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Expression Analysis of the *ADH* Genes in *Arabidopsis* Plants Exposed to PEG-induced Water Stress

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Abstract In plants, ethanolic fermentation occurs during limited oxygen condition and under certain environmental stresses. Many of the observations reported on the *ADH1* gene during environmental stress conditions were obtained from studies that used single isoforms of the gene *ADH1* even though many isoforms of the genes are known to be operational in plants based the complete genome sequence of more than 20 different plant species. Here, the *Arabidopsis* plants were exposed to polyethylene glycol-induced drought stress and the whole set of *ADH* (EC.1.1.1.1) genes as well as the enzyme activity of ADH in response to PEG-induced water stress condition was presented and discussed. At enzyme levels, both the root and leaf NADH-ADH activities were increased 5.9 and 4.4 folds when treated with 5% (w/v) PEG-20,000. At gene level, the majority of the *ADH1* gene *AT1G77120* and two of the *ADH*-like genes (*AT1G64710* and *AT5G24760*) were up-regulated in the leaf and root. The result suggests that *ADH1* together with other two more *ADH*-likes genes were responsive in the leaf and root operating along side during the PEG-induced water stress and these evidences support the conclusion that the capacity of ethanolic fermentation was enhanced in response to drought.

Keywords: *Arabidopsis, PEG induced water stress, RWC, proline, chlorophyll, alcohol dehydrogenase*

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1. Introduction

The ADH enzyme involved in ethanolic fermentation pathway and this pathway considered the oldest metabolic process used by cells to generate energy for their metabolic activity (Muller et al. 2012) in completely anaerobic with harsh physical and chemical environments (Tadege et al. 1999; Ismond et al. 2003 and Kato-Noguchi et al. 2010). Of the divergent alcohol dehydrogenase enzymes, the classic alcohol dehydrogenase (ADH, alcohol: NAD+ oxidoreductase, EC 1.1.1.1) is a Zn-binding enzyme that acts as a dimer and relies on an NAD (P) co-factor to interconvert ethanol and acetaldehyde. Under anaerobic condition, limiting oxygen inhibits the TCA cycle and damages the mitochondrial ATP generating machinery. Under this condition, plant cells switch to ethanolic fermentation to generate NAD for glycolysis that generates 2 mole of ATP per mol of glucose (Perata et al. 1993). The cofactor NAD^+ generated as a by-product of this process is what makes ethanolic fermentation important for the survival of living systems under anaerobic condition (Tedege et al. 1993).

As the earth atmosphere change over time, one of the main enzymes of the pathways i.e. alcohol dehydrogenase (ADH) was also evolved, adapting to new environments from time to time. As a consequent, the enzyme was very responsive to their dynamic environment and there are many experimental evidences that showed how sensitive this enzyme was to changes in its internal and external environments. In plants, the activities of the ADH enzymes were up-regulated not only in low oxygen conditions (Ismond et al. 2003; Mustroph et al. 2003; Tesniere et al. 2006; Kato-Noguchi 2006, 2010; and Kumutha et al. 2008) but also in other environmental stresses like dehydration, low temperature, or chemical treatments in different plant species (Matton et al. 1990; Dolferus et al. 1994; deBruxelles et al. 1996; Kato-Noguchi 2001; Peters and Frenkel 2004 and Kato-Noguchi et al. 2007). The involvement of ADH enzymes in plant adaptation to abiotic stress was also evident at the gene level in which the *ADH1* gene were up regulated in plants grown under low oxygen levels (Shiao et al. 2002). In addition, the expression of *ADH1 gene* was induced by many abiotic stresses including cold and osmotic stresses (Christie et al. 1991 and Conley et al. 1999), wounding (Kato-Noguchi 2001), dehydration and water deficit (Dolferus et al. 1994 and Senthil-kumar et al. 2010), low temperature (Peters et al. 2004 and Kato-Noguchi et al. 2007), and abscisic acid treatment (de Bruxelles et al. 1996). These observations support the notion that the ADH enzyme engage in plant adaptation to environmental stress. Several plant species exposed to environmental impacts, such as water deficit, SO_2 fumigation, ozone exposure (Kimmerer et al. 1982) and low temperature

(Kato-Noguchi et al. 2007), at ambient or even at elevated oxygen concentration (Ismond et al. 2003) produce considerable amounts of ethanol to response environmental stress. Some suggests that fermentative enzymes are involved in plant general adaptation to stress (Ismond et al. 2003; Kato-Noguchi et al. 2006 and Tesniere et al. 2006).

