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ORIGINAL ARTICLE

Oil palm leaves and roots differ in physiological response, antioxidant enzyme activities and expression of stress-responsive genes upon exposure to drought stress

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Abstract The seedling stage is a critical period for survival under drought stress. To identify biochemical and molecular drought response changes, oil palm seedlings were exposed to different levels of drought severity. Total chlorophyll, total soluble protein and total proline content were measured while expression of stress responsive genes was quantified using qPCR. The diminishing total chlorophyll (chl) content and the ratio of chl_a to chl_b (chl_a:chl_b) were observed to be significant. The significant reduction of chl_a was closely related to photosystem II deficiency. Based on the effects of drought on chlorophyll content, the samples can be categorised into mild (7 days of water withholding; DWW), moderate (14 DWW) and severe (21, 28 and 35 DWW). Sample at 21 DWW was used to represent the severe stage. Genes encoding ethylene responsive binding protein, late embryogenesis abundant (*LEA*), dehydrin (*DHN*), cold-induced, heat shock protein 70 and metallothionein type 2 were differentially up-regulated in the leaves, while in the roots only *LEA* and *DHN* were up-regulated. The proline content increased gradually in both vegetative tissues, while the total soluble protein content was affected by increasing drought severity. The activity of catalase was highest in the roots at the severe drought stage, while guaicol peroxidase activity was shown to be highest in the leaves under mild drought. These findings provide new insights into stress tolerance mechanisms of oil palm seedlings and can be used to develop stress tolerant oil palm through classical breeding and genetic engineering.

Keywords *Elaeis guineensis* · Drought · Transcription factor · Stress responsive genes (SRGs) · Enzymatic and non-enzymatic antioxidant · Vegetative tissues

Introduction

Since the 1970s, drought intensity, duration and affected areas have shown an increasing trend every year. At the same time, the percentage of areas in the world that are subjected to extreme drought is expected to rise from 1 to 30 % in the twenty-first century as a consequence of global warming and the *El Nino* phenomenon (Wang et al. 2014). Drought leads to significant crop yield losses and increased risk of forest fires. Further, it can exacerbate and intensify land degradation and desertification (Hillel and Rosenzweig 2002; Wang et al. 2014). This can be catastrophic if it happens in oil crop growing regions, which include crops such as oil palm, soybean, rapeseed, sunflower, olive and corn.

Vegetable oil is essential in many food preparations and serves as the raw material in the production of various food and non-food products. Among the oil crops, oil palm

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which is grown in tropical countries like Malaysia and Indonesia has the distinction of being the highest producer of vegetable oil (Hadi et al. 2015). The oil palm tree needs full sunlight and sufficient water for optimum growth and production. In the field, the water source is mainly rainfall. The optimum annual rainfall for the palm to achieve maximum production and yield is 1800 mm. If rainfall is below this level, the palm becomes susceptible to water deficits, which cause vegetative damage under mild and moderate drought stress conditions and ultimately death when the drought stress condition is severe (Nodichao et al. 2011). Such a scenario would have a negative impact on oil palm plantations. It is, thus, necessary to know the mechanisms involved in drought stress response for sustainable agricultural development.

Photosynthesis is a key process in primary metabolism as the process can fix CO_2 gas from the atmosphere and it is the main source of carbon in the plant biological system. Under drought stress, photosynthetic activity decreases as CO_2 diffusion to the chloroplast decreases resulting in metabolic limitation. Numerous in vivo studies established that drought may impair and damage the oxygen evolving complex, photosystem II (PSII), and degrade the D1 protein. The D1 protein is the reaction centre protein in PSII and it has the ability to bind to chlorophyll P680, pheophytin, β-carotene and manganese (Huseynova 2012). Diminishing photosynthetic apparatus, like the chlorophyll pigment, can lead to decreasing photosynthetic activity. The effects of drought on the photosynthetic apparatus and its activity have been extensively studied in various plant species, including maize (Shao et al. 2010), soybean (Hao et al. 2010), strawberry (Caulet et al. 2014) and wheat (Zivcak et al. 2014). Photosynthetic performance has become a very informative physiological indicator due to its extreme sensitivity to environmental stress. The classical measurements of photosynthesis by gas exchange and chlorophyll analysis are now widely used methods in the study of plant response to various environmental stress conditions (Massacci et al. 2008).

Reduction in photosynthesis may result in excess light excitation energy leading to photo-oxidative damage. Excessive excitation energy in PSII can result in impairment of photosynthetic functions and accumulation of reactive oxygen species (ROS), culminating in oxidative stress (Huseynova 2012). To overcome such situations, Harb et al. (2010) reported that when faced with drought conditions, plants exhibit either drought escape or drought resistance mechanisms, where the latter includes drought avoidance and drought tolerance mechanisms. Drought escape is described as the ability of plants to complete their life cycle before severe stress sets in. Drought avoidance is the mechanism by which plants maintain their high tissue water potential in confronting with drought. This includes modification of root traits, reduction of water loss through reduced epidermal conductance and reduced evaporative surface through leaf area. Drought tolerance refers to the plant's ability to withstand water deficit with low tissue water potential. However, plants have also been known to develop other mechanisms, such as, production of compatible solutes such as proline and glycinebetaine, accumulation of enzymatic antioxidants such as catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) and non-enzymatic antioxidants such as ascorbate and glutathione (Anjum et al. 2012; Sekmen et al. 2014).

