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Enzymatic Activities and Gene Transcript Levels Associated with the Augmentation of Antioxidant Constituents during Drought Stress in Lettuce

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Abstract: Efficient cultivation methods were investigated to promote the branding of products in plant factories. Moderate stress can enhance plant constituents that are beneficial for human health, without reducing yield. Dehydration stress in lettuce rhizospheres increased some antioxidants, including L-ascorbic acid (AsA) and polyphenols. In this study, the major factors contributing to the augmentation of antioxidant constituents were investigated. The drought treatment resulted in increased hydrophilic oxygen radical absorbance capacity (ORAC) values but not hydrophobic ORAC values. Both activities of antioxidant enzymes (superoxide dismutase, SOD, and ascorbate peroxidase, APX) were elevated under drought conditions. RNA-seq analysis revealed 33 upregulated and 115 downregulated differentially expressed genes, and 40 gene ontology enrichment categories. A *dehydrin* gene was the most significant among the upregulated genes in response to drought stress. Dehydrin protects plant cells from dryness through multiple functions, such as radical scavenging and protection of enzymes. Real-time PCR validated the substantial increase in some *dehydrin* paralogs with root desiccation. In conclusion, the enhancement of antioxidant levels by drought stress is likely not due to the induction of antioxidant enzyme genes, but due to increased enzymic activities. These activities might be protected by dehydrins encoded by the upregulated paralogs under drought stress.

Keywords: ascorbate peroxidase; dehydrin; oxygen radical absorbance capacity; plant factories; rhizosphere; RNA-seq; superoxide dismutase

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1. Introduction

Recently, plant factories have become common in several countries. They produce many vegetables at a time and year-round; for example, 10,000 heads of lettuce in a day. The products are chemical-free, fresh, and clean [1]. Moreover, with growing public health awareness, nutritious and functional foods are expected by consumers and supplied by various plant factories. Since plant factories are equipped with artificial light and air conditioning facilities, environmental conditions can be controlled more tightly than in open-field culture and greenhouse farming. These closed conditions can guarantee the useful nutrients in the products. Severe environmental stresses inhibit the growth of plants, whereas moderate stresses can increase the various components expressing tolerance mechanisms. Some salubrious antioxidants, such as L-ascorbic acid (AsA), glutathione, alpha-tocopherol (vitamin E), and polyphenols, were enriched in response to oxidative stress brought about by environmental stimuli [2–5]. These compounds can detoxify reactive oxygen species in plants, but they also show a beneficial activity for human health.

Leafy greens are generally low in sugars compared to fruits. Sugars are efficient sweetening materials and carbohydrates, and an energy source. AsA, better known as vitamin C, was initially identified as the factor preventing scurvy and is effective in many diseases, such as cancer, cardiovascular disease, age-related macular degeneration, cataracts, and the common cold [6,7]. Polyphenols modulate factors such as gene expression, antioxidant function, and detoxification, and have an anti-inflammatory and anticancer activity [8,9]. Some leafy vegetables include nitrates, as an undesirable substance, at high concentration. The European Union has made regulations to limit the nitrate levels in foodstuffs [10]. In the human body, nitrate can be reduced into nitrite, which has the possibility of transforming into carcinogenic nitrosamines [11]. It has also been reported that nitrite may be responsible for methaemoglobin formation [12].

Kratky [13] developed a hydroponics systems without the circulation of a solution, by lowering the water level to give airspace to roots during the entire growth period. The optimization of exposure conditions in this system can impose drought stress on crops, assuming a use in plant factories. We found that the fresh weight of hydroponic lettuce remained steady for the first 7 days with 4 cm of airspace, but decreased significantly beyond 7 days [14]. This method could increase the AsA, polyphenol, and sugar content of leaf lettuce (*Lactuca sativa* L. var. *crispa*), without reducing yield. It could also decrease undesirable nitrate–nitrogen content. Furthermore, applying this treatment to four other leafy vegetables was effective for increasing AsA. Leaf lettuce is the most frequently used vegetable in plant factories, because of its ease of growing, based on its photosynthetic properties, growth rate, and compactness. The genome sequence project of lettuce has been completed [15], which brought comprehensive gene analysis using the reference genome.

Understanding the main reason for the augmentation of antioxidant constituents by drought stress will arrow us to apply cultivation techniques and achieve breeding targets. In this research, we investigated the enzymic activities and gene expressions related to the antioxidant mechanisms in lettuce. Functions other than antioxidants were also discussed, based on the genes differentially expressed under dehydration stress.

