Moderate level of toxic boron causes differential regulation of microRNAs related to jasmonate and ethylene metabolisms in Arabidopsis thaliana

DOĞA SELİN KAYIHAN

CEYHUN KAYIHAN

YELDA ÖZDEN ÇİFTÇİ

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Doğa Selin KAYIHAN[1], Ceyhun KAYIHAN[2], Yelda ÖZDEN ÇİFTÇİ[1],*

[1]Department of Molecular Biology and Genetics, Faculty of Science, Gebze Technical University, Çayırova, Kocaeli, Turkey
[2]Department of Molecular Biology and Genetics, Faculty of Science and Letters, Başkent University, Etimesgut, Ankara, Turkey

Abstract: Earlier our colleagues detected that the genes related to jasmonate (JA), ethylene, and cell wall modification were significantly regulated under boron (B) toxicity in wheat. Determination of regulation mechanisms of these novel genes under B toxicity is very important in *Arabidopsis thaliana* as a model plant. As key regulators, the microRNAs (miRNAs) regulate gene expression at the posttranscriptional level and respond to numerous abiotic stresses in plants. In this study, expression levels of miRNAs such as miR159, miR172, miR319, and miR394 targeting JA and ethylene-related transcription factors and also miR397 targeting laccase were determined in *Arabidopsis thaliana* under toxic B conditions. Stem-loop quantitative reverse transcription polymerase chain reaction was used to amplify mature miRNAs for expression analyses. Expression levels of miRNAs targeting transcription factors related to JA and ethylene metabolisms were induced remarkably in moderate B toxicity (condition 1B) but not in severe B toxicity (condition 3B). Most remarkable regulations were obtained in miR172 and miR319 in *Arabidopsis thaliana*. Expression level of miR397 did not remarkably change under B toxicity, indicating a lack of posttranscriptional regulation of laccase related to cell wall modification. Moreover, miRNAs targeting transcription factors related to JA and ethylene metabolisms might be oxidative stress-adaptive responses of *Arabidopsis* to B toxicity.

Key words: *Arabidopsis thaliana*, boron toxicity, ethylene, jasmonate, miRNA, posttranscriptional regulation

1. Introduction

Boron (B) is an essential micronutrient for plants (Warington, 1923). However, a high level of B is one of the important abiotic stress factors in the world and it negatively affects plant development and crop yield (Landi et al., 2012). Many countries, especially those having arid or semiarid soils, are suffering from yield loss due to excess B (Nable et al., 1997). B can be readily toxic for many plants even when the B level is only slightly higher than required for growing (Mengel and Kirkby, 2001). B toxicity causes alteration of cell wall structure and disruption of cell division and development due to B binding to the ribose moieties of biological molecules (Reid et al., 2004). Typically, toxic B leads to inhibition of shoot and root growth, and to leaf burn in the tips and margins of old leaves, which is distinguished by chlorotic or necrotic zones as visible symptoms (Bennett, 1993; Fitzpatrick and Reid, 2009).

To date, numerous studies investigating components of B stress-responsive mechanisms in plants have been conducted at physiological and biochemical levels. In these

physiobiochemical studies, increased level of anthocyanin and flavonoid in tomato (Cervilla et al., 2012), higher phenolic content in sweet basil (Pardossi et al., 2015), and significant increases in activities of superoxide dismutase (SOD ; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and ascorbate peroxidase (APX; EC 1.11.1.11)) in chickpea (Ardic et al., 2009), and soybean (Hamurcu et al., 2013) were determined under toxic B conditions. On the other hand, recent molecular studies via omics technologies have provided important insight**s** into responses of toxic B and possible relationships with biological pathways (Öz et al., 2009; Kayıhan et al., 2017). Importantly, they proposed that B homeostasis is regulated by changes in transcription factors such as WRKY, MYB, and NAC. It was also found that B toxicity induced jasmonate (JA)- and ethylene-related genes in wheat (Kayıhan et al., 2017) and barley (Öz et al., 2009). Also, many genes associated with cell wall modification were significantly regulated in wheat under B toxicity conditions (Kayıhan et al., 2017). Interestingly, the genes related to JA, ethylene, and cell wall modification were significantly regulated in only