Previous reports showed the involvement of different stress responsive genes (SRGs) which play different roles in plant stress responses. Altered expression of various kinds of SRGs such as *LEA*, *HSP70*, *MET*, *cold-regulated* (*COR*) and *ABA-responsive element binding* (*AREB*) transcription factor is one of the key mechanisms in plants to adapt in extreme environmental conditions (Liu et al. 2014; Khan et al. 2015). HSP70 protein functions as a molecular chaperone in protein folding, protein transportation across the plasma membrane, regulation in protein degradation and the prevention of irreversible protein aggregation (Chen et al. 2013). LEA is involved in membrane stabilization and preventing crystallization of cellular components in extreme conditions. Dehydrin belongs to the Late Abundant Class 2 (LEA 2) protein (Liu et al. 2013; Sasaki et al. 2013) and is known to protect the plant's protein from denaturation under unfavorable conditions (Vaseva et al. 2014). Metallothionein is involved in repairing DNA damage, regulation of metal ions homeostasis and involved in ROS scavenging in extreme conditions (Nishimura et al. 2013). CI is a member of COR proteins which are widely expressed at low temperatures and under freezing conditions (Wanner and Junttila 1999).

To gain a clear understanding of the reactions exhibited by oil palm seedlings under different levels of drought stress, we conducted a study with pigment and proline composition, total soluble protein content, antioxidant enzyme activity and gene expression as biochemical–molecular indicators in response to different levels of drought in the root and leaf tissues. These results may help in the development of a new variety of stress tolerant oil palm plants.

Materials and methods

Plant materials

Drought stress treatment was carried out using 3-month-old *tenera* (Dura × Pisifera) oil palm seedlings. The seedlings were bought from the Sime Darby R&D Centre, Banting,

Selangor. They were left to acclimatize for 2 months in the Glasshouse, Universiti Putra Malaysia, before being subjected to the drought treatments. They were watered every day, while, fertilizer was given every fortnight. Acclimatization and drought treatment was conducted at ambient temperature.

Drought treatment of the oil palm seedlings

For drought treatment, 5-month-old oil palm seedlings were used. The seedlings were grown in black plastic polypot (5 l, 17.78 cm diameter) containing clay soil. Water was withheld from the seedlings for 7, 14, 21, 28 and 35 days, except for the control seedlings. The control seedlings were watered every day. The roots were cut from the base of the stem, while the leaves were cut from the petiole. Both leaves and root tissues were washed with tap water to remove the soil and dirt. Then they were further cleansed with 2 % Clorox solution and rinsed three times with distilled water.

The experiment was carried out using completely randomized design (CRD). Each treatment and control comprised 5 plants. The same plants were used for all analysis.

Estimation of chlorophyll content

Leaf samples (0.5 g) were ground using a mortar and pestle in 10 ml of 80 % (v/v) acetone. Calcium carbonate (0.5 mg) was added to prevent the formation of pheophytin and the extract was filtered using Whatman paper No. 1. The excess samples were washed with acetone until the samples became colourless. The extracts were combined and made up to 20 ml and the absorbance was read at 645 nm (the maximum wavelength for chl_b in acetone extract) and 663 nm (the maximum wavelength for chl_a in acetone extract). The content of chl_a, and chl_b were calculated using the method established by Harborne (1973).

Estimation of proline content

The proline content was determined according to the method described by Bates (1973) with minor modifications. Five hundred milligrams of leaves and roots, respectively, was homogenized in 10 ml of aqueous sulfosalicylic acid. The homogenate was centrifuged at high speed of 13,362×*g* for 30 min to separate the supernatant and biological debris. Two millilitres of clear supernatant were extracted and reacted with a mixture of 2 ml ninhydrin solution and 2 ml glacial acetic acid, in a test tube. The reaction mixture was incubated for 1 h at 100 °C. After 1 h the reaction mixture was incubated on ice for 5 min to terminate the reaction. Four millilitres of toluene were then added to the mixture and mixed vigorously by pipetting up and down. The chromophore containing toluene was then aspirated from the aqueous phase and warmed to room temperature. The absorbance reading was taken at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis.

Crude protein extraction and total soluble protein estimation

Crude protein was extracted using a method described by Ozturk and Demir (2002) with minor modifications. Frozen leaves or roots (0.5 g each) were ground in liquid nitrogen. The chilled powder was then mixed in potassium phosphate buffer pH 7.5 containing 5 mM dithiothreitol at 4 °C. In this study, high speed acceleration was used to separate the supernatant and biological debris. The supernatant was centrifuged at 13,362×*g* for 15 min at 4 °C. The supernatant was collected and re-centrifuged at 13,362×*g* for 15 min at 4 °C. The total soluble protein content was measured using the Bradford assay based on Bradford (1976).

CAT, POD and APX assay

The CAT and POD activity were determined using the procedure of Ozturk and Demir (2002). One unit of CAT activity was defined as the amount of enzymes catalysing the decomposition of 1 μmol H_2O_2 per min per mg protein (at 240 nm for 3 min). One unit of POD was determined as the amount of enzymes catalysing the oxidation of 1 μmol of guaicol/min/mg protein (at 470 nm for 3 min). The ascorbate peroxidase was assayed according to the method from Jebara et al. (2005). The APX activity was defined as 1 μmol of ascorbate oxidized per min per mg protein (at 290 nm for 3 min).