2. Materials and Methods

2.1. Plant Material

Leaf lettuce (*Lactuca sativa* L. var. *crispa*) 'Frillice' (Snow Brand Seed Co., Ltd., Hokkaido, Japan) was selected as a material, owing to its frequent use in plant factories.

2.2. Hydroponics and Drought Stress Treatment

Plants were grown and stressed as described in Koyama et al. [14]. Briefly, 5-day seedlings were transferred to a hydroponic system using Otsuka-SA nutrient solution with EC of 1.8 $mS \cdot cm^{-1}$ (OAT Agrio Co., Ltd., Osaka, Japan) and cultivated for an additional 23 days. The drought stress was given to the rhizosphere by lowering the water level by 4.0 cm in the solution tub for 7 days. The solution was continuously aerated with an air pump. The growth conditions were maintained in a thermostatic room under a 14 h light/10 h dark photoperiod with cool-white fluorescent lamps (approximately 140 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photosynthetic photon flux) at 20 °C, and subjected to rhizosphere drought stress before harvest.

2.3. Assay of ORAC

ORAC values were measured using an OxiSelect ORAC Activity Assay (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacturer's protocol; 0.5 g frozen leaves were homogenized with 1 mL water, and the homogenate was centrifuged at 12,000× *g* for 10 min at 4 °C. The supernatant was recovered, and the insoluble pulp was washed with 0.5 mL water. The wash was combined with the previous supernatant after centrifugation at 12,000× *g* for 10 min at 4 °C. The pooled supernatant was adequately diluted and used for the hydrophilic fraction in the assay. The pulp was further extracted by adding four times (*w*/*v*) its volume of pure acetone and mixing at room temperature for 50 min.

The extract was centrifuged at 12,000× *g* for 10 min at 4 °C, and the acetone extract was recovered and directly used for the hydrophobic fraction in the assay. These extracts were stored at −80 °C before performing the assay; 25 µL extract or antioxidant standard was thoroughly mixed with 150 µL fluorescein probe solution in a 96-well microtiter plate, and it was incubated at 37 °C for 30 min; 25 µL free radical initiator solution was added to each well start the reaction. Immediately, the mixture reaction was started, by analyzing the fluorometric with an excitation wavelength of 480 nm and an emission wavelength of 520 nm at 37 °C, using a fluorescence microplate reader (SH-9000Lab; Corona Electric Co. Ltd., Hitachinaka, Japan). The data were read in increments of approximately 3 min for a total of 60 min. Trolox antioxidant standard curves were prepared by measuring the solution in water or 50% acetone. Each ORAC value in the hydrophilic fraction and hydrophobic fraction can be calculated from the results of the fluorometric analysis, and those results were combined as the total ORAC value.

2.4. Determination of Antioxidative Enzyme Activities

For obtaining the enzyme extracts, 1 g of frozen leaves was homogenized with 2.5 mL of 25 mM potassium phosphate buffer, pH 7.8, containing 0.4 mM EDTA, 1 mM AsA, and 2% (*w*/*v*) polyvinylpolypyrrolidone. Homogenates were centrifuged at 9000× *g* for 20 min at 4 °C, and the supernatants were used for enzymatic determination after filtering through four layers of gauze. SOD activity was determined using a SOD Assay Kit—WST (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's protocol [16]; 200 µL WST working solution and 20 µL enzyme working solution supplied by the manufacturer was added to 20 µL enzyme extracts and incubated at 37 °C for 20 min. Then, the absorbance was measured at 450 nm using an absorption spectrophotometer (UV-160A). The fifty percent inhibition activity of SOD could be calculated from the results of the absorbance. APX was measured via a modification of the method of Nakano and Asada [17]. Fifty-microliter enzyme extracts were blended with 940 µL of 25 mM potassium phosphate buffer, pH 7.0, containing 0.1 mM EDTA, and 0.25 mM AsA. The oxidation reaction of ascorbate was started by adding 10 µL 10 mM H_2O_2, and the decrease in 290 nm absorbance value was measured using an absorption spectrophotometer (UV-160A). APX activity was calculated from the results of the absorbance using an extinction coefficient of 2.8 $mM^{-1} \cdot cm^{-1}$. The protein content of the enzyme extracts was measured using Protein Assay CBB Solution (Nacalai Tesque Inc., Kyoto, Japan), with bovine serum albumin as a standard; 2.5 mL CBB solution diluted to one-fifth with water was mixed with 50 µL enzyme extracts or standard and was left to stand for 10 min at room temperature. Then, the absorbance was determined at 595 nm using an absorption spectrophotometer (UV-160A).

