1 **Barley Long Non-Coding RNAs and Their Tissue-Specific Co-expression Pattern**
2 **with Coding-Transcripts**

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1 **Abstract**

2 Long non-coding RNAs (lncRNA) with non-protein or small peptide-coding potential
3 transcripts are emerging regulatory molecules. With the advent of next-generation
4 sequencing technologies and novel bioinformatics tools, a tremendous number of
5 lncRNAs has been identified in several plant species. Recent reports demonstrated roles
6 of plant lncRNAs such as development and environmental response. Here, we reported
7 a genome-wide discovery of ~8,000 barley lncRNAs and measured their expression
8 pattern upon excessive boron (B) treatment. According to the tissue-based comparison,
9 leaves have a greater number of B-responsive differentially expressed lncRNAs than the
10 root. Functional annotation of the coding transcripts, which were co-expressed with
11 lncRNAs, revealed that molecular function of the ion transport, establishment of
12 localization, and response to stimulus significantly enriched only in the leaf. On the
13 other hand, 32 barley endogenous target mimics (eTM) as lncRNAs, which potentially
14 decoy the transcriptional suppression activity of 18 miRNAs, were obtained. Presented
15 data including identification, expression measurement, and functional characterization
16 of barley lncRNAs suggest that B-stress response might also be regulated by lncRNA
17 expression via cooperative interaction of miRNA-eTM-coding target transcript
18 modules.

19
20 **Keywords:** Boron, endogenous target mimicry, *Hordeum vulgare*, long non-coding
21 RNA, novel transcript discovery and annotation

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1 **1. Introduction**

2

3 Long non-coding RNAs (lncRNA) are known as non-protein coding transcripts longer
4 than 200 nt. Although, the information about their functions is so limited, studies
5 revealed that they have several direct and indirect roles in the transcriptional, post-
6 transcriptional or post-translational processes such as gene expression, chromatin
7 modification, transcriptional regulation, and conformational changes in proteins
8 (reviewed by Liu et al., 2015). They act as the precursor of micro-RNA (miRNA) and
9 short interfering-RNA (siRNA). For instance, five lncRNA in Arabidopsis (npc34,
10 npc351, npc375, npc520, and npc523) matched with 24-nt siRNAs from both strands,
11 suggesting these lncRNAs are siRNA precursor. Moreover, it was reported that plant
12 miRNAs, miR869a and miR160c, which were derived from lncRNAs of npc83 and
13 npc521, respectively (Ben Amor et al., 2009). Additionally, it was also discovered that
14 miR675 is derived from a mouse lncRNA, H19, and extensively expressed in
15 embryonic liver (Dey et al., 2014; Keniry et al., 2012).

16

17 Recently, short peptide-coding sequences were discovered in the non-coding regions of
18 plant primary-miRNAs (pri-miRNA) and called as miRNA-encoded peptide (miPEP),
19 which increases the transcription of pri-miRNA (Lauressergues et al., 2015; Waterhouse
20 and Hellens, 2015). Therefore, it was suggested that some plant lncRNAs might have
21 peptide coding potential (Liu et al. 2015). In addition, miRNA activity can be regulated
22 by endogenous target mimicry (eTM) molecules, being a type of lncRNAs (Karakulah
23 et al., 2016). Such an example, an endogenous lncRNA called *Induced by Phosphate*
24 *Starvation 1 (IPS1)* of *Arabidopsis thaliana* binds to miR399 to inhibit the cleavage of

1 the miR399 target transcript (Franco-Zorrilla et al., 2007). Other than these functions,
2 plant lncRNAs were found to be involved in many regulatory mechanisms, such as
3 histone modeling (Heo and Sung, 2011), promoter modification (Ding et al., 2012;
4 Zhou et al., 2012), protein re-localization (Sousa et al., 2001), and alternative splicing
5 (Bardou et al., 2014).

6
7 To date, lncRNAs have been identified extensively in mammals, in which human
8 genome includes more than 56,000, and mice have almost 46,000 lncRNAs (Xie et al.,
9 2014). Publicly available databases such as LncRNADB (<http://lncrnadb.com>), a
10 database for functional lncRNAs, harbor functionally annotated lncRNAs, of the
11 majority belong to the human. A few databases were so far released for plant lncRNAs;
12 such as the Green Non-Coding Database (GreeNC, <http://greenc.sciencedesigners.com>)
13 (Paytuví Gallart et al., 2016) and CANTATAdb (<http://cantata.amu.edu.pl>) (Szczesniak
14 et al., 2016), which provides information for around 45,117 lncRNAs from several plant
15 species. RNA-sequencing or deep transcriptome analysis is an important technology,
16 which provides information not only for protein coding transcripts, but also for non-
17 coding RNAs (such as miRNA, siRNA, piwi-interacting RNA (piRNA), and small-
18 nucleolar RNA (snoRNA) as well as lncRNAs). In addition, it allows distinguishing
19 lncRNAs expressed in different tissues or cells.