* Correspondence: yelda75@yahoo.com

a sensitive wheat cultivar when compared to a tolerant wheat cultivar (Kayıhan et al., 2017). These genes might participate in the B toxicity response in plants. However, answering the question of how genes are regulated during stress is as important as finding novel genes (Zhang, 2015). Hence, microRNAs (miRNAs), as key regulators of gene expression in a posttranscriptional manner, are good candidates for exploring the stress network. These noncoding small RNAs, with a nucleotide length of 21 to 24, have a wide distribution in plants. To date, only two studies related to determination of B toxicity-responsive miRNAs were performed in plants. These included a high-throughput sequencing strategy in barley (Ozhuner et al., 2013) and *Citrus* (Huang et al., 2016). In spite of many advantages of miRNA sequencing, there is a need to further evaluate these noncoding transcripts for functional relevance (Chugh and Dittmer, 2013). Stem-loop (SL) quantitative reverse transcription polymerase chain reaction is an important strategy to detect and amplify mature miRNAs (Kramer, 2011; Gautam et al., 2016). The SL primer is designed as a hairpin structure and it has a 3′ overhang complementary to the miRNA. Then miRNA-specific primers and a universal primer are used for amplification of mature miRNA in PCR (Balcells et al., 2011).

In this study, by means of the SL method, expression levels of miRNAs such as miR159, miR172, miR319, and miR394, targeting JA- and ethylene-related transcription factors, and also miR397, targeting laccase (related to cell wall modification), were determined in *Arabidopsis thaliana* exposed to 1 mM and 3 mM boric acid. To the best of our knowledge, our work is the first report on SL analysis that demonstrates the quantification of miRNAs in plants exposed to toxic B.

2. Materials and methods

2.1. Growth conditions and boron treatments
Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) seeds were surface-sterilized with 70% EtOH solution for 2 min and then with 15% NaOCl for 10 min. The seeds were rinsed three times with distilled water and finally transferred to MS media (Murashige and Skoog, 1962) including normal (100 μM) and toxic levels of B (1 mM and 3 mM H_3BO_3). Following the vernalization period for 3 days at 4 °C, germination and growth were carried out at 22 °C in a growth chamber providing a 16/8-h light photoperiod. After 2 weeks, seedlings were harvested and used for further analyses.

2.2. Quantitative real-time PCR conditions
miRNA expression was determined by quantitative real-time PCR (qRT-PCR) method (Varkonyi-Gasic et al., 2007). Firstly, mixtures of 12 μL involving 1 μg of RNA, RNase-free water, and 2 μM SL primer mix were prepared.

They were then incubated for 5 min at 65 °C followed by incubation on ice for 2 min. Subsequently, 5X reaction buffer, RiboLock RNase inhibitor (20 U/μL), 10 mM dNTP, and reverse transcriptase were added to the mixtures. They were incubated at 16 °C for 30 min followed by pulsed reverse transcription of 60 cycles at 30 °C for 30 s, 42 °C for 30 s, and 50 °C for 1 s. The tubes were subsequently incubated for 5 min at 70 °C. For qRT-PCR analysis, 1 μL of cDNA, 7 μL of 2X Master Mix (Thermo Scientific), and 0.3 μM final concentration of primers were supplied to a total volume of 12 μL with nuclease-free water. The qRT-PCR conditions were initiated with denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s. The melting curve was analyzed at 60–95 °C after 40 cycles.

Normalization was made by using the actin gene (*ACT2*) (Kayıhan et al., 2016) and $2^{-\Delta Ct}$ was used for determination of fold change of each comparison. The miRBase database was used to obtain miRNA sequences. SL-reverse transcription and forward primers were specifically designed according to the protocol of Varkonyi-Gasic et al. (2007). Primer sequences for miRNAs are given in the Table.

2.3. Data statistics
Experiments were performed as four biological replicates (n = 4). The data were statistically analyzed by using nonparametric versions of the t-test. They were shown as mean with standard error (SE).

3. Results
The expression level of miR159 was increased twofold under 1B treatment; however, it was stable under 3B treatment as compared to the control (Figure). The 1B treatment caused a threefold increase in miR319 expression, whereas a 1.3-fold increase in miR319 expression was determined under 3B treatment in *Arabidopsis thaliana*. Moreover, there was a significant difference between miR319 expressions of 1B and 3B conditions. Following 1B treatment, miR394 expression was increased more than 2.5-fold; however, it was slightly increased after 3B treatment, but not significantly. Similarly, miR172 expression was induced remarkably (almost fourfold) by the 1B condition, whereas it was slightly increased in the 3B condition. Also, there was a significant difference between miR172 expressions of 1B and 3B conditions. Moreover, a 1.3-fold increase was observed in miR397 expression under 1B and it was increased 1.4-fold after 3B when compared to control conditions. However, there was no significant difference between miR397 expressions of the 1B and 3B conditions (Figure).