2.5. RNA Extraction

RNA was extracted automatically with a Maxwell 16 Automated Purification system (Promega, Madison, WI, USA), as described in Ishibashi et al. [18]. Frozen leaves were pulverized with a multibead shocker (Yasui Kikai, Osaka, Japan). Then, 100 mg of powdered sample was used for extraction, according to a manual of Maxwell RSC Plant RNA kit (Promega) and using the Maxwell purification system "RNA-PLANT" protocol. The resulting RNA samples were stored at −80 °C.

2.6. RNA-Seq Analysis

Biological repetitions with three totally independent RNAs were bulked for each treatment. The total bulked RNA was submitted to a custom service (GENEWIZ Japan, Tokyo, Japan) for cDNA library preparation and sequencing reactions. A standard cDNA library was prepared with polyA-selected mRNA. The cDNA libraries were sequenced on the HiSeq platform (Illumina, San Diego, CA, USA), in a 2 × 150 bp paired-end configuration, with 6.0 Gb pF data per sample. Bioinformatic analysis was performed using scientifically recognized algorithms by GENEWIZ, Inc. Filtered short sequence reads were mapped to the lettuce reference database; lactuca sativa v8 supplied from "Lettuce Genome

Resource" website at UC Davis genome center. Fragments per kilobase per million read (FPKM) were calculated on the basis of read counts from HT-seq (V 0.6.1) [19]. Both the *p*-value and *q*-value (false discovery rate) between differential expressions were calculated. Differentially expressed genes (DEGs) were determined by the criteria of a fold change greater than 1.5 and a *q*-value of less than 0.05. Phylogenetic trees were generated using the CLC Main Workbench (version 8.1) sequence analysis program (QIAGEN Aarhus A/S, Aarhus, Denmark).

2.7. Real-Time PCR

Quantitative real-time PCR was performed according to Ishibashi et al. [18], with slight modifications. According to the manufacturer's protocol, cDNA was synthesized using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan) from 100 ng of total RNA. Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) using Light Cycler 480 (Roche Diagnostics, Basel, Switzerland). Primers were designed for *LsDHN3* (F:TGATTGTGCAGTCGGAGGAT, R:GCTCATCTCCACCAGGAAGC), *LsDHN5* (F:CCACTTCACTCTACCACCGA, R:CCAATGTTGCTGCTGGACAA), *LsDHN12* (F:AAGAGAAGCTTCCTGGACATCA, R:ACATGGCTTGTGACCCATCA), and *LsDHN4* (F:GGCCACCATGGAGTTAGTGG, R:GGGGGACGCTTTGATGCTC), and Actin (F:ACCCTGTTCTTCTCACTGAG, R:CAGCCTGGATAGCAACATAC). The thermal cycling conditions were as follows: 95 °C for 30 s, three-step amplification with 40 cycles of 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s, premelt holding at 95 °C for 10 s, and melting at 58 °C–97 °C at 0.1 °C/s. All data were normalized against each actin transcript level.

3. Results and Discussion

3.1. Effect of Drought Stress on the Biosynthesis of Antioxidants

Lettuce roots were exposed to the air during the last 7, 9, 12, and 14 days of the culture period (Figure S1). A gradual decrease in fresh weight was observed as the number of exposed days increased. The upper part of the root area began browning in all the treatments, except the control. From these results we confirmed that the drought stress was given to the lettuce by exposing the roots to the air. Under optimized drought conditions (7 days of drought treatment with 4 cm of air space), both AsA and polyphenols were increased without a significant yield reduction [14]. Drought stress in the roots triggers oxidative stress in the whole plant, because of changes in the root absorption and leaf photosynthesis rates [20]. Since oxygen radical absorbance capacity (ORAC) values are the index for showing antioxidant activity, they were compared between the control and drought-stressed samples. Total ORAC values were composed of more than 90% hydrophilic and less than 10% hydrophobic ORAC values (Figure 1). The hydrophilic ORAC value was significantly increased with desiccation. However, hydrophobic ORAC values showed no significant difference between the control and drought-stressed plants. In lettuce, the major hydrophilic antioxidants are phenolic acid and AsA, while the hydrophobic antioxidants are carotenoids and alpha-tocopherol (vitamin E) [21]. It has been reported that the amount of hydrophilic anti-oxidants is higher than that of hydrophobic anti-oxidants. This is consistent with our drought conditions, where both polyphenols and AsA were enriched [14]. Another study also showed that chicoric acid is increased by drought stress in lettuce, but alpha-tocopherol is not [22]. To understand the effect of antioxidative enzymes, SOD (superoxide dismutase), and APX (ascorbate peroxidase) on ORAC values, each activity was measured using both control and drought-stressed samples (Table 1). Consequently, the dehydration treatment significantly activated both SOD and APX by 1.12 and 1.36 times when compared with the control, respectively ($p < 0.01$). This was consistent with the increased polyphenols and AsA under drought conditions [14]. Some reports stated that SOD activity is affected by drought stress and whether it rises or falls depends on the species, growth stage, and the stress period [23,24]. Superoxide dismutase catalyzes the dismutation of superoxide radicals to oxygen and H_2O_2 [25]. In lettuce subjected to drought stress by lowering the water level by 4 cm for 7 days, the defense mechanism of SOD might

