Expression pattern of drought-responsive genes in burley tobacco under in vitro water deficit

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Abstract. Understanding molecular mechanism in tobacco adaptation to drought stress become indispensable strategy for the development of high-yield tolerant varieties. Profiling the pattern of drought-related gene expression is a promising strategy to preliminary evaluates the plant response to drought stress. This study aims to investigate the expression pattern of drought-responsive genes in burley tobacco under in vitro water deficit. In this study, we used three burley varieties including Marakot, Jepon Mawar and MKY. Drought stress treatment was applied in vitro using MS medium supplemented with several concentration of PEG 6000 (Polyethylen Glycol) (0%, 10%, 20% and 30%). The expression profile of drought-related genes including \( NtLTP1 \), \( NtABA2 \) and \( NtERD10B \) were investigated using quantitative Reverse Transcriptase-PCR (qRT-PCR). The results showed that \( NtABA2 \) and \( NtERD10B \) genes were parallely overexpressed following an increase concentrations of PEG in var. Jepon Mawar and var. MKY. However, the highest mRNA relative expression of these genes was occured in var. MKY treated with 30% of PEG, which increased 1.7 and 1.64-fold compared to control, respectively. \( NtLTP1 \) was down-regulated in var. Jepon Mawar following an increase of PEG concentrations. In contrast, \( NtLTP1 \) was up-regulated in var. MKY. Meanwhile, var. Marakot demonstrated similar pattern of all gene expressions, where treatment of 20% PEG induced highest expression compared to the other treatments. The overall result suggests that var. MKY is likely more tolerant to drought stress followed by var. Jepon Mawar and var. Marakot, respectively.

1. Introduction
Plant often perceives many environmental conditions including both biotic (pathogen, herbivores, etc) and abiotic (high temperature, low level of available nutrients, salinity, extreme pH condition, high concentration of heavy metals, cold and drought stress, etc) [1, 2]. Among them, drought stress is considered as the most influencing environmental stress that affect plant productivity. The incidence of low plant productivity due to the inability of the plant to escape is increasing. It also coincides with global climate change, which results in an unpredicted seasonal phenomenon [3].

Plants develop many interesting strategies to cope with extreme environmental stress. These include morphological, physiological and molecular adaptations. Plants are responding the environmental cues by re-designing their root architectures, inducing the stomatal closure, producing high level of cuticular wax, reducing the chlorophyll, inducing the formation of antioxidant to scavenge the reactive oxygen species (ROS), which is usually over-produced during the environmental stress [1]. Plant gene regulations have been extensively studied using both proteomic and
transcriptomic approaches to better understand the plant molecular responses during environmental stresses. Transcriptomic approaches include measuring the expression of some transcript species such as mRNAs, non-coding RNAs and small RNAs under particular environmental stresses [4,5]. Previous studies have demonstrated that some genes might be used as drought stress markers [6]. Some of these genes include NtLTP1 (Lipid Transfer Protein), which is involved in epicuticular wax deposition [7, 8], NtABA2, which encodes an important enzymes involved in the biosynthesis of abscisic acid hormone [9] and the NtERD10B that encodes the protein, which acts as important osmolites [10].

Solanaceae family is considered as one of important plant family that has been long-term cultivated. It comprises around 3,000 species, where tobacco is the most economically important species among the non-edible Solanaceae family. Not only serves as the main source for cigarette industry, tobacco is being utilized in the pharmaceutical industry and bio-energy sources [11]. Some interesting tobacco varieties have been identified and cultivated in Indonesia, such as Prancak 95, Dixie Bright, Marakot, Jepon Mawar, and MKY. Each variety possesses unique characteristics, both morphological and physiological characters. The genetic diversity that is responsible for diverse morpho-physiological characters also plays a central role in regulating the plant adaptation to environmental conditions. Therefore, each variety might possess distinct responses against environmental stress [2]. To date, many research have been conducted to study plant adaptation [5,6] but little is known about the molecular responses of Indonesian tobacco varieties against environmental adverse condition, including drought stress.

