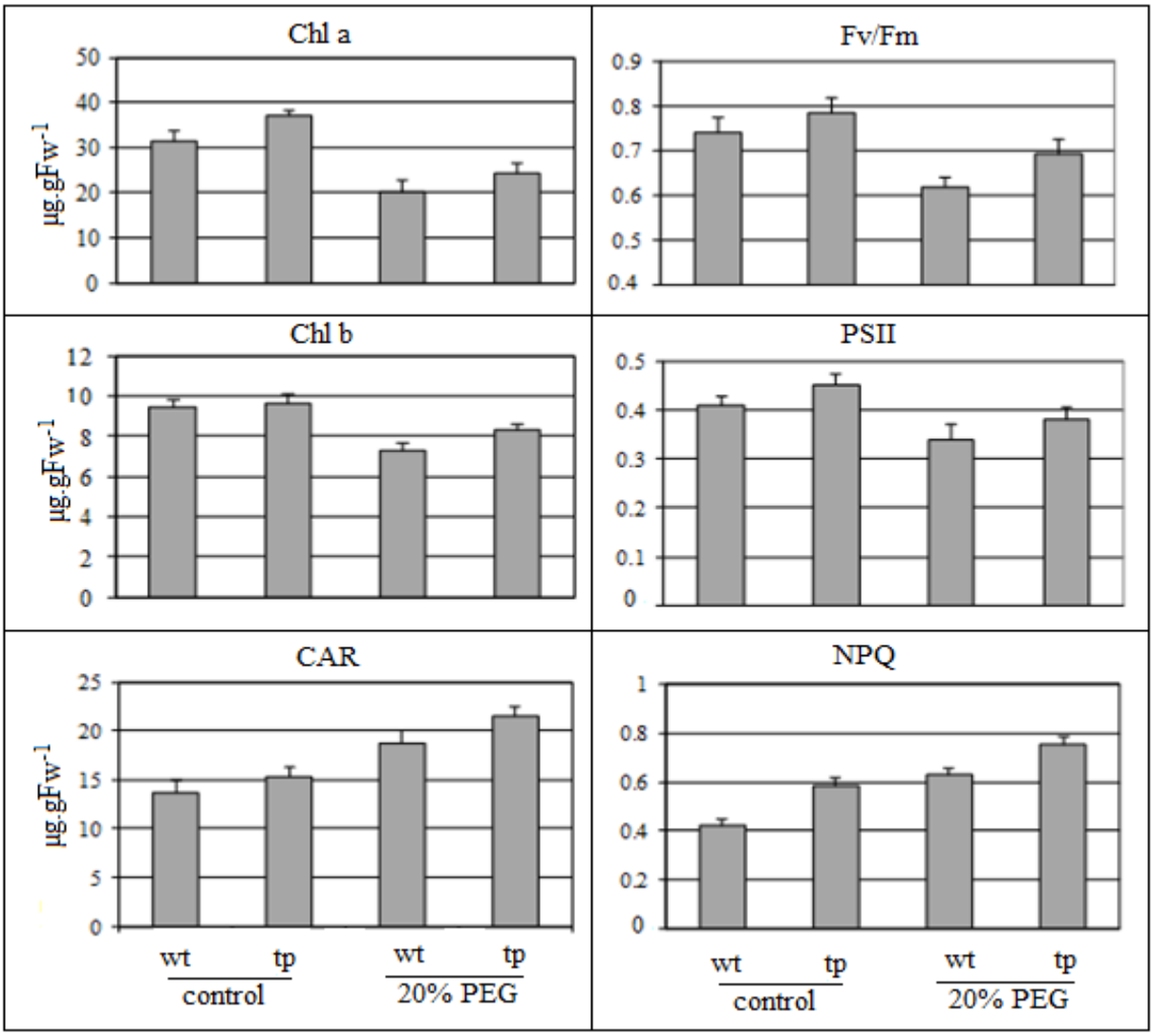

Fig. 4. Effect of water stress on chlorophyl pigments and photosynthesis activity: changes on chlorophyl a,b , carotenoids contents and Fv/Fm on plants under control and PEG (20%) treatments.