Total RNA extraction and cDNA synthesis

Total RNA from the roots and leaves of the oil palm seedlings were isolated using a method described by Prescot and Martin (1987) with minor modifications. Total RNA extracted from individual plant under the same treatment was combined together and subjected to DNase I treatment. The reaction mixture contained 2 μg RNA, 20U of DNase I and 10x reaction buffers with $MgCl_2$ (Fermentas, USA). Then, phenol:chloroform:isoamylalcohol (P:C:I) extraction was carried out by adding an equal volume of P:C:I (25:24:1, v/v) to precipitate the nucleic acid. Further cleanup of the total RNA was performed using the RNA clean up method established by Qiagen, Germany. The first-strand cDNA was synthesized from the treated total RNA tissues using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, USA), following the manufacturer's instructions.

Estimation of expression level of SRGs in oil palm tissues by quantitative real-time PCR (qPCR)

All the primers for genes encoding *EREBP*, *EABF*, *CI*, *DHN*, *HSP70*, *LEA* and *MET2*, (Table 1) were designed by using Primer3 (v.0.4.0) (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for qPCR. The nucleotide sequence of *EABF* and *EREBP* were obtained from Omidvar et al. (2012) and Omidvar et al. (2013), respectively, while the other stress-responsive genes studied were identified by reference to the oil palm EST database (http://palmoilis.mpob.gov.my/index.php/palmoilis/palmgenes-private). In this study, seven endogenous controls (*GAPDH*, *β-actin*, *cyclophilin*, *α-tubulin*, *β-tubulin*, *PD569* and *EA1332*) were tested across the treated and control samples. Among them, *GAPDH*, *β-actin*, *cyclophilins*, *PD569* and *α-tubulin* were stably expressed throughout the control and treated samples. Afterwards, *GAPDH*, *cyclophilins* and *α-tubulin* were further used in gene expression analysis. The qPCR was performed using Power SYBR® Green PCR master Mix (Applied Biosystem, USA).

Statistical analysis

The data were analysed using SAS version 9.2 (SAS Institute, Cary, NC). The comparison of means was performed using Tukey's test at 5 % probability.

Results

Effect of drought severity on total chlorophyll content

Photosynthetic activity is dependent on the chloroplast which contains chlorophyll pigment to absorb energy from sunlight. From the analysis (Table 2), chl_a was shown to decrease significantly ($P \leq 0.05$) in response to drought, while, chl_b did not show any trend of change. The chl_a content measured was 2.79 (control), 1.72 (7 DWW), 1.70 (14 DWW), 1.23 (21 DWW), 1.27 (28 DWW) and 0.72 (35 DWW). The total chlorophyll (TC) content dropped by as much as 0.92, 0.93, 0.78, 0.80 and 0.63-fold at 7, 12, 21, 28 and 35 DWW, respectively, compared to the well-watered seedlings. A similar pattern was observed for the ratio of chl_a and chl_b where it decreased significantly with the progress of drought. The fold changes were 0.36 (7 DWW), 0.35 (14 DWW), 0.28 (21 DWW), 0.27 (28 DWW) and 0.16 (35 DWW). The leaves still looked greenish under mild and moderate stress, even though the chlorophyll pigments had started to degrade.

Total proline accumulation under different drought stress severity levels

As shown in Fig. 1, proline content was found to accumulate at high levels in vegetative tissues of seedlings

Table 1 List of primers used for quantitative gene expression analysis

Primers name	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
GAPDH	GGTGACAGCAGGTCCAGCAT	ATCAAAGCCAGGCAAGCATC
β-actin	CAAGTCATGTAGGGTTGC	ACTAGGCTGGCAAAGTTCAT
Cyclophilin	CGTGATGGAGCTGTATGCTG	AACGTCGACCCCTTGTAGTG
α-tubulin	GCCTTCGAGCCATCTTCTATGAT	AGGCAGCAAGCCATGTACTTAC
β-tubulin	CGAGCTTATCGACTCCGTTC	AGTGCCTCCTCCCAAAGAAT
PD569	ATCAACCACTCAATCTTCTGG	CTTCTGCGTTCATCTTTTGC
EA1332	TTAAGAATGCTCGGGAAAGG	CTACTTCTGTCTGCAATTTTGG
EREBP	TGGGATGCAAGGGTTCTATC	CACAGTGAGACATCGCCATC
EABF	CCGGTTCTGTCCCTTTCATA	GTGAGACACCGCCATCATTA
CI	GGGTTTTACCCTGGAAGAGC	CTTTGGACATTCGCTTGGAG
DHN	AGTACGGCAACCCGATCC	AGTACGGCAACCCGATCC
HSP70	GACAAACGCTCTGTCGATGA	GCCTCATCTGGGTTGATGTT
LEA	CAAGCAGAAAGGTTGGAAGC	GTCTTCTCCATGCCGGACT
MET2	GACGCAAAAGCCTCAAAGAC	GCCGGTTATTTTTAGCACCA

CI cold-induced, *DHN* dehydrin, *EABF* ABA-responsive binding factor, *EREBP* ethylene-responsive element binding protein, *EA1332* unknown protein, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *HSP70* heat stress protein 70, *LEA* late embryogenesis abundant, *MET2* metallothionein type 2, *PD569* manganese superoxide dismutase

Table 2 Chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (TC) content and Chl_a:Chl_b ratio in the leaf tissues of oil palm seedlings treated with drought stress

Days of water withholding	Chl_a (mg/g FW)	Chl_b (mg/g FW)	TC (mg/g FW)	Chl_a:Chl_b
Control	2.79^{a}	1.08^{b}	3.87^{a}	2.58^{a}
7	1.72^{b}	1.85^{a}	3.56^{ba}	0.93^{b}
14	1.70^{b}	1.90^{ba}	3.60^{bc}	0.90^{b}
21	1.27^{c}	1.75^{ba}	3.02^{bc}	0.73^{b}
28	1.27^{c}	1.82^{ba}	3.09^{bc}	0.70^{b}
35	0.72^{d}	1.72^{b}	2.44^{c}	0.42^{b}