As previously reported, some genes are responsive to drought stress at the transcriptional level. The expression of these responsive genes might be decreased or increased when the plants are exposed to drought stress. It indicates that the dynamic expression of the genes is resulted as a consequence of the environmental stress. In addition, this molecular regulation certainly controls plant adaptation against drought stress [12]. Based on the above information, investigating the gene expression pattern could be utilized to preliminary evaluate plant response to drought stress. Even though many studies on the tobacco response to drought stress has been widely reported, until now, there is no research have been reported about the expression pattern of drought responsive genes of some burley tobacco, especially var. MKY, Marakot and Jepon Mawar. Therefore, this present study aims to investigate the expression pattern of drought-responsive genes in burley tobacco under in vitro water deficit.

2. Methods

2.1. In vitro culture of tobacco and drought stress treatment

Tobacco seeds used in this study include Nicotiana tabacum var. Marakot, Jepon Mawar and MKY. The seeds were obtained from PT. Sadhana. The seeds were sterilized by mixing in 1% NaOCl solution for 5 min and were subsequently washed with distilled water for 3 times. The seeds were then grown in the Murashige and Skoog (MS) medium (100 seeds per medium) [13] and kept in culture chamber with 12/12 h of light/dark photoperiod at 22°C. Seedlings aged 18-20 days after germination (dag) were moved to the MS medium containing Polyethylene glycol (PEG) 6000 at different concentrations (0%, 10%, 20% and 30%, w/v). After 8 days, the seedlings were collected and subjected to further analysis.

2.2. Total RNA extraction and quantification

Seedlings were treated as routine preparation prior to total rna extraction [14]. Briefly, samples were freezeed using liquid nitrogen and then ground directly into powder. Subsequently, total RNA extraction was carried out using Total RNA Mini Kit (Plant) (Geneaid) according to the protocols provided by the manufacturer. Quantification of total RNA was done using Nano Drop (Thermo Scientific™ NanoDrop 2000) and the concentration was expressed as ng/μL.
2.3. Gene expression analysis

Gene expression was analyzed using quantitative real time PCR (qRT-PCR). qRT-PCR was carried out using KAPA SYBR® FAST Universal One-Step qRT-PCR Kit (Kapa Biosystem) according to the manufacturer’s protocols. The concentration of total RNA used in this analysis was 20 ng/μL. Three drought responsive genes were analyzed including NtABA2 (accession no: EU123520), NtERD10B (accession no: AB049336), and NtLTP1 (accession no: AAT45202). Meanwhile, NtEF-1α (accession no: AF120093) was used as gene of reference. The primers were designed using PrimerQuest Tool (Integrated DNA Technologies), NtABA2 {5’- GTGGGATTGGAGGGTTAGTT -3’ (forward) and 5’- GGTCCTCTATATTGCCCTTCTC -3’ (reverse)}; NtERD10B {5’- ACGGACGAATACGGCAATC-3’ (forward) and 5’- TCTCCTTAATCTTCTTCATCC-3’ (reverse)}; NtLTP1 {5’- CATTGTGGTTGTTGTGTG -3’ (forward) and 5’- TGGAAAGGCTAATCTTGTAGG-3’ (reverse)}; NtEF-1α {5’- TGAGATGCACCACGAAGCTC -3’ (forward) and 5’- CCAACATTGTCACCAGGAAGTG -3’ (reverse)}. The PCR condition were reverse transcription at 42 °C for 5 min, enzyme inactivation at 95 °C for 3 min followed by 39 cycles of 95 °C for 3 s, 61.3 °C for 20 s and extension step at 61.3 °C for 20 s. The gene expression was calculated using ΔΔCt method [15]. All the relative mRNA levels were normalized to the NtEF-1α level in the same samples. All gene expression analysis was done in triplicate.

3. Results and discussion

3.1. Effect of in vitro water deficit on the expression of NtABA2 gene

NtABA2 encodes the ZEP (zeaxanthin epoxidase) enzyme that serves to catalyze the epoxidation of zeaxanthin to violaxanthin in the biosynthesis of ABA in plants. Furthermore, violaxanthin is converted to xanthoxin via neoxanthin intermediates. This reaction is catalyzed by NCED (9-cis-epoxicarotenoid dioxygenase). Subsequently, two enzymatic reactions complete the final stage of ABA synthesis. Based on these pathways, ZEP is a pivotal enzyme that functions in the early stages of ABA biosynthesis [16]. The results showed that each tobacco variety had a different response to PEG-induced drought stress. At 10% and 30% (w/v) of PEG, the expression of NtABA2 in var. Jepon Mawar and Marakot were decreasing compared to control. However, the expression of NtABA2 in var. Jepon Mawar is higher than that observed in var. Marakot on any given treatment. Whereas, var. MKY showed an increase of NtABA2 expression following increased of PEG concentrations, compared to control (Figure 1). The highest expression of NtABA2 is observed in var. MKY treated with 30% (w/v) PEG, which was 1.7-fold compared to untreated tobacco (control).