The alcohol dehydrogenase (ADH) enzyme in the model plant *Arabidopsisis* encoded by *ADH1*. There are other *ADH*-like genes which may also contribute to the ADH enzyme activity. With the *Arabidopsis* genome completed, only a single class-P alcohol dehydrogenase locus as *ADH1* gene (class P alcohol dehydrogenase) and other seven genes were annotated as alcohol dehydrogenase-like genes that display in different loci of *Arabidopsis* genome which are spread over three chromosomes (The ArabidopsisGenome Reannotation. 2011). Accordingly, the Uniprot web site presented that both *ADH1* and these seven alcohol dehydrogenase-like genes encoded the ADH enzyme (EC 1.1.1.1) that belongs to the homology of biochemical function and all these genes are localized in cytoplasm. In addition, information on the Plant Metabolic Network (PMN), alcohol dehydrogenase genes *ADH1*, *ADH2* and other eight isozymes (EC.1.1.1.1) catalyze reduction of alcohol dehydrogenase to ethanol in *Arabidopsis* (Figure1).

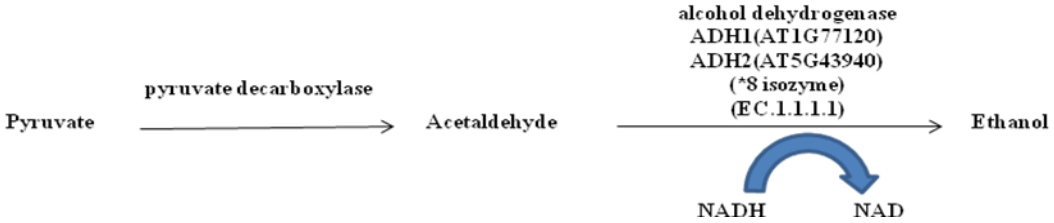

Figure 1. Fermentative enzymes, substrate and product.

Since *Arabidopsis* have many ADH enzyme (EC.1.1.1.1) encoded genes; the involvement and role of drought-responded *ADH* genes under PEG-induced water stress are unknown. Important question yet to be determined is there any other *ADH* in *Arabidopsis* plants responding to drought condition. Here, the expression levels of different *ADH* genes in the *Arabidopsis* plants grown under drought-related water stress were evaluated and discussed.

2. Material and method

2.1. Plant Materials and Experimental Growth Conditions

Arabidopsis thaliana (wild) used in expression analysis was the ecotype Columbia-0 obtained from The Arabidopsis Biological Resource (TAIR) Centre at The Ohio State University, USA. For experimentation, the plant was grown in a hydroponic system using the method established by Smeets et al. 2008. Briefly, the seeds were surface sterilized and germinated on MS plates according to the established protocol (TAIR). Fourteen-day-old seedlings were transferred to the hydroponic system equipped with an aeration system provided by a small aquarium pump. The nutrient was of Hoagland's with replacement done every week. This system was established in a growth room at 22˚C with a light scheme of 16 h light and 8 h dark.

2.2. PEG-induced Water Stress Treatment

In this experiment, four-week-old plants at their vegetative stage were subjected to 5% (w/v) PEG 20,000 (polyethylene glycol, MW 20,000, Sigma-Aldrich) treatment. The plants were initially exposed to 1 % (w/v) PEG and then the PEG level was gradually increased by 1% for every 24 h until the maximum of 5% PEG treatment was reached at fifth day of treatment. Gradual introduction of the PEG would allow the plants to adapt to the treatment gradually rather than being shocked by a single high dose of the chemical.

2.3. Experimental Procedure

Ideally, the treated plants should be in a moderately water-stressed condition before the analysis could be done. The stress parameters analyzed were relative water content (RWC), proline and chlorophyll (Chl). The same stress plant samples were used for determination of ADH enzyme activities and expression analysis of the *ADH* genes.

2.4. Relative Water Content

Relative water content (RWC) was measured by using the method established by Weatherley (1950) in order to determine the level of water content in the leaf samples of the PEG-treated *Arabidopsis* plants. Three fully expanded rosettes leaves were sampled and pooled as one replication. The weight was read to the nearest mg, and the relative water content was determined using the equation below.

$$RWC(\%) = \left[\frac{W - DW}{TW - DW}\right] x100$$

W = fresh weight;

TW = turgid weight; DW = dry weight

2.5. Proline Content

Proline content was determined by using the colorimetric method described by Bates et al. (1973). The absorbance was then taken at 520 nm and toluene was used as a blank. Proline concentration was estimated based on standard curve and calculated as follow:

[(μg proline/mL × mL toluene) / 115.5 μg / μg mole]/ [(g sample)/5] = μmoles proline/g of fresh weight material

2.6. Chlorophyll Determination

Chlorophyll content was used as an indicator for photosynthetic capacity of the plant. In this experiment,

chlorophyll content was estimated by using the method established by Porra (2002). Fresh leaf material was extracted with 80% (v/v) acetone and absorption of this solution was measured spectrometrically at 663 and 646 nm. The different types of chlorophyll molecules were determined using the following equations:

$$Chlorophylla(\mu g/mL) = 12.25(A_{663}) - 2.55(A_{646})$$
$$Chlorophyllb(\mu g/mL) = 20.31(A_{646}) - 4.91(A_{663})$$
$$Total\ Chlorophyll(\mu g/mL) = 17.76(A_{646}) + 7.34(A_{663})$$

Table 1. *ADH1* and *ADH*-like genes analysed in current study.

	Entry	locus name	protein name	length	function	sub celluar logation
1	ADH1 P06525	AT1G77120	alcohol dehydrogenase class-P EC.1.1.1.1	379	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
2	Q8VZ49	AT1G64710	putative alcohol dehydrogenase alcohol dehydrogenase -like-4 EC.1.1.1.1	380	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
3	Q9SK86	AT1G22430	alcohol dehydrogenase -like-1 EC.1.1.1.1	388	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
4	Q9SK87	AT1G22440	alcohol dehydrogenase -like-2 EC.1.1.1.1	386	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
5	A1L4Y2	AT1G32780	alcohol dehydrogenase -like-3 EC.1.1.1.1	394	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
6	Q0V7W6	AT4G22110	alcohol dehydrogenase -like-5 EC.1.1.1.1	389	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
7	Q8LEB2	AT5G24760	alcohol dehydrogenase -like-6 EC.1.1.1.1	381	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm
8	Q9FH04	AT5G42250	alcohol dehydrogenase -like-7 EC.1.1.1.1	390	alcohol dehydrogenase (NAD) activity nucleotide binding oxidoreductive activity zinc ion binding	cytoplasm

Table 2. Sequence of the specific primers used to amplify the *ADH* gene.

Name of *ADH* isoform	Primer sequence 5' - 3'
AT1G22430	F: CCGTTGGACTCGCTGTTGCTGA
	R: TGTCGATCCCCAACCGACGTGA
AT4G22110	F: GCGTGGAGCCGGCAAGATCA
	R: ACCCGTGCGAGTGCTGGAGA
AT1G22440	F: AGCGGTTGCAGAAGGAGTCAGA
	R: TTACCCGATCCCGGGCGAGT
AT5G42250	F: TCGGATTAGCGGTTGCAGAGGGT
	R: GAACCCGGCTTGTCCACCCC
AT1G32780	F: CCGTTGGACTCGCTGTTGCTGA
	R: TGTCGATCCCCAACCGACGTGA
AT1G64710	F: TGCAGTTTCAACTTTCAAAGCCAGC
	R: CGCTCTCCACTATCCCTGCTGC
AT1G77120	F: AGGCGCTGCAGAAGGTGCTA
	R: TGCAACACCCCAGCCATCGT
AT5G24760	F: CATAACCTGCAACGCGGCGG
	R: TGCAATGCCGACATGACCCAC
Actin2	F: AGTTGTAAGAGATAAACCCGCCTAT
	R: CCAGCCTTCACCATACCGGTAC

2.7. ADH Enzyme Assay

The activity of ADH enzyme was determined using crude enzyme extract. The plant sample 0.5 g was deep-frozen in liquid nitrogen and then ground to powder by using mortar and pestle. The powder was transferred to a 15 mL protease-free Falcon tube before 5.0 mL of extraction buffer was added to it for homogenization by

vortexing. The extraction buffer was 50 mM Tris-HCl at pH 8.0 supplemented with 15 mM dithiothreitol (DTT). The homogenate was centrifuged at 12,000 x *g* for 15 min at 4 ℃, and the supernatant was used as enzymes source. The reductive activity of ADH at 340 nm was determined by using the method described by Kimmerer (1987). Enzyme activity was expressed as nmol NADH oxidized min^{-1} mg^{-1} total protein.