Means with different letters are significantly different at $P \leq 0.05$ by Tukey's Test

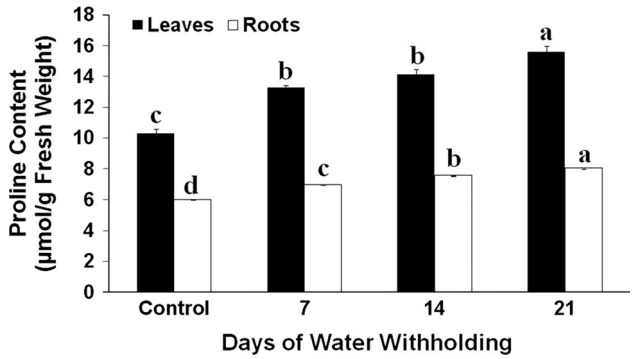


Fig. 1 Proline content in leaf and root tissues of oil palm seedlings subjected to drought stress. Data are mean ± SEM of five replicates. *Different letters* significant difference at $P \leq 0.05$ by Tukey's range test

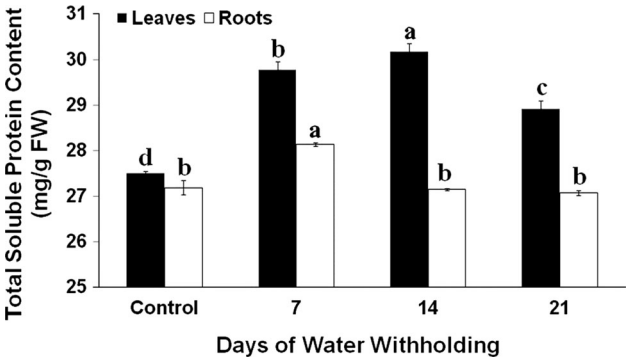


Fig. 2 Total soluble protein content in leaf and root tissues of oil palm seedlings subjected to drought stress. Data are mean ± SEM of five replicates. *Different letters* significant difference at $P \leq 0.05$ by Tukey's range test

subjected to drought stress. In the leaves, proline increased significantly at $P \leq 0.05$. The increase in proline content in the leaves was recorded as 1.29, 1.37 and 1.51-fold under mild, moderate and severe conditions, as compared to the well-watered seedlings. The same trend was observed for the proline content in the roots. However, the amount of proline content in the roots was much lower than that in the leaves. The fold increases were determined as 1.16-, 1.26- and 1.34-fold at mild, moderate and severe stress conditions, respectively. This finding indicates that proline plays a vital role in drought stress (Fig. 1).

Total soluble protein content under different drought stress severity levels

Total soluble protein content was found to increase in both vegetative tissues (Fig. 2). It was shown that the total soluble protein content was the highest at 14 DWW, which was 1.09-fold higher than that in the leaves of the control plants. At severe stage (21 DWW) the total soluble protein content decreased significantly to 1.05-fold. In the roots the highest accumulation of total soluble protein content was at 7 DWW (1.03-fold). Under moderate and severe stage conditions, the total soluble protein content dropped to basal levels.

Pattern of antioxidant enzyme activities (CAT, POD and APX) under drought stress

A major impact of drought stress is cellular oxidative damage. It can cause accumulation of ROS. In plants, elevated ROS without an efficient scavenging system may lead to high injury index and finally death of the whole plant. Therefore, in our work, we determined the activity of three kinds of antioxidative enzymes, the CAT (EC 1.11.1.6), guaicol POD (EC 1.11.1.7) and APX (EC 1.11.1.11) (Fig. 3).

In the leaves, CAT activity (Fig. 3a) under mild drought (12.05 nmol/min/mg protein), showed a significant increase ($P \leq 0.05$) compared to the control (7.36 nmol/min/mg protein) but the activity was not detected under moderate and severe conditions. In the roots, the CAT activity showed no significant changes under mild drought conditions, but steadily increased under moderate (1.31-fold) and severe stress conditions (1.52-fold).

The guaicol POD activity in the leaves (Fig. 3b) was found to increase sharply under mild stress (3.81-fold) but dropped under moderate (2.37-fold) and further reduced under severe drought conditions (2.06-fold), even though the levels were still higher than that for the control. In the roots, the guaicol POD activity significantly increased

under mild (1.60-fold) and further increased under moderate (1.74-fold) and severe stress conditions (2.69-fold).

A different trend was shown by APX (Fig. 3c). The APX activity in the leaves showed a gradual decrease compared to the control. The activity was reduced to 0.57- and 0.75-fold under mild and moderate stress conditions, respectively. A similar trend was shown in the roots. The APX activity diminished significantly under mild and moderate stress conditions, to 0.73- and 0.25-fold than that of the control roots, respectively. No activity of APX was observed under severe stress conditions, both in the leaves and roots.

Expression pattern of SRGs in dehydrated oil palm seedlings

Drought stress transiently increased expression of different kinds of SRG transcripts in the stressed oil palm seedlings. As shown in Fig. 4, overall, most of the genes studied were up-regulated in the leaf tissues more than in the root tissues under different levels of drought conditions. The genes were categorized into two groups, the transcription factor and SRGs. In the expression analysis, the fold change was determined relative to the expression observed in the well watered plants.