Numerous transgenic studies revealed a positive effect of *DHNs* genes expression on plant stress tolerance (Hara *et al.*, 2003; Saavedra *et al.*, 2006; Yin *et al.*, 2006; Brini *et al.*, 2007; Kumar *et al.*, 2014;). *DHNs* may perform important protective functions in plant cells, such as protecting the structure and stabilizing the plasma membranes and raise the activity of stress-sensitive enzymes against drought stress (Allagulova *et al.,* 2003; Sun *et al.,* 2009). Preservation of membrane integrity and stability under abiotic stress conditions is a main component of environmental stress tolerance in plants (Levitt, 1980; Filippou *et al.,* 2011; Elsheery and Cao, 2008). Transgenic plants over expressing *DHNs* showed less lipid peroxidation and leakage rate values (Shekhawat *et al.,* 2011, Xing *et al.*, 2011). The K-segments of *DHNs* can form amphiphilic α-helix, which may control the interaction of DHNs with lipid, plasma membranes and with hydrophobic sites of partially denatured proteins to prevent protein-protein aggregation of plasma membranes under drought stress (Close

1996, 1997; Campbell and Close, 1997; Allagulova *et al.*, 2003). Tobacco expressing spinach *CAP85* and *CAP160 DHNs* revealed lower level of electrolyte after frost test which indicates a reduction of freezing injury in transformed plants (Kaye *et al.,* 1998). Stimulating H_2O_2 production in wt plants under stress comparing with tp plant suggesting a lack of an enhanced capacity for protection from oxidative damage induced by drought stress in this wt.