2.8. Gene Expression Analysis by Reverse Transcription

The leaf and root RNAs of PEG treated and control sample were prepared by using the method of Krapp et al. (1993). Expressions of the ADH genes were determined by measuring the respective transcripts of the genes generated *in vitro* through reverse transcription reactions coupled with PCR (RT-PCR). The respective cDNAs were synthesized by using the protocol accompanied with the QuantiTect Reverse Transcription Kit (Quaigen). First-strand cDNA synthesis was performed in a 28 µL reaction using 4 µg total RNA and 1 µL of Quantiscript Reverse Transcriptase enzyme. Together with this kit was a procedure to remove any possible genomic DNA contamination which was carried out for all of the RNA preparations.

PCR was performed using 0.5 - 2 µL of the cDNA in a total volume of 25 µL. The PCR reaction mix contained 1 x PCR buffer, 0.2 mM deoxynucleotide (dNTP), 2.0 mM magnesium chloride ($MgCl_2$), 0.5 µM of each primer, and 0.5 U of *Taq* DNApolymerase (Fermentas, USA) in a total volume of 25 µL. PCR was carried out using the following PCR programme: 95 ℃ for three min, 30 cycles of 95 ℃ for 30 sec, 55 ℃ for 30 sec and 72 ℃ for 60 min, and then 72 ℃ for five min. The primers used for the gene's expression analyses are listed in Table 2. The gene-specific primers were designed using the Primer-BLAST software of the NCBI based on the exon-exon junction regions of the genes to differentiate any products amplified from contaminated genomic DNAs. The internal control used in this analysis was *Actin-2*. PCR conditions were standardized using gene-specific primers for *Actin-2*.

2.9. Data Analysis

Since *Arabidopsis* plants are small, ten of experimental plants were harvested and pooled as one biological sample for molecular and biochemical analyses. Three biological samples were analyzed for these experiments which were run on a completely randomized design (CRD). The data was analyzed by using the computer program SAS 9.2 and mean differences were tested for significance using *t* test at 0.05 level of probability for comparing control and PEG treatment of wild plants.

3. Result

3.1. Biophysical Evidence of PEG-induced Water Stressed

A pre-requisite for studying the effect of water-limited condition on various aspects of plants is that the experimental plant must be in a *real* water-stressed state. In order to confirm that the PEG-treated plant used in the present study was water-stressed, the level of water content in the plant was evaluated. In this experiment, water-stressed plants were obtained by exposing the plants to polyethylene glycol (PEG 20000) in the Hoagland's solution using a combination of hydroponic system.

In the present study, relative water content (RWC) of the PEG-treated wild plants was decreased when treated with 5% (w/v) PEG. In the treated plants, the level of RWC dropped to 75% from the initial level of 85% as measured in the control plant (Table 3). Hence, exposing *Arabidopsis* plants grown in hydroponics with 5% (w/v) PEG 20000 successfully induced to the moderate water-stressed state required for the study. Apart from RWC, two common metabolites (proline and chlorophyll) often used as biochemical markers for water-stress conditions, were also monitored to further confirm the water-stressed state of the plants. The treated plants had lower content of total chlorophyll (Table 3). Another biochemical marker measured was proline which level in the treated plants was higher than was higher than the control (Table 3). These observations (increase in proline, decrease in chlorophyll, drop in RWC) confirmed that the treated *Arabidopsis* plants were in moderate water-stressed states; therefore, allowing them to be used for studying of *ADH* gene expression.

Table 3. PEG-induced water-stressed Arabidopsis plants were confirmed by RWC, proline and chlorophyll contents. Data are means (± SD) calculated from three independent experiments. Significant differences between control and treatment were indicated by *P*< 0.05 based on the *t* test.

	RWC (%)	Proline content (µmol g^{-1} FW)	Chlo a (μg^{-1} mL)	Chlo b (μg^{-1} mL)	Total Chlo (μg^{-1} mL)
Control	84.82 ±1.38[a]	2.36 ±0 .98[b]	5.14 ±0.16[a]	2.76 ±0.59[a]	7.90 ±0.73[a]
Treated (5% PEG)	75.33 ±1.92[b]	11.62 ±5.37[a]	4.55 ±0.19[b]	1.85 ±0.29[a]	6.39 ±0.48[b]

2.10. Biochemical Response of PEG-treated Water Stress

To determine the behaviour of alcohol fermentative pathway in plants when water was limited, changes in the activities of ADH were monitored in the *Arabidopsis* wild plants treated with PEG. Since the enzyme is involved in the ethanolic fermentation of acetaldehyde to ethanol, the activity would reflect the pathway's behaviour under this condition.