4. Discussion
After plant miRNAs were discovered as important posttranscriptional regulators in 2002 (Llave et al., 2002)

Table. Primer list for *Arabidopsis thaliana* miRNAs.

Primer name	Sequence 5′ to 3′
miR397_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATCAA
miR397_F	CGATATTCATTGAGTGCAGCG
miR172_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATGCAG
miR172_F	CGGCGGAGAATCTTGATGATG
miR159_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAGAGC
miR159_F	CGGCGGTTTGGATTGAAGGGA
miR319_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGAG
miR319_F	CGGATATTGGACTGAAGGGAG
miR394_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGGT
miR394_F	GGTAGTTTGGCATTCTGTCC
Universal_R	GTGCAGGGTCCGAGGT
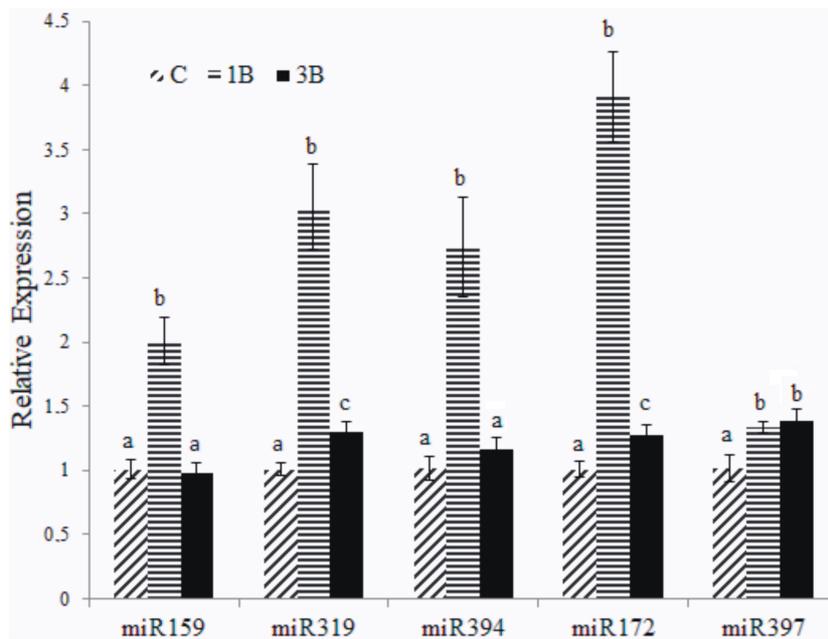
Figure. Relative expression level of miRNAs in *Arabidopsis thaliana* in response to toxic B. C: Control, 1B: 1 mM H_3BO_3, 3B: 3 mM H_3BO_3. Values are mean ± SE (n = 4). Different letters indicate significant differences for each miRNA (P < 0.05).

and suggested to have important roles in plant growth and development (Bartel, 2004), further investigations revealing their contribution to environmental stress responses (Jones-Rhoades and Bartel, 2004; Zhang et al., 2005) made miRNAs a hot topic. These studies suggest that they are involved in the adaptive responses of plants to numerous environmental stresses (Hsieh et al., 2009; Wu et al., 2010). However, only a few miRNAs that change in response to B toxicity have been determined in barley (Ozhuner et al., 2013) and *Citrus* (Huang et al., 2016).

To date, no reports on toxic B-responsive miRNAs have been published for *Arabidopsis thaliana*. In this study, the expression level of miR397 was first determined in *Arabidopsis thaliana* under 1 and 3 mM B conditions. It was suggested earlier that excess B could alter cell wall structure (Reid et al., 2004; Ghanati et al., 2005). Supportively, Kayıhan et al. (2017) determined differential regulation of genes related to cell wall modification in wheat cultivars. Therefore, we investigated the regulations of miR397 targeting the laccase gene, *LAC*, which encodes

a Cu-containing enzyme involved in secondary cell wall integrity while plants are responding to an abiotic stress (Liang et al., 2006). However, the miR397 expression level was not dramatically changed under either B toxicity condition. Thus, we can suggest that B toxicity might not affect cell wall modification at the posttranscriptional level in *Arabidopsis thaliana*. Contrarily, remarkable induction of miR397 in a B-sensitive *Citrus* cultivar in response to high B supply was recently revealed (Huang et al., 2016). Therefore, further investigations are required to understand the differential regulation of miR397 in plants.