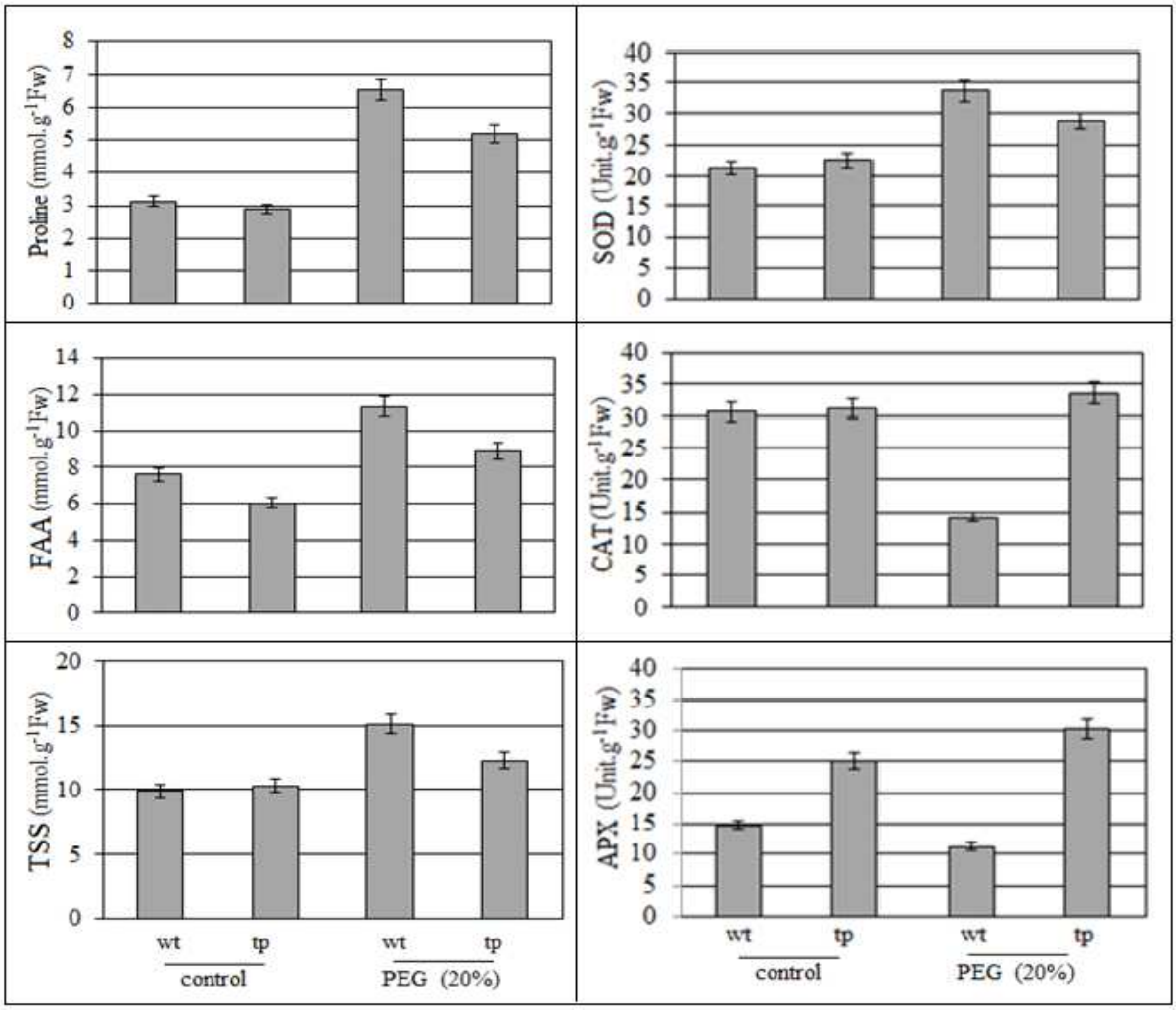

Fig. 5. Changes in osmolytes contents and antioxidant avtivities: proline, FAA, TSS, SOD,CAT and APX for both wt and tp plant under control and PEG (20%) treatment under control and PEG(20%) treatmrnts.

In this study, *JcDHN-2* overexpression prevents excessive accumulation of H_2O_2 under stress condition. Reactive oxygen species (ROS) scavenging function of DHNs was reported which can be mediated by direct interactions between the a residue and the ROS species. Thus, it causes oxidation of the residue (Hara *et al.,* 2003, Hanin *et al.,* 2011).Moreover, DHNs can function as antioxidants (e.g., CuCOR15 and CuCOR19 in *Citrus unshiu*) (Hara *et al,.* 2001, Hara *et al.*, 2005). Because of most DHNs function as molecular chaperons (Close, 1997) we could exclude that ROS level could be reduced by antioxidant enzymes protected by JcDHN-2 expressed in pt plants under stress. Pattern of antioxidant enzymes activities showed good participation of SOD, CAT and APX in tp plants during stress treatment which can play role in ROS scavenging. Low activity of both CAT and APX in wt plants under drought stress caused losing of drought tolerance. CAT might be responsible for elimination of excess ROS during water stress (Mittler, 2002; Helaly *et al.,* 2017; Helaly *et al.,* 2018)

and remain more active for a greater duration of drought stress (Rivero *et al.,* 2007). Accordingly, losing of CAT and APX activities in wt plants with increasing the activity of SOD resulted in accumulation of super oxide free radicals which cause severe effects on cell membrane and components. In our results, APX activity showed significant increase in tp plants under drought stress. APX and POD might be responsible for the fine modulation of ROS for signaling. Various stressful conditions of the environment have been shown to induce the activity of GPX (Kovalchuk, 2010), POD, APX, and GR (Benešová *et al.,* 2012, Omar *et al.,* 2012) in tolerant species.

It appears that there was an association between the higher antioxidant capacity and higher tolerance to drought stress in our transgenic rice which supports the idea of protection role of DHNs to antioxidant enzymes expressed in pt plants under water stress. Positive role of overexpression of DHNs on relative water content and drought yield index as associated traits with drought tolerance occurred in set of Korean barley cultivars (Park *et al.,* 2006). Also, the correlation between higher accumulation of DHNs transcript and drought tolerance was found in two differently tolerant cultivars of wheat (Labhilili *et al.,* 1995). DHNs contain high proportions of hydrophilic aa and change their conformation in response to the changes in their ambient micro-environment (Hanin *et al.,* 2011) which led to changes in protein function. (Tompa, 2002; Tompa *et al.,* 2005).

In conclusion, we here presented that rice transgenic plants overexpressing *JcDHN-2* gene showed higher tolerance to water stress condition induced using 20 % PEG 6000. Water stress tolerance in tp plants was accompanied with stability of membrane, increasing of photosynthetic parameters and good participation of some antioxidant enzymes. These results prove the protection role of DHNs under water stress condition.

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