Exposing the plants to 5% (w/v) PEG enhanced the activities of the fermentative enzymes NADH-ADH (Figure 2). The increase in ADH activities was observed in both root and leaf of the plants. In the roots, the magnitude of a marked increase in enzyme (NADH-ADH) demonstrated an increase of 5.9 fold (Figure 2A) whereas leaf NADH-ADH enzyme showed 4.4-fold (Figure 2B) in activity. In short the increase in the levels of the activity of the NADH-ADH enzymes was observed in the PEG-induced stress plants.

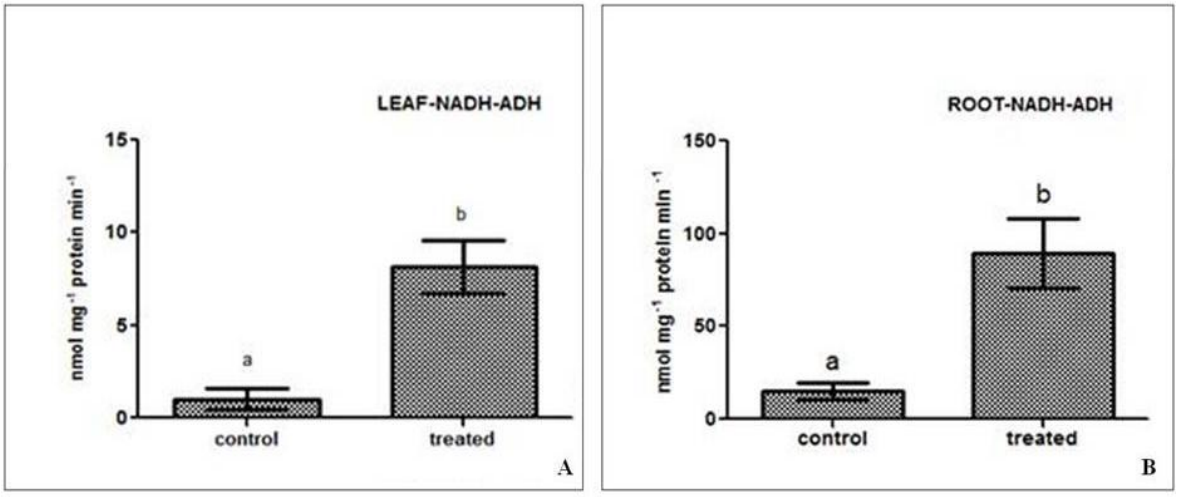

Figure 2. Effect of 5% (w/v) PEG on the NADH-ADH activities in leaves and roots of *Arabidopsis*. Four-weeks-old plants were slowly exposed to 5% (w/v) PEG. The fermentative enzyme activities of NADH-ADH in leaves (A) and roots (B) were measured following the treatment. Means ± SE were calculated from three independent experiments. Significant differences at $p < 0.05$ were indicated by different letters.

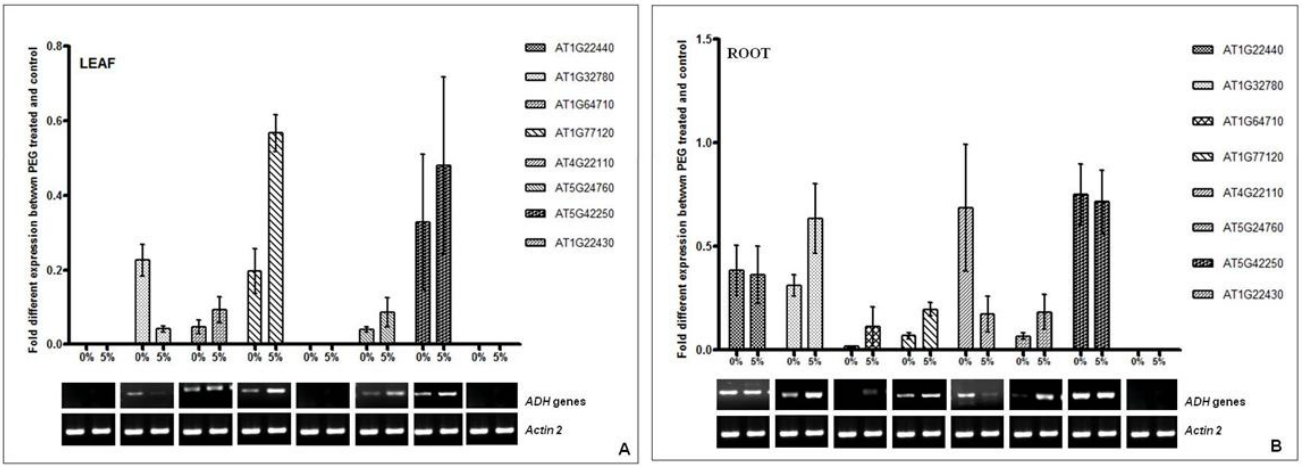

Figure 3. Expression patterns of the *ADH1* and *ADH-like* genes in the PEG-treated *Arabidopsis* plants. The levels of the transcripts were determined in the leaf (A) and in root (B). The transcript of the *Actin-2* gene was used as a loading control.