The transcription factor genes, analysed in this study were *EREBP* and *EABF*. Under drought stress, no mRNA accumulation was recorded for *EABF* in both vegetative tissues. However, *EREBP* was found to be highly up-regulated under drought conditions, in a distinctive manner. Under mild stress, high accumulation of *EREBP* transcripts was found in the leaves at 4.81-fold higher than in the control seedlings. However, the expression level was significantly down-regulated under moderate stress (0.33-fold). A different scenario was observed for the roots, whereby the expression level decreased sharply under mild (0.05), moderate (0.35) and severe (0.01) stress conditions, even though there was a slight increase in expression under moderate compared to mild stress.

Five SRGs (*LEA, DHN*, *HSP70*, *CI* and *MET2*) showed differential expression in a distinctive pattern in the vegetative tissues under different drought stress levels. Expression of *LEA* in the leaves was observed to gradually increase under mild (12.70-fold), moderate (92.63-fold) and severe stress (977.76-fold) conditions. A similar scenario was observed for the roots, where *LEA* was expressed at increasing levels under mild (3.28×10^4-fold) and moderate stress conditions (5.53×10^3-fold). No expressions were detected under severe stress conditions.

DHN belongs to group II of the LEA protein. In this study, *DHN* was expressed in a distinctive manner compared to the expression of *LEA*. *DHN* was highly up-regulated in the leaves as compared to the roots. Under mild and moderate stress, *DHN* was up-regulated by as much as 9.19-fold and 9.85-fold, respectively, in the leaves. In the dehydrated roots, *DHN* was down-regulated under mild stress (0.68) and moderate stress (0.03) but up-regulated under severe stress (1.62) conditions.

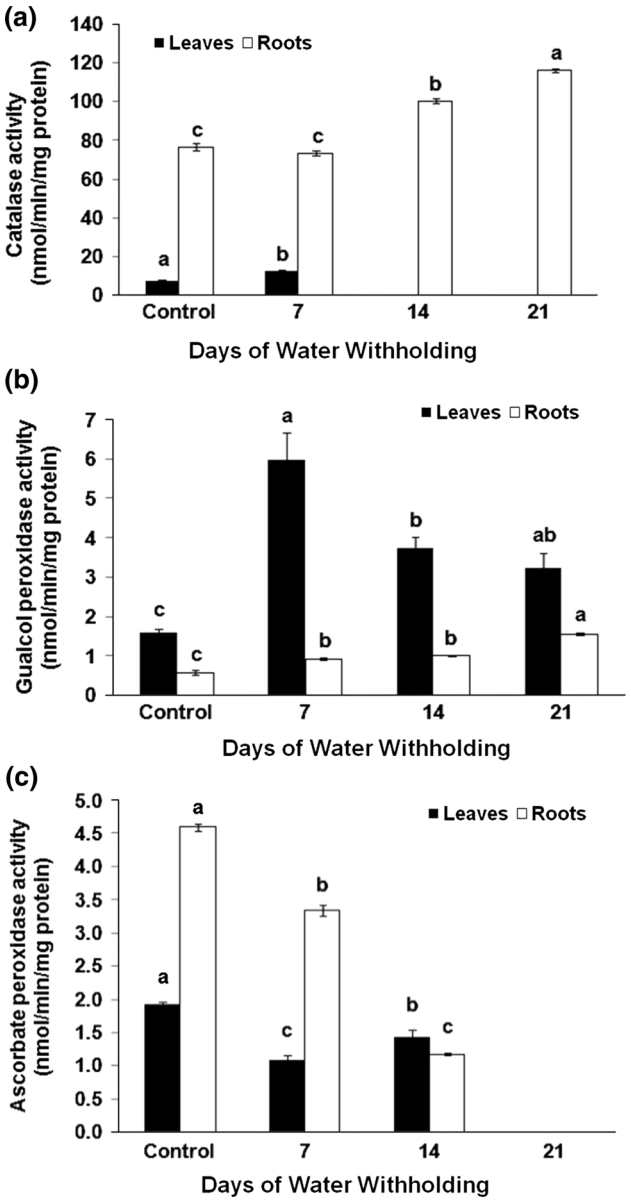
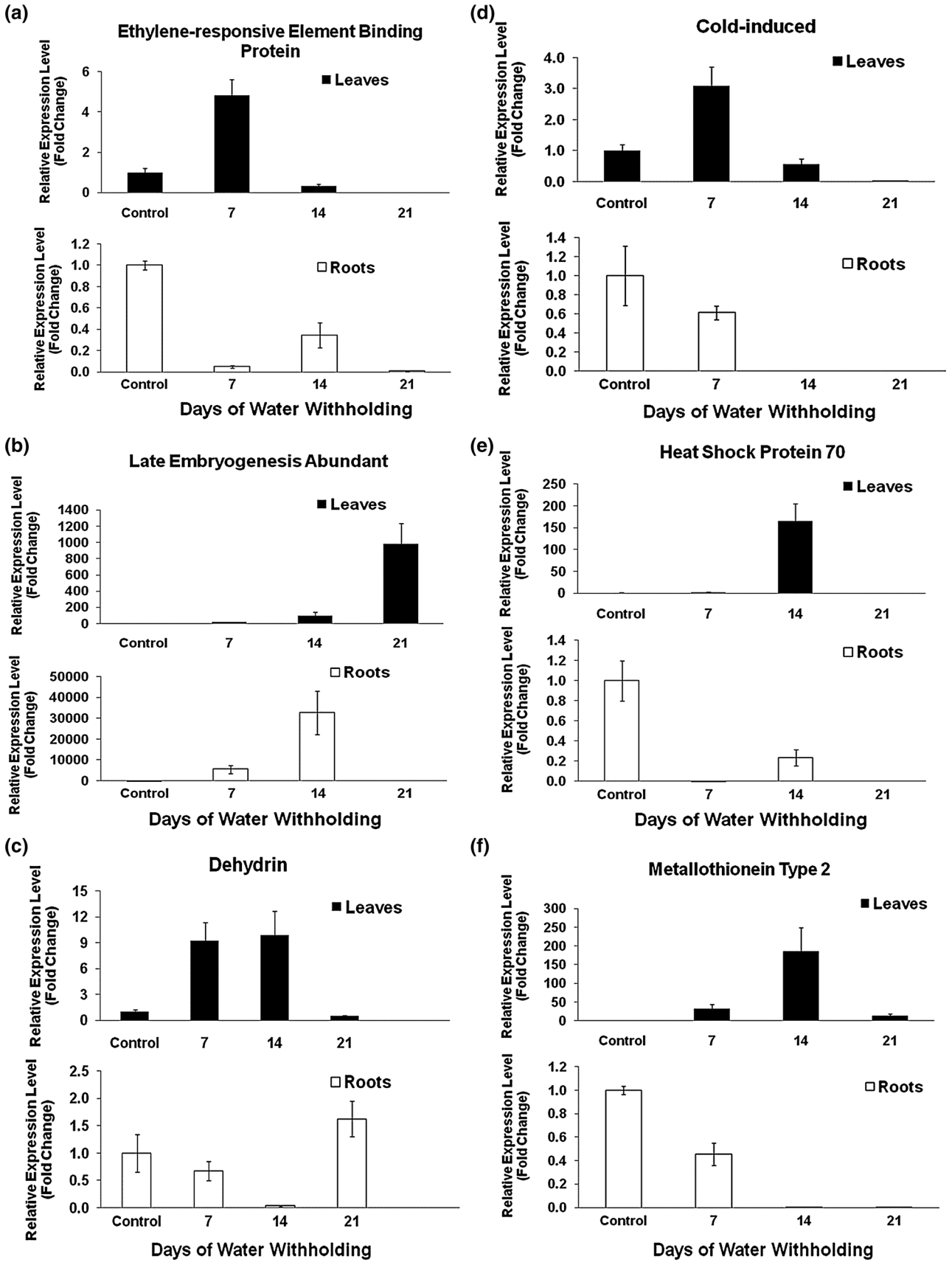