![Figure 1](image_url)  
Figure 1. Tobacco NtABA2 gene expression during in vitro water deficit.
The dynamic gene expression patterns is possibly due to overall changes in physiological responses to stress, which might depend on the genotype of the tobacco. ABA biosynthesis generally increases following the severity of stress [17]. Previous study has showed that ABA augmentation is associated with an increase of ABA2 expression and consequently result in better resistance of a plant to drought stress [18]. In addition, ABA2 mutant study has also demonstrated that the plant hormone ABA plays a significant role in plant adaptation during severe drought condition [19]. ABA also serves to activate several other drought responsive genes including ERD10 [20]. In addition, ABA functions in the stomatal closure regulation to prevent water loss through transpiration during water deficit. Based on our results, var. MKY is thought to have the best response to drought stress compared to other varieties, followed by var. Jepon Mawar and var. Marakot. This is indicated by a regular pattern of NtABA2 expression in response to elevated level of PEG concentrations. Nevertheless, other study showed that there will be other genes involved in the plant adaptation mechanism against drought stress [21].

3.2. Effect of in vitro water deficit on the expression of NtERD10B gene

NtERD10B encodes the Late Embryogenesis Abundant (LEA) protein family (Group II LEA). The ERD gene is defined as an inducible gene and is rapidly activated under drought stress conditions [19]. LEA proteins are expressed in all plant organs and accumulated in high amounts when the plants are facing the drought stress [22]. Our results demonstrated that the NtERD10B gene expression profile showed different patterns in each variety against different level of PEG-induced drought stress concentration. Var. Marakot showed similar NtERD10B pattern of expression with those observed in NtABA2. While, the expression of NtERD10B in var. Jepon Mawar and var. MKY under water deficit also showed the same profile as observed in NtABA2. The expression of NtERD10B increased in a dose-dependent manner. However, the highest expression of NtERD10B was found in var. MKY, treated with 30% (w/v) of PEG (1.64-fold) compared to control (Figure 2).

![Figure 2](image.png)

**Figure 2.** Tobacco NtERD10B gene expression during in vitro water deficit.

Similar pattern of expression between NtERD10B and NtABA2 in this study, support the previous data, which stated that NtABA2 induce the expression of other drought-responsive genes, including NtERD10B [19]. Our results are in accordance with previous study that also reported an increase of ERD10B expression. Furthermore, upregulation of this gene could reach 10-fold compared to untreated tobacco plant. Indeed, dramatic increase of ERD10B expression could also take place when plant is undergoing combined environmental stresses [23]. Enhance expression of this gene is followed by increase of transpiration rate and it is consequently reflected with an elevation of the ABA production in the plants. The latter might then induce stomatal closure [24]. It is also worth noting that
overexpression of plasma membrane intrinsic proteins in *Medicago falcata* resulted in upregulation of *ERD10B* and thus enhanced the plant tolerance against drought stress [25].

The function of ERD10B in plant defence mechanism against drought stress include membrane protector, antioxidant, metal ion binding, protein stabilizer and water-binding molecule [26]. As protein stabilizer, LEA proteins have the capacity to protect proteins from inactivation and aggregation during drought stress. Its role as a stabilizer is supported by its ability to retain enzyme activity after partial dehydration or drought. During drought condition, membrane protection is important to maintain cellular and organelles integrity. The LEA protein has a charged amino acid residue, which allows it to absorb the ions. LEA proteins scavenge the ROS (reactive oxygen species) or ROS-producing metal ions. The production of ROS is increasing when the plants are suffering from environmental stress. Therefore, plants produce more antioxidants for minimizing the negative effect of an increase of ROS [2]. The function of LEA protein, including dehydrin, as an antioxidant is facilitated by the interaction of the amino acid residues of the protein with both the ROS and metal ions. This interaction could further results in oxidated amino acid residues and the formation of covalent bond between the metal ions and the LEA proteins [26]. Based on the relative mRNA expression of the *NtERD10B* gene on all three tobacco varieties, var. MKY is thought to be more responsive to drought stress followed by var. Jepon Mawar dan var. Marakot.