work against toxic reduced oxygen species. APX activity is increased in response to various biotic and abiotic stresses in several plant species [26]. The drought stress treatment of the roots in this study made H_2O_2; detoxified using ascorbate for a reduction reaction as a function of APX, and as reported by Jaleel et al. [25]. Lettuce plants might activate this antioxidant mechanism in response to dehydration stress by increasing substances and enzymatic activities to protect themselves from injury by reactive oxygen species.

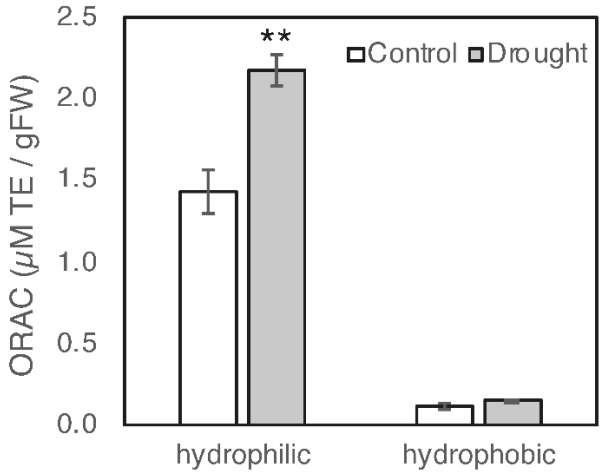

Figure 1. Effects of drought stress on the value of oxygen radical antioxidant capacity (ORAC) in lettuce. Each seedling was treated by lowering the water level by 0 cm (control, white bars) or 4 cm (drought stress, gray bars) for 7 days. ORAC value in the hydrophilic fraction (**left**) and a hydrophobic fraction (**right**) was measured using Trolox equivalent to the standard. The vertical bar indicates a standard error of nine replicates of the total ORAC value. ** Significant differences between control and drought stress at $p = 0.01$ with Student's *t*-test.

Table 1. Effects of drought stress on the activities of enzymes for biosynthesis of antioxidants.

Component	SOD (Units/mg/Protein)	APX (μmol/mg Protein/min)
Control	148.82 ± 3.51 [1]	0.014 ± 0.00072
Drought	167.10 ± 3.87 ** [2]	0.019 ± 0.00087 **

[1] Data represent the mean (±standard error; $n = 6$). [2] ** Significant differences at $p = 0.01$ with Student's *t*-test.

3.2. Effect of Drought Stress O Then Transcription of Genes Related to Antioxidant Mechanisms

Transcriptome analysis was invoked to understand the simultaneous expression of genes, including *SOD* and *APX*, during the drought treatment. RNA-seq resulted in a total of 42.6 million (control) and 47.5 million reads (drought stress), whose mapping rates were 93.8% and 93.3%, respectively. Mahmoudi et al. [27] reported the expression of *SOD* genes encoding three isozyme groups in lettuce: Fe-SOD, Mn-SOD, and Cu/Zn-SOD. The protein sequences of LsSODs were obtained from a lettuce genome database (*Lactica sativa* v5.0 genome) using the BLASTP search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 21 October 2021)) and using amino acid sequences of eight AtSODs (AF061518, M55910, Y12641, AF061852, X60935, AF061519, AF061520, and AT1G12520) as queries (Table S1). The candidate LsSOD sequences were confirmed by searching copper/zinc and iron/manganese SOD domains via the PfamScan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/ (accessed on 21 October 2021)). Eleven *SOD* gene paralogs comprised three *Mn-SOD* (LsMSD), four *Fe-SOD* (LsFSD), three *Cu-Zn SOD* (LsCSD), and one *Cu-SOD* (LsCCS) in the lettuce genome (Figure 2A). None were upregulated by drought stress conditions with a significant difference, according to the criteria of $\log_2$ fold change greater than 1.5 and a *q*-value of less than 0.05 (Figure 2B). This result showed a similar tendency to salt-stressed lettuce without clear induction by NaCl [27]. Regarding