B toxicity was previously found to induce JA- and ethylene-related genes (Öz et al., 2009; Kayıhan et al., 2017). Thus, the expression levels of miR159, miR172, miR319, and miR394 were assessed in *Arabidopsis thaliana* in order to understand posttranscriptional regulation of JA- and ethylene-related transcription factors under high B conditions. First, miR172 was upregulated by 1B. It regulates flowering time and floral organ identity in *Arabidopsis thaliana* by targeting APETALA2/ETHYLENE RESPONSE FACTORS (AP2/ERFs) (Zhao et al., 2007). These transcription factors, as key components of ethylene signaling cascades, contribute to homeostasis by negative or positive regulations in response to environmental stresses (Phukan et al., 2017). In addition to ethylene-responsive genes, JA-responsive genes are regulated by ERFs (Ou et al., 2011). miR159, miR319, and miR394 were among the JA-related miRNAs. According to psRNATarget analyses, miR159, miR319, and miR394 target MYB (Myeloblast), TCP (Teosinte branched 1, Cycloidea, and Proliferating cell nuclear antigen factors), and F-box transcription factors in *Arabidopsis thaliana*, respectively. In this study, the expression levels of these JA-related miRNAs were dramatically increased under the 1B condition. However, they were dramatically decreased in the 3B condition when compared to the 1B condition. On the other hand, the expression levels of miR159 and miR319 were specifically repressed by carbon deficiency condition in *Arabidopsis thaliana* (Liang et al., 2015). miR319 is one of the negative regulators of the leaf senescence mechanism in plants and the miR319-regulated clade of *TCP* transcription factor genes facilitates the biosynthesis of JA, which then accelerates leaf senescence (Schommer et al., 2008; Liang et al., 2015). Liang et al. (2015) suggest that carbon starvation induces leaf senescence by suppressing the expression of miR319. In this study, 1 mM B might not have induced the leaf senescence mechanism due to upregulation of miR319 expression. Instead, miR319 might positively regulate leaf growth under 1 mM B because TCP transcription factors, the target of miR319, function throughout leaf development to coordinate the balance between leaf growth, which they negatively regulate, and leaf senescence, which they positively regulate. On the

other hand, 3 mM B might induce the leaf senescence mechanism due to stable levels of JA-related miRNAs, mainly miR319. Supportively, Schommer et al. (2008) suggested an altered senescence behavior and miR319/TCP transcription factors have a role in this dynamic process. Likewise, genes related to protein degradation were found to be differentially expressed following B toxicity stress in wheat cultivars, suggesting programmed cell death in senescing leaves (Kayıhan et al., 2017). As a result, severe B toxicity stress (3B) can induce the leaf senescence mechanism in *Arabidopsis thaliana*. The visual phenotype of the plants confirmed this interpretation because partial yellowing and growth inhibition were gradually increased with increasing toxic B level (data not shown). Therefore, differential regulation of JA- and ethylene-related miRNAs might also be associated with toxic B-mediated oxidative stress because in our previous report a higher lipid peroxidation level was observed in 1B treatment than 3B in *Arabidopsis thaliana* (Kayıhan et al., 2016). Similarly, Hamurcu et al. (2013) found higher antioxidant activity under 12 mg B kg^{-1} than under 2 mg B kg^{-1} and thus malondialdehyde content as a lipid peroxidation marker increased under 2 mg B kg^{-1} but decreased under 12 mg B kg^{-1} in soybean. Also, in our previous report, lower increment in SOD activity and H$_2$O$_2$ content was observed after 1B and this was verified by lower increase in expression of *MSD1* and *CSD1* and higher increase in expression of miR398 targeting the *CSD* gene under 1B (Kayıhan et al., 2016). This means that posttranscriptional regulation of JA and ethylene metabolisms might be coordinately regulated with posttranscriptional regulation of SOD in *Arabidopsis thaliana* under B toxicity. For this reason, differential regulations of miR159, miR319, miR394, and miR172 under only 1B condition may be an oxidative stress-adaptive response rather than a protective antioxidant mechanism in *Arabidopsis thaliana*.

In conclusion, in this study, expression levels of miRNAs targeting transcription factors related to JA and ethylene mechanisms were determined in *Arabidopsis thaliana* exposed to toxic B conditions. Accordingly, leaf senescence might be induced by 3 mM B toxicity but not 1 mM B toxicity as verified by differential regulation of JA- and ethylene-related miRNAs. Also, posttranscriptional regulation of JA and ethylene metabolisms might coordinately be regulated with antioxidant machinery in plants exposed to toxic B because our findings were in accordance with our previous results including biochemical and transcriptional regulations of protective antioxidant mechanism under toxic B.

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