Fig. 3 Effect of drought stress on the activities of antioxidant enzymes: **a** catalase; **b** guaicol peroxidase; and **c** ascorbate peroxidase. Data are mean ± SEM of three replicates. *Different letters* significant difference at $P \leq 0.05$ by Tukey's range test

Fig. 4 Expression profile of stress-responsive genes in the leaves and ▶ root tissues of oil palm seedlings subjected to drought stress. **a** *EREBP* transcription factor; **b** late embryogenesis abundant (*LEA*); **c** dehydrin (*DHN*); **d** cold-induced (*CI*); **e** heat shock protein 70 (*HSP70*) and **f** metallothionein type 2 (*MET2*)


(a)
Ethylene-responsive Element Binding Protein
Relative Expression Level (Fold Change)
Leaves
Roots
Control
7
14
21
Days of Water Withholding
(b)
Late Embryogenesis Abundant
(c)
Dehydrin
(d)
Cold-induced
(e)
Heat Shock Protein 70
(f)
Metallothionein Type 2

The *CI* was slightly up-regulated under mild stress (3.10-fold) followed by a reduction in expression to 0.55-fold under moderate and 0.02-fold under severe stress, in the leaves. In the roots, the *CI* transcript was down-regulated under severe stress (0.24-fold). None of the transcripts were detected under moderate and severe stress conditions.

The chaperone protein, *HSP70* was slightly up-regulated under mild stress (2.64-fold) followed by a sharp up-regulated under moderate stress (165.04-fold), in the leaves. In the roots, the expression was down-regulated under mild and moderate stress. None of the mRNA transcripts accumulated under severe stress in both vegetative tissues.

The *MET2* was gradually up-regulated under mild (31.27-fold) and moderate (185.25-fold) stress conditions in the leaves. However, under severe stress the expression decreased but was still higher than the basal level (13.61-fold). In the roots, the expression level was down-regulated under mild stress (0.46-fold) and none of the transcripts were detected under moderate and severe stress conditions.

Discussion

The decrease in chlorophyll content observed in our study during drought stress has been demonstrated in many species, including dehydrated oil palm seedlings. Similarly, the different studies also showed that the chlorophyll degradation status depends on the duration and severity of drought stress. Cha-um et al. (2013) reported that degradation of Chl_a, Chl_b and TC in oil palm seedlings showed decreasing trends with increasing severity of drought stress; 42, 20, 13 and 6 % of soil water content. By mimicking water deficit using PEG on oil palm seedlings, Cha-um et al. (2010) showed that Chl_a, Chl_b and TC significantly dropped; $-0.42 < -0.98 < -2.15$ MPa. The ratio of Chl_a and Chl_b sharply decreased in oil palm seedlings under a combination of drought and nutrient stress (Sun et al. 2011). In this study, chl_a was more severely affected by drought, as compared to chl_b. However, different observation was observed by other researchers (Cha-um et al. 2010, 2011, 2013; Sun et al. 2011) in which they found that chl_b content dropped with progress of drought stress. The different observation might be due to a few factors such as the method used for stress treatment, chlorophyll measurement and age of the plants. The decrement of Chl_a content clearly showed that the PSII of oil palm seedlings is severely affected as drought progresses. Now, with the availability of a sensitive and non-invasive method of measuring chl_a as reported by Rakic et al. (2015), it is possible to use chl_a as a reliable indicator for drought stress condition. This is because the fluorescence is used directly to measure PSII status without causing damage and with minimal stress to the plants.