APX genes, 10 paralogs were found in the BLAST search using eight AtAPX amino acid sequences (AT1G07890, AT3G09640, AT4G35000, AT4G09010, AT4G35970, AT4G32320, AT1G77490, and AT4G08390) as queries (Figure 3A; Table S1). All candidate LsAPX sequences were assessed for the presence of the conserved Haem-peroxidase domain (IPR002016) using InterProScan (http://www.ebi.ac.uk/interpro/ (accessed on 21 October 2021)), and peroxidase domain (PF00141) using the PfamScan. Almost all transcriptions of *APX* genes were not significantly increased by drought stimulus when compared with the control (Figure 3B). These *SOD* and *APX* genes might have been expressed constantly, or otherwise increased early in the treatment. Most of the *SOD* and *APX* genes were not induced after three hours in osmotic stress with polyethylene glycol (data not shown). The promoter region of these genes might not have *cis*-elements by which the transcription factors could upregulate the expressions under drought stress. However, the transcript levels were not drastically reduced under desiccation stress, so the amounts of the two proteins should have been maintained. These results suggest that the increased enzymatic activities of both SOD and APX are posttranslational, rather than transcriptional, during stress adaptation.

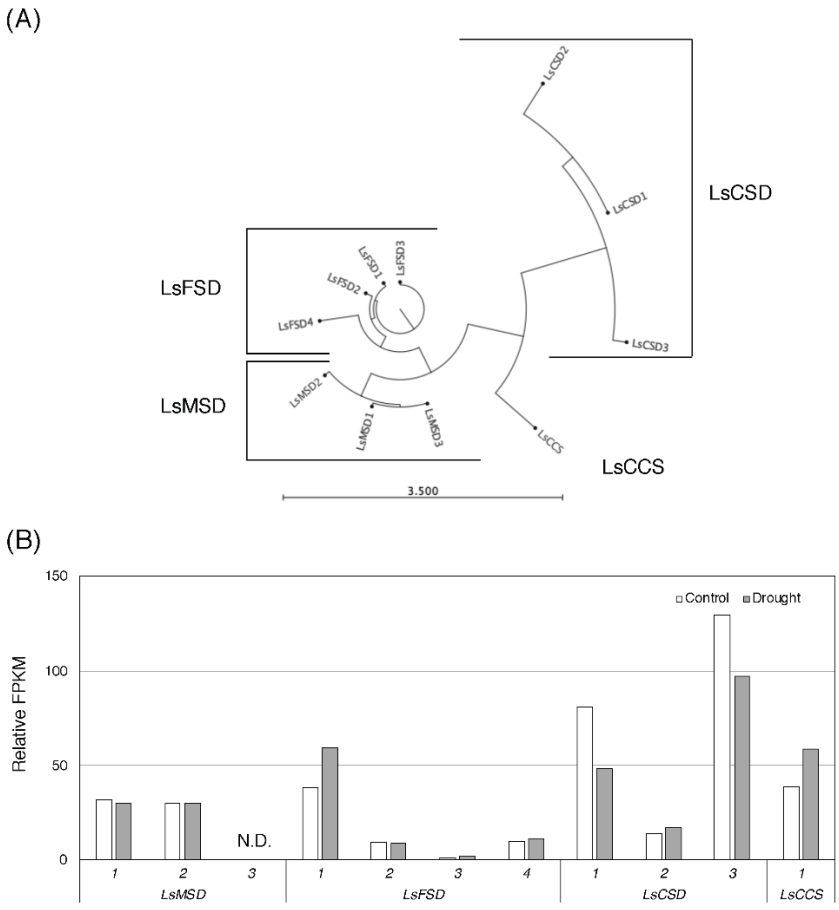

Figure 2. Phylogenetic tree of SOD and relative expression of *SOD* genes during dehydration in lettuce. The protein sequences of 11 SODs were obtained from a lettuce genome database (*Lactuca sativa* v8.0 genome), and a phylogenetic tree was constructed (**A**). SODs were divided into four groups: Mn-SOD (LsMSD), Fe-SOD (LsFSD), Cu-Zn SOD (LsCSD), and Cu-SOD (LsCCS). The relative expression of *SOD* was compared via RNA-seq (**B**). The expression levels of *SOD* were evaluated with the fragments per kilobase per million reads (FPKM) between the control (white) and drought stress (gray). Each relative FPKM was calculated on the basis of the control treatment of *LsFSD3*.