### 3.3. Effect of in vitro water deficit on the expression of *NtLTP1* gene

*NtLTP* encodes Lipid Transfer Protein (LTP). LTP is encoded by a large multigenous family and these genes have been characterized in several crops including tobacco [27], *Oryza sativa* (52 members), *Triticum aestivum* (156 members) [28] and in *Arabidopsis thaliana* (110 members), of which 15 are named LTP1 to LTP15 [29]. The results of the qRT-PCR showed that the three tobacco varieties exhibited different expression pattern of the *NtLTP1* under in vitro water deficit. The pattern of *NtLTP1* expression on var. Marakot and var. MKY is similar to the expression of *NtABA2* and *NtERD10B* of both varieties. The expression of *NtLTP1* seems to be dose-dependent manner in var MKY, where the highest expression was showed in 30% (w/v) of PEG (1.52-fold compared to control). Meanwhile, we observed dynamic and irregular pattern of expression in var Marakot. All var. Marakot treated with different concentration of PEG exhibited low expression of *NtLTP1* (figure 3). Interestingly, in contrast to var. MKY, the *N. tabacum* var. Jepon Mawar exhibited different pattern of expression. The *NtLTP1* was down-regulated following an increase of PEG concentration. Highest level of expression was showed in this variety under 10% (w/v) PEG treatment (1.52-fold compared to control). This value was similar to that observed in var. MKY under 30% (w/v) of PEG treatment (Figure 3).

![Figure 3](image-url)  
**Figure 3.** Tobacco *NtLTP1* gene expression during *in vitro* water deficit.
Decrease and increase of relative mRNA expression values is related to the stress resistance mechanism developed by different plants [30]. Upregulation of *NtLTP1* has been reported previously to be an example of plant response to drought stress. This is tightly associated with an enhance plant tolerance to drought stress [31]. In addition, variation in gene expression levels in response to drought stress also indicate that each plant possess their own limitation to this type of stress [32]. The LTP1 protein is involved in the intercellular lipid transfer trafficking, accumulation of wax and cutin deposition by bringing the epicuticular components to extracellular matrix. Furthermore, this protein is also involved in the embryogenesis, defence mechanism against pathogens, and crop adaptation to various environmental conditions [31]. In addition, another study also showed that LTP1 is involved in the secretion of lipid from glandular trichomes in *Nicotiana tabacum*. The latter could increase plant resistance to aphids [8]. Furthermore, LTP is considered as a pathogenesis-related protein family (PR-14) and is believed to be involved in several physiological processes including resistance to plant diseases [33].

The LTP has been reported to be induced by drought stress [34] and it will be upregulated in response to both osmotic and pathogenic stress [31]. The expression of *NtLTP1* gene in another species of Nicotiana (*Nicotiana glauca* L. Graham) increases when exposed to drought stress. Consequently, it increases the deposition of the cuticular wax. This elevated LTP1 might be due to plant strategy to prevent water loss through transpiration [7]. Based on the relative mRNA expression of the *NtLTP1* gene on all three treated varieties, var. MKY is thought to be responsive to drought stress followed by var. Jepon Mawar dan var. Marakot.

4. Conclusion
The present study demonstrated that molecular genetic factor plays important role in plant defense mechanism against drought stress. It was reflected with the distinct pattern of drought responsive genes in three treated tobacco varieties (*N. tabacum* var. MKY, Jepon Mawar and Marakot). Interestingly, *N. tabacum* var. MKY is found to be the most responsive variety compared to other treated varieties. However, evaluating other molecular genetic aspects is still required in order to complete our understanding on plant defence mechanism against water deficit condition.