3.2. Expression Analysis of the *ADH* genes in Response to PEG- Induced Water Stress

To identify the *ADH* genes that are responded to PEG-induced water stress, the transcript levels of the genes were compared using RT-PCR. The samples used for the expression analysis were taken from the same experiment used for the enzyme and metabolites analysis experiment. The *Actin-2* gene was used a loading control as the expression of this gene was not affected by PEG-induced water stress condition and its transcript levels in the leaf and root were also constant (Figure 3). In general, the expression of the *ADH* genes varied in response to the treatment depending on organ and expression pattern. In the leaf, *ADH1* and three *ADH*-likes genes (AT1G77120, AT1G64710, AT5G24760, and AT5G42250) were up-regulated and one *ADH*-likes gene (AT1G32780) was down-regulated (Figure 3A). The transcript level of the *ADH1* gene (AT1G7712) was the highest among the expressed *ADH* genes. Interestingly, three of the *ADH-likes* genes (AT1G22440, AT4G22110 and AT1G22430) were not expressed in the leaf. In the root, a group of closely-related genes *ADH1* and three of *ADH-like* genes (AT1G77120, AT1G32780, AT1G64710, and AT5G24760) were up-regulated and only one (AT1G22110) was down-regulated (Figure 3B). Two of the *ADH-like* genes (AT1G22440 and AT5G42250) were not responded to the treatment as no changes were observed on their transcript levels after the treatment. Only one of the *ADH-like* genes (AT1G22430) was not expressed in the leaf and in the root. Together, *ADH1* and two of the *ADH-like* genes (AT1G77120, AT1G64710 and AT5G24760) were up-regulated in both roots and leaves of the PEG-induced stress plants (Figure3A and B).

4. Discussion

4.1. Exposing *Arabidopsis* Plants to 5% PEG Turned the Plant to Moderate Water Stressed State

Drought is a complex stress phenomenon that is difficult to model in any growth system. Various forms of drought induction system are applying in a growth system used to study plant response to drought including soil drying and hydroponics. In the present study, hydroponic system was chosen for the growth system because it allows easy access to the root system of the plant as well

as provides constant water potential throughout the whole root system. These features are hardly obtained if one uses soil drying. In soil drying, one common technical difficulty is to maintain uniform and constant water potential throughout the whole soil profile (Munns et al. 2010); for example, transmission of nutrient in the soil was reduced at low soil water potential (Nye and Tinker 1977), soil could easily become saturated at the bottom of a pot (Passioura 2006). Applying water stress in hydroponic is often used as a way of overcoming the problems of heterogeneity, drainage, and inconstant water potential (Munns et al. 2010). Therefore, hydroponics with constant aeration is a major scientific modeling tool for studying abiotic stress tolerant including drought (Shavrukov et al. 2012). However, without constant aeration, a hydrophonic system would become anaerobic especially at the root tips and this will affect plant metabolism and the way plant response to drought. The use of water stress agents to mock drought condition in plants is feasible because the stress agents "steal" water molecules from being taken up by plants, reducing the level of cellular water content in the plants, a condition similar to drought. In this study, water-limited stress was generated by using PEG as the water stress agent. Other chemicals like mannitol is sometimes used for the same purpose (James et al. 2008; Rahnama et al. 2010 and Rai et al. 2010) but PEG-20000 is the most common because this polymer is too large to enter the plant cells (Carpita et al. 1979 and Oertli 1985). Therefore, PEG was widely used in the study of various aspects of drought tolerance in plants (Verslues et al. 2006; Zhang et al. 2006 and Maruyama et al. 2008).