20
21 Boron (B) is an essential micronutrient for plants, and its unfavorable concentration
22 negatively affects plant growth and productivity where the soils having with insufficient
23 or excess B (Unver et al., 2008). The range of sufficient B concentration in soil is so
24 limited, thus generally plants suffer from either B-deficiency or -toxicity problem that is

1 common in agricultural soils of around the world (Camacho-Cristobal et al., 2015).
2 However, plants growing in B-contaminated soils must tolerate the excess level of B to
3 survive. In the last decade, many studies conducted to understand the cellular
4 mechanisms underlying to balance cellular B content in plants (Miwa and Fujiwara,
5 2010; Tombuloglu et al., 2015). Facilitated transport of boric acid (a regular form of B
6 in soil) by transporter channels was suggested to be the molecular regulatory
7 mechanism. Several B-importer and exporter proteins have recently been identified as
8 B-transporters to regulate its cellular homeostasis (Miwa and Fujiwara, 2010). On the
9 other hand, novel sequencing-based approaches to discover the transcriptional response
10 at genome-wide level are being extensively utilized in plants faced with unfavorable
11 environmental conditions. In this context, to quantify gene expression and to annotate
12 coding-transcripts, we performed a high-throughput genome-wide transcriptome
13 analysis on barley tissues treated with excess B (1 mM), previously (Tombuloglu et al.,
14 2015). In addition, we also screened B-responsive miRNA expression pattern (Ozhuner
15 et al., 2013); identified MYB type transcription factors (TF) (Tombuloglu et al., 2013)
16 and water channel Aquaporins (AQP) (Tombuloglu et al., 2016) to understand B
17 homeostasis of barley. These studies helped to observe the main or possible players
18 involved in B regulation. Besides the emerging evidences suggested that the molecular
19 regulatory mechanism is so complicated and not only limited to activity of those coding
20 transcripts. LncRNAs being the new players were also be discovered as regulatory
21 molecules on the regulation of gene expressions.

22

23 To date, a large set of RNA-seq libraries was used to identify lncRNAs in genome-wide
24 scale or tissue/condition/inoculation-specific manner (Chen et al., 2016; Li et al., 2014;

1 Liu et al., 2012). Stress-responsive lncRNAs were examined from the RNA-seq data of
2 the plants under the distinct type of stress conditions as well (reviewed by Chekanova,
3 2015; Shafiq et al., 2016; Zhang et al., 2013). Qi et al., 2013 identified 584 lncRNAs,
4 which were responsive to drought stress in foxtail millet. 125 putative lncRNAs were
5 identified in wheat, responsive to powdery mildew infection and heat stress (Xin et al.,
6 2011). Detailed examination of a large set of poplar (*Populus trichocarpa*) RNA-seq
7 data revealed 504 lncRNAs in response to drought (Shuai et al., 2014). Additionally,
8 Huang et al reported over 12,000 barley lncRNAs, of them 604 were *Fusarium* head
9 blight inoculation responsive (Huang et al., 2016). However, no such a study to profile
10 expression level of lncRNAs under the B-excess as one of the abiotic stress conditions
11 was conducted till now. In this study, we identified and quantitatively compared the
12 expression of B-responsive barley lncRNAs from four transcriptome datasets. Tissue-
13 specific (root and leaf) and excess B-responsive lncRNAs, which were co-expressed
14 with coding-transcripts, discovered and comprehensively analyzed.