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References
[1] Wang W, Vinocur B and Altman A 2003 Planta 218 1-14
[2] Jadid N, Maziyah R, Nurcahyani DD and Mobarokah NR 2017 AIP Conf. Proc. 1854 020018
[3] Zhang S, Kang H and Yang W 2017 PLOS ONE 12 e0182012
[4] Wang Z, Gerstein M and Snyder M 2009 Nat. Rev. Genet. 10 57-63
[5] Wehner G, Balko C, Humbeck K, Zyprian E and Ordon F 2016 BMC Plant Biol. 16 3
[6] Jiang Q, Zhang J-Y, Guo X, Monteros MJ and Wang Z-Y 2009 Int. J. Plant Sci. 170 969-78
[7] Cameron KD, Teece MA and Smart LB 2006 Plant Physiol. 140 176-83
[8] Choi YE, Lim S, Kim H-J, Han JY, Lee M-H, Yang Y, Kim J-A and Kim Y-S 2012 Plant J. 70 480-91
[9] Audran C, Borel C, Frey A, Sotta B, Meyer C, Simonneau T and Marion-Poll A 1998 Plant Physiol. 118 1021-8
[10] Rai A, Suprassana P, D'Souza S F and Kumar V 2012 PLOS ONE 7 e32658
[11] Apollonio D and Glantz SA 2017 Am. J. Public Health 107 1636-42
[12] Rejeb IB, Pastor V and Mauch-Mani B 2014 Plants 3 458-75
[13] Murashige T and Skoog F 1962 Physiol. Plant. 15 473-97
[14] Jadid N, Mardika R K, Nurhidayati T and Irawan M I 2016 AIP Conf. Proc. 1744 020042
[15] Livak K J and Schmittgen T D 2001 Methods 25 402-8
[16] Xiong L, Lee H, Ishitani M and Zhu J-K 2002 J. Biol. Chem. 277 8588-96
[17] Zhou S, Medlyn B, Sabate S, Sperlich D, Prentice IC and Whitehead D 2014 Tree Physiol. 34 1035-46
[18] Yue Y, Zhang M, Zhang J, Tian X, Duan L and Li Z 2012 J. Exp. Bot. 63 3741-8
[19] Xiong L, Lee B-H, Ishitani M, Lee H, Zhang C and Zhu J-K 2001 Genes Dev. 15 1971-84
[20] Alves MS, Reis PAB, Dadalto SP, Faria JAQA, Fontes EPB and Fietto LG 2011 J. Biol. Chem. 286 20020-30
[21] Bartels D and Sunkar R 2005 CRC Crit. Rev. Plant Sci. 24 23-58
[22] Battaglia M, Olvera-Carrillo Y, GarciaRRubio A, Campos F and Covarrubias AA 2008 Plant Physiol. 148 6-24
[23] Mackova H, Hronkova M, Dobra J, Tureckova V, Novak O, Lubovska Z, et al. J. Exp. Bot. 64 2805-15
[24] Vysotskaya LB, Veselov SY and Kudoyarova GR 2010 J. Exp. Bot. 61 3709-17
[25] Zhuo C, Wang T, Guo Z and Lu S 2016 BMC Plant Biol. 16 138
[26] Hanin M, Brini F, Ebel C, Toda Y, Takeda S and Masmoudi K 2011 Plant Signal. Behav. 6 1503-9
[27] Masuta C, Furuno M, Tanaka H, Yamada M and Koiwai A 1992 FEBS Lett. 311 119-23
[28] Boutrot F, Chantret N and Gautier MF 2008 BMC Genomics 9 86
[29] Arondel V, Vergnolle C, Cantrel C and Kader J-C 2000 Plant Sci. 157 1-12
[30] Zhou R, Yu X, Ottosen C-O, Rosenvist E, Zhao L, Wang Y, et al. 2017 BMC Plant Biol. 17 24
[31] Jung H W, Kim W and Hwang B K 2003 Plant Cell Environ. 26 915-28
[32] Ramakrishna A and Ravishankar G A 2011 Plant Signal. Behav. 6 1720-31
[33] Buhot N, Gomes E, Milat M-L, Ponchet M, Marion D, Lequeu J, et al. 2004 Mol. Biol. Cell 15 5047-52
[34] Colmenero-Flores JM, Campos F, GarciaRRubio A and Covarrubias A A 1997 Plant Mol. Biol. 35 393-405