To evaluate the level of cellular water content in plants, various methods are available but the most commonly used is relative water content (RWC). Relative water content of a plant reveals the level of cellular water in the plant and it is widely used to quantify water deficits in leaf tissues (Oliver et al. 2010). In the present study, the RWC of the PEG-treated *Arabidopsis* plant dropped to 75% from the normal level of 85% as shown in the control. This reduction level caused the plant to be moderately water-stressed based on an earlier study on RWC of *Arabidopsis* plants (Gigon et al. 2004). The study characterized water-stressed states of *Arabidopsis* plants into four categories: mild, moderate, severe and very severe of water stress. The plant is considered in a mild state of water-stressed when the level of RWC reduced to 82% from the normal fully-watered plant. Further reduction of the RWC to 73%, 47% and 17% caused the plant to be moderately, severely and very severely water-stressed, respectively. Using this scale, the level of water stress in the plants treated with 5% PEG in the present study was moderate. Apart from monitoring the level of RWC, the status of the water-stressed plants can also be evaluated by measuring the stress parameters known to be affected by water stress. Common stress parameters are proline and chlorophyll. Drought stress decreases the plant photosynthesis (Kawamitsu et al. 2000) and chlorophyll content (Ommen et al. 1999) which is one of the biochemical markers used to indicate the photosynthetic capacity of a plant (Negeswara et al. 2001). Drought-related water stress inhibits plant photosynthetic processes as a result of damage occurring to chlorophyll and other photosynthetic apparatus (Iturbe Ormaetxe et al. 1998) caused by active oxygen species (Smirnoff and Cumbes 1998). In this study, the *Arabidopsis* plant exposed to 5% (w/v) PEG demonstrated lower chlorophyll content than the control, indicating a reduction in the capability of the stress plant to carry out photosynthesis under water-limited condition.

Another metabolite that is sensitive to limited-water conditions is proline. There were many studies showed that proline involved in cell osmo-regulation and acts as a compatible osmolyte which can protect subcellular structures from the impact of dehydration as well as stabilizing proteins, membranes and macromolecules (Vanrensburg et al. 1993; Hong et al. 2000 and Mafakheri et al. 2010) and maintaining NADP for reducing damage of photosynthetic apparatus during drought condition (De Ronde et al. 2004). In Addition it levels were also increased in water-stressed plants (Alexieva et al. 2001 and Choudhary et al. 2005). In the present study, the stressed *Arabidopsis* plant behaved the same way accumulating high levels of proline following the 5% (w/v) PEG treatment.

Together, the drop in the levels of RCW and chlorophyll content as well as the increase in proline strongly support the observation in the present study that *Arabidopsis* plants treated with 5% PEG was in a moderate state of water stress, and therefore, the plant was appropriately used for studying the effects of water-limited stress in *Arabidopsis* plants.

4.2. Enhanced Ethanolic Fermentation in the Water-Stressed Plants

One experiment carried out in this study was on the impact of water-limited stress to plant ethanolic fermentation which is the oldest metabolic pathway, surviving environmental changes billions of years ago when the world atmosphere was anaerobic. While the current atmosphere is no longer anaerobic, there are situations where anaerobic condition may be re-generated in the cells and their environment. For example, low level of oxygen or hypoxia may be generated during flooding depending on how deep the root system was submerged in water; occurring of ethanolic fermentation was common in the situation (Shiao et al. 2002; Tesniere et al. 2006 and Sairam et al. 2008). The pathway plays a vital role during this condition as it is the only way to generate NAD^+ for the continuation of glycolysis (Tadege 1999 and Agarwal et al. 2007). In plants, switching to the ethanolic pathway for energy generation is not limited to low oxygen level only, sometimes this also occurs in response to other stresses such as wounding (Kato-Noguchi et al. 2001), low temperature (Kato-Noguchi et al. 2006, 2007) and altering sugar metabolism during infection (Tadege 1998). In addition, ethanolic fermentation enhanced disease resistance by altering their sugar metabolism during infection, suggesting that fermentation might be an important switch to regulate carbohydrate metabolism under stress condition (Tadege 1999). All these evidences back the suggestion that ethanolic fermentation contributed in dynamic environmental conditions. Under different environmental stresses, the main enzymes of the pathways ADH and PDC seemed to response swiftly to any chemical or physical changes to their environmental conditions. Many studies that analysed the activities of the

two enzymes reached to the same general conclusion that ethanolic fermentative enzymes were sensitive to their environments. Physical and chemical changes in the plant growth environments would normally give the same observation such as from high to low temperature (Christie et al. 1991 and Kato-Noguchi et al. 2001), dehydration, ozone, wounding, and SO_2 treatment (Kimmerer and Kozlowski 1982; Dolferus et al. 1994; Desikan et al. 2001 and Seki et al. 2002).