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2. Materials and Methods

2.1. Identification of barley lncRNAs

To study global expression profiling of barley lncRNAs under boron stress condition, we utilized four transcriptome libraries each including pooled RNAs from three biological replicate previously generated by our group (SUB337351 and SUB2170217) (Tombuloglu et al., 2015). Briefly, a total of 208,249,690 clean sequencing reads from four paired-end libraries (50_leaf; 52, 422,032, 50_root; 52,168,358, 1000_leaf; 52,305,062, and 1000_root, 51,354,238) were utilized in this study. We first removed the adapter sequences and low-quality reads from the sequencing reads with Trimmomatic v0.36 (Bolger et al., 2014), and these clean reads were aligned to the barley reference genome (ASM32608v1 assembly) by TopHat2 v2.1.1 with default parameters (Kim et al., 2013). Afterward, genome-aligned reads were assembled *ab initio* using popular transcriptome analysis suit Cufflinks v2.2.1 (Trapnell et al., 2010) to build potential transcript structures. All gene transfer format (GTF) files produced in the assembly step were merged with Cuffmerge tool, and transcript features were queried against the Ensembl Plant database (release 33) (Kersey et al., 2016) by Cuffcompare to discover previously unannotated transcript sequences. Among all unannotated transcripts, including coding and non-coding sequences, we obtained lncRNAs as follows: (i) we first tested coding potential of each transcript individually using TransDecoder (<https://transdecoder.github.io/>), and filtered out those with an open reading frame having more than 100 amino acids, (ii) we then removed transcripts which were shorter than 200 nucleotides in length, (iii) to remove housekeeping lncRNA species, we queried all potential lncRNAs against to non-coding RNA family database, Rfam (v12.1) (Nawrocki et al., 2015), with Infernal tool (v1.1.1; cutoff E-value ≤ 0.001) (Nawrocki et al., 2009). Then, potential miRNA precursors, tRNAs etc were removed and were not included for further analysis, and (iv) we aligned all potential lncRNA transcripts to the Swiss-Prot database (release 2017_01) (The UniProt, 2017) using Blastx (v2.5.0; cutoff E-value ≤ 0.001) (Camacho et al., 2009) to eliminate transcripts with potential protein-coding ability.

1 2.2. Expression pattern analysis of coding and non-coding RNAs

2 Transcript abundances in each library were measured with Kallisto v0.43.0 (Bray et al.,
3 2016). Then the transcripts expressed <1 Transcripts Per Million (TPM) in all libraries
4 were considered as transcriptional noise and were removed from further downstream
5 analysis steps. Differentially expressed transcripts within each group (leaf and root
6 samples) were determined by calculating fold changes of TPM values in RNA-seq
7 datasets. Transcripts with differential expression values ≥ 2 fold-changes in compared
8 datasets were classified as boron responsive. The Gene Ontology (GO) analysis of
9 differentially expressed genes was performed using online GO analysis toolkit, agriGO
10 applying default parameters (Du et al., 2010).

11

12 2.3. Co-expression analysis of lncRNAs with coding mRNAs and prediction of 13 endogenous target mimicry (eTM) sequences

14 We predicted putative functions of differentially expressed lncRNAs with “guilt-by-
15 association” approach, which employed in previous studies for lncRNA annotation
16 (Rinn and Chang, 2012) (Guo et al., 2013) (D’Haene et al., 2016). To reveal potential
17 lncRNA-mRNA associations, we identified co-expressed mRNA-lncRNA pairs with
18 Spearman's correlation test in R v3.1.0 statistical computation environment (Team,
19 2016). Then, co-localized mRNA-lncRNA pairs on the reference genome were
20 identified with Bedtools v2.25.0 (Quinlan, 2014). We considered the mRNA-lncRNA
21 pair as co-expressed if the Spearman's rho is equal or greater than 0.90 ($p\text{-val} < 0.01$)
22 between the expression values of coding and lncRNA transcripts, and as co-localized
23 when the distance between two transcripts were less than 10 kb. To dissect putative
24 eTM sequences among the transcripts annotated as lncRNA, we employed our analysis

1 pipeline previously introduced by our group (Karakulah et al., 2016). In the eTM
 2 sequences analysis pipeline, we utilized mature miRNA sequences of barley collected
 3 from miRBase (release 21) (Griffiths-Jones, 2006). To identify potential target
 4 transcripts of barley miRNAs, we utilized psRNATarget, an online miRNA target
 5 analysis tool (Dai and Zhao, 2011) as previously described previously (Akdogan et al.,
 6 2016; Bakir et al., 2016; Eldem et al., 2012; Inal et al., 2014; Yanik et al., 2013).

7

1 **3. Results and discussion**

2

3 *3.1. Barley lncRNA identification*

4 After the adapter sequence trimming and removal of low-quality reads, the mean library
5 size of four sequencing libraries included in the study was over 20 million (min:
6 18223418, max: 22171430). Additionally, we observed an average of 82.35 % (min:
7 78.7 %, max: 90.9 %) overall read mapping for the alignment step, and considered all
8 sequencing libraries had sufficient quality to perform an *ab initio* transcriptome
9 reconstruction analysis. The run of Cufflinks pipeline was led to the identification of as
10 many as 34,000 previously unannotated intergenic transcripts, of which 10,439 were the
11 lack of coding potential and more than 200 bp in length (Table S1). When we filtered
12 out the transcripts expressed at low levels (< 1 TPM in all samples), we obtained 8,009
13 intergenic putative lncRNAs in the final list, which were distributed almost equally to
14 all barley chromosomes (Figure 1A). However, chromosome 2 was observed to be the
15 richest one in terms of the total number of lncRNAs it harbors. In this study, a total
16 number of ~8,000 barley lncRNAs were identified which is smaller than that of human
17 (~56,000) (Xie et al., 2014) and mouse (~46,000); higher than fruit fly (~3,300) (Chen
18 et al., 2016), and poplar (2,542) (Shuai et al., 2014) (Table S1). Actual numbers of
19 lncRNAs can be altered depending on sample examined. In this analysis, four RNA-Seq
20 libraries were used to detect total lncRNAs. More lncRNAs can be found from barley
21 genome by increasing the number of RNA-Seq sets from distinct tissues and/or
22 conditions. In general, low expression levels of most lncRNAs compared to protein-
23 coding genes make it more difficult to detect lncRNAs (Mercer et al., 2011). Generally,
24 they are excluded from the total lncRNA pool resulting fluctuations of total lncRNA