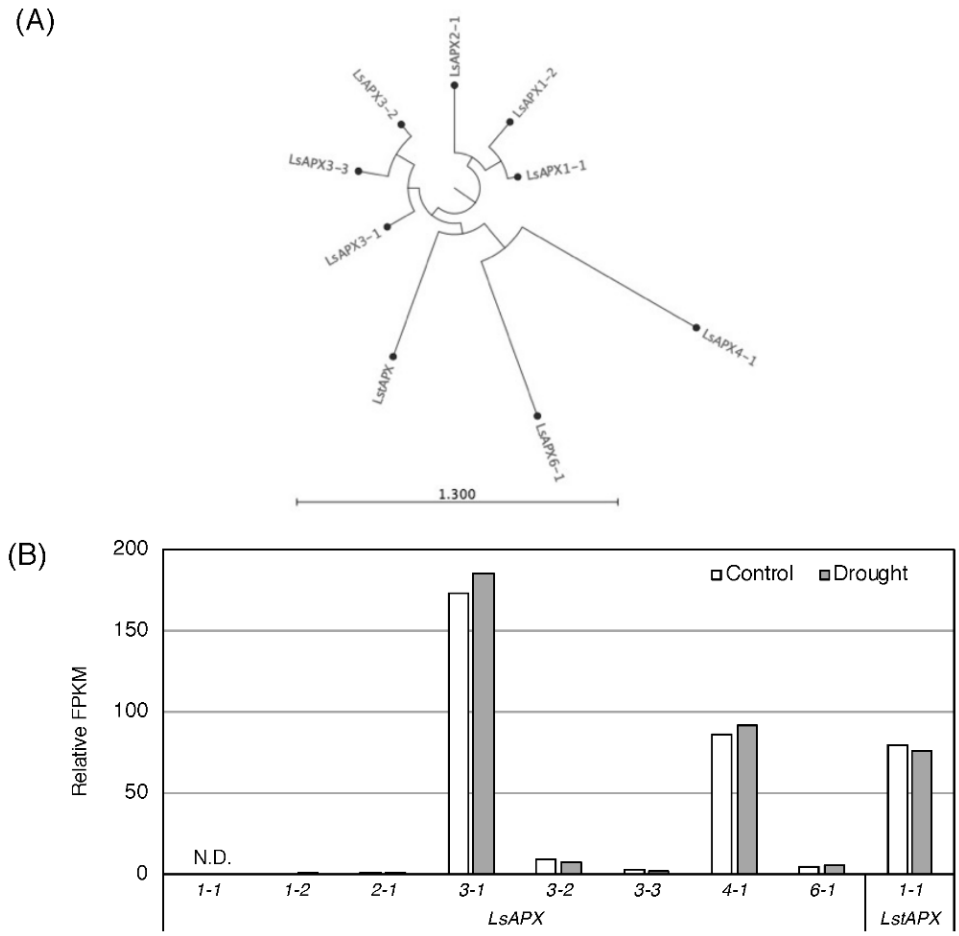

Figure 3. Phylogenetic tree of APX and relative expression of *APX* genes during dehydration in lettuce. The protein sequences of 9 APX were obtained from a lettuce genome database (*Lactuca sativa* v8.0 genome), and the phylogenetic tree was constructed (**A**). The relative expression of *APX* was compared via RNA-seq (**B**). The expression levels of *APX* were evaluated with fragments per kilobase per million reads (FPKM) between the control (white) and drought stress (gray). Each relative FPKM was calculated on the basis of the control treatment of *LsAPX2-1*.

3.3. Differentially Expressed Genes under Drought Conditions

Focusing on the differentially expressed genes (DEGs), 33 upregulated and 115 downregulated genes were found, with significance according to the criteria of $\log_2$ fold change greater than 1.5 and a *q*-value of less than 0.05 (Table S2). Table 2 extracts the DEGs from both the top 5 rankings of upregulation and downregulation. Gene ontology enrichment (GO) analysis picked out 40 GO categories with p-value ≤ 0.05 (Figure S2 and Table S3). The major GO categories corresponding to the three ontology groups were O-methyltransferase activity (molecular functions), oxidation-reduction process (biological process), and microtubule (cellular components). Jasmonate O-methyltransferase (JMT) is an enzyme that catalyzes the methylation of jasmonate into methyljasmonate. *JMT* gene could respond to signals produced in response to environmental stimuli [28]. *OsJMT1* was upregulated by drought stress to produce methyljasmonate, leading to a reduction of rice grain yield [29]. On the contrary, two *JMT*s were decreased under drought stress at seven days in our study (Table 2). This might be due to differences, such as stress periods and vegetative/reproductive organs. Regarding the oxidation-reduction process as the second GO category, it was difficult to predict its mechanism under stress, because so many types of enzymes were included in this category. The third GO category was microtubule, which has been reported as a sensor for stress perception [30]. The other categories that could be interpreted with up/down in DEGs were nitrate reductase (molecular functions)