ABA is well established as a mediator to stomatal closure when the plant is exposed to drought stress (Cominelli et al. 2010) as well as ethylene (Beguerisse-Diaz et al. 2012). Tanaka et al. (2005) reported that ethylene can hamper stomatal closure by inhibiting the ABA-signaling pathway. Oil palm EREBP belongs to the ethylene responsive element (ERE)-binding protein, in which its expression in the leaves is induced by ethylene treatment. *In vivo* and in vitro analysis of DNA–protein binding of EREBP have shown that the protein can bind to both ERE and the dehydration-responsive element (DRE/CRT) (Omidvar et al. 2013). This shows that *EREBP* gene is involved in crosstalk between ethylene and ABA signaling pathways. Hence, in the present study, the enhanced expression of the *EREBP* transcription factor under mild stress might be involved in the inhibition of the ABA-signaling pathway as a repressor protein. EREBP might be helping to maintain the turgidity of the guard cells, to remain open under drought so that the plant is able to continuously fix atmospheric CO_2 and therefore prolong the photosynthesis activity to supply carbon. However, the expression of *EREBP* was sharply down-regulated under moderate and severe stress conditions. It signifies that the stomata aperture is predominantly closed under such conditions. To the best of our knowledge, this is the first report that relates *EREBP* role in regulating the stomata opening under water stress condition.

Expression of *EABF* gene was not detectable in the leaves and root tissues under drought stress. However, the expression of *EABF* was up-regulated in mesocarp tissue of ripening oil palm fruits both under drought and ABA treatments (Omidvar et al. 2012). The 9-cisepoxy-carotenoid dioxygenase (NCED) is an important protein involves in the regulation of ABA in higher plants. Expression of *CsNCED2* in citrus leaves was not detectable under drought treatment. But, *CsNCED2* was expressed in flavedo of citrus fruit at the ripening stage under fruit dehydration treatment (Rodrigo et al. 2006). Thus, *CsNCED2* could be a potential candidate to be regulated by *EABF* in ripening fruit tissue under drought stress.

LEA, *DHN*, *HSP70*, *CI* and *MET2* were regulated in a different way in response to different drought stress severity levels in both vegetative tissues of the oil palm seedlings. Under mild drought, the seedling tissues start to dehydrate. Hence, the plants begin to develop a defence system as an adaptation strategy to survive under the stress conditions. In the leaves, the increased expression of *LEA*, *DHN*, *CI*, *HSP70* and *MET2* under such conditions is suggested to facilitate the plants in preventing cellular proteins, including those involved in photosynthesis from

aggregation and to stabilize the cell membrane. The presence of *HSP70* might be associated with the disturbance of the plant's cooling system. The reduced transpiration due to stomatal closure might hamper evaporative cooling as well as photosynthesis of the oil palm seedlings. In this study, these genes were further up-regulated under moderate stress except for *CI*, while under severe stress *LEA* and *MET2* was continuously up-regulated. The sharp increase in the expression level of *LEA* under severe stress showed that the seedlings were possibly dealing with severe destabilization of the membrane. The remarkable increase in the expression of SRGs in the leaves, as compared to the roots, suggests that they cooperate in maintaining the photosynthetic apparatus from damage and degradation under different drought severity conditions.

Under stress conditions, it is a critical response from the oil palm roots to search for sources of water and minerals. As soil dehydration progresses, the stability of the plasma membrane of these roots worsens (Farooq et al. 2009). Up-regulation of *LEA* under mild and moderate stress in oil palm seedlings' roots could possibly prevent membrane aggregation and oxidative burst due to electrolyte leakage. The expression of *LEA* in roots under drought stress conditions was also observed in other plants such as *Populus deltoids* (Cohen et al. 2010), *Gossypium herbaceum* (Ranjan et al. 2012) and *Tamarix hispida* (Gao et al. 2014). In this study, *CI* was up-regulated in the oil palm seedlings' roots under mild drought conditions. Similar finding was also observed for cold-regulated protein with a molecular mass of 85 kDa (COR 85) (Kazuoka and Oeda 1992), while the peanut cold-stress related gene, *Gsi83,* was up-regulated under different levels of drought severity in the leaves and roots (Ding et al. 2014). The COR85 was also reported to be involved in drought, exogenous application of ABA and wounding response (Kazuoka and Oeda 1994). Therefore, the findings to date suggest the possible involvement of *CI* in crosstalk between the cold and drought signaling pathways.

Some of the genes that were shown to be differentially expressed in oil palm seedlings under different drought severity have also been reported in other plant species. In *Erianthus arundinaceus HSP70* showed enhanced expression under water stress (Augustine et al. 2015). In addition, *MET2* was found to be the one of the most abundant gene expressed under drought condition in *Ammopiptanthus mongolicus*. Studies carried out using insect resistant transgenic maize showed that *HSPs*, *LEAs*, *dehydrin*, *ERF/AP2*, *WRKY*, *NAC* and *MYB* were expressed under drought condition (Gulli et al. 2015). Some of the genes that showed altered expression levels in oil palm seedlings under drought stress are commonly observed to be differentially expressed under different types of abiotic stress. For example, Liu et al. (2013) reported that, cold and drought treated *A. mongolicus* seedlings induced accumulation of *CORs*, dehydrin, *LEAs*, 14-3-3 proteins and glyoxalases betaine-aldehyde dehydrogenase (*BADH*). Expression of the same genes may suggest the presence of unifying mechanisms for tolerance and survival of the plants.