Ethanolic fermentation in the *Arabidopsis* roots is particularly sensitive to drought stress condition as the PEG-induced water stress enhanced the activities of the ADH enzyme in this study. Roots sense the occurrence of low oxygen supply during when soil becomes compact as a result of dryness. Even 21% of limited oxygen concentration, ethanolic fermentative pathway occurs and ethanol could be produced by root tips (Kimmerer 1990). In addition, decreasing cellular water content of the root cells was normally accompanied with a reduction in water potential in the cells which dropped the cellular pH that made the cytoplasm acidic. More specifically, Fan and Neumann (2004) explained that cell wall pH was significantly lower within 0-3 mm from the root apex. This acidic environment triggered ethanolic fermentation (Roberts 1984; Wu et al. 1996; Fan and Neumann 2004). Therefore, these situations of root region under PEG-induced water stress may switch to the ethanolic fermentation and ADH enzyme activity was increased.

Back to what happened in the leaves of the water stressed plants of the present study. As mentioned earlier, the ethanolic pathway was greatly induced in response to PEG treatment which might be connected to the physiological response of the leaf and a synchronies transport of metabolites between the root and leaf organs. When a plant was drought stressed, the transpiration rate in the leaves of the plant was increased which led to the increase in the pH of leaf sap promoting ABA accumulation which would directly regulate the stomata movement (Chave et al. 2003 and Liu et al. 2004). In plants, ABA plays an important role for mediating stomatal behaviour at moderate water stress (Liu et al. 2004). Water-limited stress triggered a reduction in stomatal conductance which restricts the movement of gases between the leaf and its environment generating a hypoxic condition inside the leaf. As a result, ethanolic fermentation was triggered PEG-induced water stress and ADH activity was increased.

4.3. Enhanced Ethanolic Fermentation was Accompanied by the Up-Regulation of some *ADH* Genes

The expression of *ADH* genes were not only induced drought stress but also by many other stresses such as cold and osmotic stresses (Christie et al. 1991 and Conley et al. 1999), wounding (Kato-Noguchi et al. 2001), dehydration (Dolferus et al. 1994), low temperature (Peters and Frenkel 2004 and Kato-Noguchi 2007), abscisic acid treatment (de Bruxelles et al. 1996), water deficit (Senthil-kumar et al. 2010), low oxygen (Shiao et al. 2002). Similarly in this study, PEG-induced water stress also increased the transcripts of *ADH* genes.

The evidence of transcript level and the higher in the ADH activity in this study implied functioning of ethanolic fermentative pathway under the PEG-induced water limited condition. This ADH enzyme (EC.1.1.1.1) is encoded by their particular genes. From the *ADH1* and *ADH-like* genes, some were up-regulated in *Arabidopsis* plants grown under PEG-induced water stress. Together with *ADH1* (AT1G77120), another two *ADH-like* genes (AT1G64710, and AT5G24760) were induced in both root and leaf organs of the plant under the PEG treatment. It could be clearly seen that, *ADH1* was not the only *ADH1* (EC.1.1.1.1) genes related to increasing of ADH activity that was functioning in the leaf and root of the treated plant but rather a group of the closely-related *ADH-like* genes were responded contributing to the pathway's overall activity under the stress condition. These transcriptional evident of accompanied together in some *ADH1* and *ADH-like* genes could be a reason for enhancement ethanolic fermentation in root and leaf under PEG-induced water limited stress condition.

At the gene level, the promoter of a gene plays a crucial role in determining how a gene responds to their environment. The promoter of the *Adh* genes was reported to be induced by exogenous ABA treatment and suggested the essential role in proper responses to environmental stimuli in the root of *Arabidopsis* (Dolferus et al. 1994; de Bruxelles et al. 1996) and soybean (Preiszner et al. 2001). As endogenous ABA levels increase as a result of dropping cellular water content, some *ADH* genes that have ABA inducible promoters would respond to the stress, enhancing the ADH activity.

5. Conclusion

The induction level of the enzyme ADH was higher under PEG induced water limited condition. Therefore, it is suggested that ADH is responsive enzyme of the ethanolic fermentative pathway under PEG-induced drought stress condition in *Arabidopsis.* The enzyme, alcohol dehydrogenase (ADH) in the model plant *Arabidopsisis* encoded by *ADH1*. In this plant, there are other *ADH*-like genes which may contribute to the ADH enzyme activity. Not all responding to drought. Some are differentially-regulated in the sense that their activity varies depending on organ. The *ADH1* and two *ADH-like* genes responded to the stress both in roots and leaves of the treated plants. Hence, the evidences (increased mRNA transcripts and enhanced enzyme activities) implied that ethanolic fermentation was active in plants grown under drought stress condition and the high level of the enzyme indicate the existing of a mechanism to regulate this and the mechanism is associated with or part of the adaptation system the plant used in order to overcome drought stress.

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