and response to water (biological process). Each category included only one DEG. *Nitrate reductase* (*NR*) gene was the fourth ranking downregulated DEG, with −3.26 $\log_2$ fold change. A decreased transcription of *NR* after dehydration treatment has been found in maize [31]. These support the result of a significant decrement of nitrate–nitrogen content by drought stress in lettuce rhizosphere in our previous report [14]. Regarding the DEGs in the GO category 'response to water', *Dehydrin* gene (Lsat_2_78000) ranked first, with a considerable value of 5.0 $\log_2$ fold change. Dehydrin belongs to the group 2 late embryogenesis abundant protein family, and its encoding genes are known for their stress-responsive function [32]. Dehydrin protects plant cells from stress with multiple functions, such as binding to phospholipids, radical scavenging, binding to water and ions, phosphorylation, binding to calcium, protection of enzymes, binding to cytoskeletons, and binding to nucleic acids [33]. As an illustration, the overexpression of a *dehydrin* gene demonstrated both the promotion of SOD activity and a decrease of H_2O_2 compared to the wild type in *Ipomoea pes-caprae* [34]. The expression profiles of lettuce *dehydrin* paralogs were compared with those of the control and drought stress treatments (Figure 4). A total of 15 genes, including Lsat_2_78000 (*LsDHN3*) were discovered to be *dehydrin* paralogs (Figure S1). Of them, eight genes showed increased expression with drought stress. On the basis of a *p*-value of $\leq$0.05, four genes (*LsDHN3*, *LsDHN4*, *LsDHN5*, and *LsDHN12*) were selected for the verification of drought-induced expression with real-time PCR. Both *LsDHN3* and *LsDHN12* confirmed their upregulation by drought treatment (Figure 5). *LsDHN3* has been reported as *lslea 1*, which accumulated in lettuce roots under drought-stressed conditions, when compared to the well-watered condition in a pot experiment [35]. This experiment was conducted for 10 days; considering 7 days of our drought treatment, *LsDHN3* could sustain its expression with long-term stress. Furthermore, it became clear that *LsDHN3* accumulates, not only in roots, but also in leaves' edible portions, against drying stress. This suggested the possibility that dehydrin protects lettuce from desiccation and enhances antioxidant enzymatic activities via its multiple functions. Although the function of *LsDHN3* in drought stress needs to be confirmed by a follow-up study, such as overexpression or knockout plants, this gene could be a suitable marker of stress level, to improve nutritional attributes in lettuce.

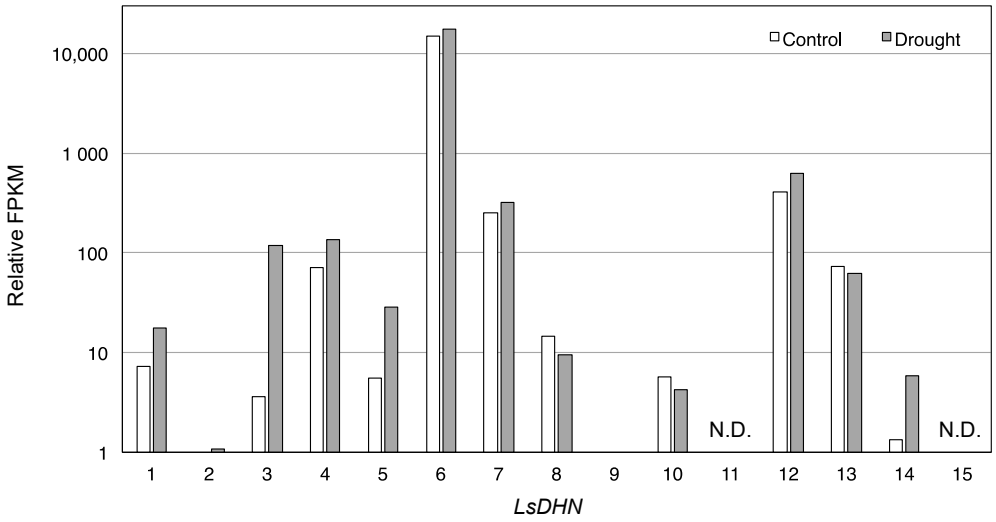

Figure 4. Relative expression of *DHN*s during dehydration in lettuce. The relative expression of *DHN* was compared via RNA-seq. The expression levels of *DHN* were compared with the fragments per kilobase per million reads (FPKM) between the control (white) and drought stress (gray). Each relative FPKM was calculated on the basis of the control treatment of *LsDHN9* and plotted on the axis of a logarithmic scale.