The activity of the CAT enzyme increased significantly in both oil palm seedling leaves and roots under mild drought. However, no CAT activity was determined in the leaves under moderate and severe drought conditions. It could be that the CAT protein was affected by the drought treatment. The CAT and POD decompose H_2O_2 by oxidation. POD activity increased compared to control under different severity of drought in both tissues which showed that POD is an essential enzyme in scavenging H_2O_2 in the oil palm seedlings. Increase in POD activity was also reported in leaves of oil palm seedlings treated with drought and low temperature stress (Cao et al. 2011). The APX catalyses the reduction of H_2O_2 to water using the reducing power of ascorbate. However, our findings showed that, APX activities were affected by drought as the activities decreased as drought conditions progressed. The decrease indicated the reduced stability of the three dimensional structure of the APX due to protein-misfolding and aggregation. The reduction of APX activities was also observed in *Phaseolus vulgaris* under saline conditions (Jebara et al. 2005) and *Poa pratensis* in response to heat stress (Du et al. 2013). A different observation was reported in *Brassica napus* L. where CAT, POD APX and SOD activities was studied under drought stress (Mirzaee et al. 2013). They reported an initial increase and later decrease in antioxidant activities in leaves and roots tissues compared to control plants with the increasing duration of stress indicating the unbalance of the active oxygen metabolism system. The high accumulation of active oxygen was able to initiate and accelerate lipid peroxidation. But, when the levels of the active oxygen exceeded the ability of antioxidant system to cope with them, damage to cellular components occurred (Cao et al. 2011).

Soluble proteins are the most abundant protein in green tissues. In C_3 plants Rubisco comprise up to 12–30 % of the total soluble proteins. Rubisco is an enzyme involved in the first step of carbon fixation in plants (Ishida et al. 2008). In our study, biosynthesis of soluble protein content was observed in the oil palm vegetative tissues under drought conditions. However, the progression of stress reduced the amount of protein accumulation. The increase in soluble protein content in an earlier response to drought might be due to the increase in accumulation of drought-related proteins, while, the decrease might partly be due to the decrease in proteins related to the photosynthetic apparatus. This observation is in agreement with the findings of Chutia and Borah (2012) who reported similar trends of protein accumulation under drought stress. The decreased of the total soluble protein content under drought

stress was also observed in cotton plants (Ananthi and Vijayaraghavan, 2012). In addition, total soluble protein content decreased in leaves and shoot tissues of *Paspalum scrobiculatum* L. when compared to that of control plants under drought stress (Ahmad et al. 2013). Accumulation of soluble protein content in root is in agreement with Zhu et al. (2007) who reported accumulation of cell wall protein in maize roots. Soluble protein content is considered to be the first nitrogenous compound affected under stress conditions (Martignone et al. 1987). Proteomic analysis in *Zea mays* suggested that seventy-eight out of 413 proteins showed a significant quantitative variation, some decreased while others increased in their protein expression after being subjected to drought conditions (Ricardi et al. 1998). Inhibition of CO_2 assimilation also contributes to the reduction of the entire protein content. Strong correlation between carbon metabolism and protein synthesis has been reported by Smith and Stitt (2007).

The maintenance of high proline content is also associated with drought tolerance. The results of this study are in agreement with other investigations involving the oil palm species. In recent studies, high accumulation of proline content was observed in leaves and roots of oil palm seedlings under drought conditions (Cao et al. 2011; Chaum et al. 2013). Proline synthesis occurs in the shoots, and then it is transported to the roots. Recently, the roots have been reported to function as sink organs for proline. This is essential for sustaining root growth at low water potentials (Zhang et al. 2014). Proline accumulates in the cytoplasm and chloroplast stroma. It is known for participating in osmotic adjustment to stabilize cell membranes and proteins. When the cells and tissues dehydrated, proline accumulation increases to stabilize the cellular structures through hydrophilic interactions and hydrogen bonding. It also works as an electron receptor in preventing photosystem injuries in dealing with ROS. Apart from that, proline is involved in buffering cellular redox potential activities, alleviating cytoplasmic acidosis, maintaining appropriate $NADP^+$/NADPH ratio and acting as a protein-compatible hydrotrope under stress conditions. Under recovery and repairing responses, proline acts as a reducing agent in supporting mitochondrial oxidative phosphorylation and the generation of ATP (Ashraf and Foolad 2007).

Conclusion

Drought adversely affects the plant metabolism in oil palm seedlings by decreasing chlorophyll content, affecting antioxidant enzyme activities and degrading total soluble protein content in leaves and roots tissues. In addition, increased in proline content and differential up-regulation of many SRGs in leaves and roots of oil palm seedlings show that, the plant system undergoes a coordinated and complex recovery process. Thus, the respective SRGs can be benefited as potential targets for the enhancement of stress tolerance in oil palm, through genetic engineering and breeding programmes to develop drought tolerant oil palm.

Author contribution statement Azzreena Mohamad Azzeme carried out the research as part of her PhD project. Siti Nor Akmar Abdullah is the project leader and main supervisor. Maheran Abdul Aziz and Puteri Edaroyati Megat Wahab are both co-supervisors.

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