Table 2. Each top-five upregulated and downregulated gene with 7 days' drought stress in lettuce identified via the RNA-seq analysis.

Gene ID	$\log_2$ Fold Change	*q*-Value	Description
Lsat_2_78000	5.01	5.6×10^{-15}	Dehydrin Rab18-like [1]
Lsat_1_129340	4.85	2.8×10^{-4}	Stigma-specific STIG1-like protein 1
Lsat_9_34320	3.72	2.0×10^{-4}	Laccase-7-like
Lsat_7_49380	2.58	1.7×10^{-10}	Berberine bridge enzyme-like 8
Lsat_4_56281	2.55	1.2×10^{-5}	Uncharacterized
Lsat_2_79621	−3.80	2.5×10^{-5}	Arylacetamide deacetylase
Lsat_9_13940	−3.60	3.4×10^{-9}	Expansin
Lsat_7_10721	−3.40	4.8×10^{-19}	Jasmonate O-methyltransferase
Lsat_7_94901	−3.26	2.7×10^{-4}	Nitrate reductase
Lsat_1_16900	−3.15	1.5×10^{-6}	Jasmonate O-methyltransferase

[1] The results from the RNA-seq were further analyzed to determine the top-5 genes with significantly differential expression according to the criteria of fold change greater than 1.5 and a *q*-value (false discovery rate) of less than 0.05.

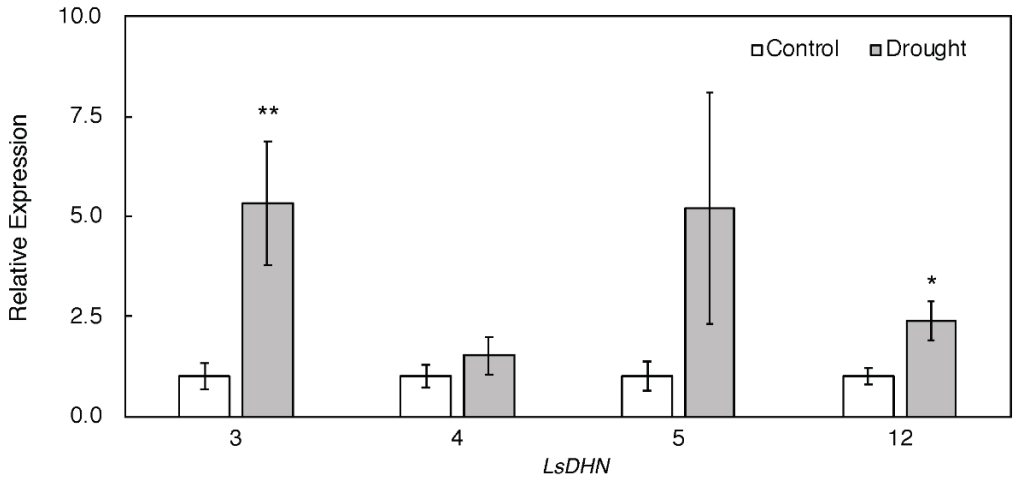

Figure 5. Verification of gene transcript levels of *DHN*s in lettuce using real-time PCR. RNA was extracted from non-stressed (white bars) or drought-stressed harvests (gray bars). The relative transcript levels of *LsDHN3*, *LsDHN4*, *LsDHN5*, and *LsDHN12* were normalized against that of the actin gene. Control levels were adjusted to 1.0. Vertical bars represent the standard errors of three biological and four technical replicates. The asterisk indicates a significant difference when compared with the control (** $p < 0.01$, * $p < 0.05$; Student's *t*-test).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7110444/s1, Table S1: List of identified genes by homology search, Table S2: List of differentially expressed genes, Table S3: List of GO enrichment categories, Figure S1: Drought stress treatment on lettuce by root exposure to the air, Figure S2: GO enrichment histogram by 7 days' drought stress in lettuce identified via the RNA-seq analysis.

Author Contributions: R.K., H.I. and Y.U. participated in the design of the experiments, interpreted the data. R.K. and Y.U. wrote the original draft. R.K., A.Y., M.I., H.I. and Y.U. reviewed and revised the manuscript. R.K. grew and treated the plant materials. R.K. and Y.U. assayed antioxidant enzymes. R.K., A.Y., M.I. and Y.U. analyzed their transcripts. R.K., A.Y., M.I. and Y.U. carried out in silico analysis. All authors have read and agreed to the published version of the manuscript.

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