number. But it is important to note that lncRNAs with low expression may have a big impact, thus extensive and a deep pipeline is required to extract lncRNA, which may possess important biological functions. In general, the distribution of lncRNAs to the barley chromosomes is proportional with its chromosome sizes, except chromosome 2, which includes the highest number (Fig1A).

3.2. Expression pattern of barley lncRNAs and coding transcripts upon excess B-treatment

As the expression profiles of lncRNAs in root and leaf samples were examined, it was determined that expression levels of lncRNAs in the samples collected from same tissue were similar to one another (Figure 1B). The hierarchical clustering analysis, however, revealed that particular lncRNA clusters were expressed at relatively higher levels specific to tissue types (Figure 1C). Differential expression analysis of both coding and lncRNA transcripts showed that there was 2 fold or more change (up- or down-regulation) in the expression of the vast amount of transcripts in response to boron stress in leaves and root tissues (Table S2). We observed that the number of common coding transcripts that were differentially regulated in both tissues was 517; in addition, the total number of differentially expressed coding transcripts in the leaf tissue was roughly doubled as compared to root tissue (Figure 2A). Similar to the differential expression analysis of coding transcripts, we detected a greater number of up- or downregulated lncRNAs in the leaves than the root samples (Figure 2B).

3.3. Functional annotation of lncRNAs co-expressed with coding transcripts

1 The GO enrichment analysis of differentially expressed coding transcripts (Table S3)
2 revealed that ion transport (GO:0006811), establishment of localization (GO:0051234),
3 and response to stimulus (GO:0050896) terms significantly enriched (FDR<0.05) only
4 in the leaf samples (Figure 2C). In addition to this, molecular function terms of ligase
5 activity (GO:0016874) and cysteine-type peptidase activity (GO:0008234) were
6 significant and specific to the leaves (Figure 2D). Based on the co-expression and co-
7 localization analysis of barley lncRNAs and coding transcripts, we observed potential
8 lncRNAs in association with the ion transports, establishment of localization, and
9 response to stimulus related transcripts (Figures 3 and 4). We determined 6 lncRNAs,
10 which strongly linked to ion transport related genes (Figure 3A). However, only one
11 lncRNA (TCONS_00061958) was showing ≥ 2 fold expressional change in both the
12 leaf and root samples (Figure 3B). A great majority of response to stimulus related
13 lncRNAs increased (≥ 2 fold) their expression in leaf samples upon the boron exposure
14 (Figure 3C-D).

15 Additionally, we detected 12 lncRNA sequences, which had similar expression patterns
16 and co-localized with “establishment of localization” related genes (Figure 4). We also
17 detected that some of the lncRNAs were only differentially expressed in tissue-specific
18 manner such as TCONS_00002116 expression in leaf under B-excess (Figure 4B).
19 However, TCONS_00061958 was found to be differentially regulated in both leaf and
20 root tissues. Studies also revealed the differential expression pattern of plant lncRNAs
21 such as Arabidopsis, wheat, and poplar in response to biotic and abiotic stresses (Liu et
22 al., 2012; Shuai et al., 2014; Xin et al., 2011). Here, we identified that barley boron-
23 responsive lncRNAs are expressed in the tissue-specific manner (Figures 2-4 and Table
24 S2). According to GO term enrichment analysis, differentially expressed coding

transcripts were categorized into three biological processes: response to stimulus (50 transcripts), ion transport (17 transcripts), and establishment of localization (52 transcripts), which were leaf specific in this analysis (Fig 2C and Table S3). These findings were consistent with our previous report (Tombuloglu et al., 2015), where the biological process, ion transport, and establishment of localization categories were found to be enriched in both leaf and root tissues.

3.4. eTM sequence discovery

Here, we predicted 32 barley eTMs, which might decoy the transcriptional suppression activity of 18 miRNAs, including conserved barley miRNAs such as miR159a, miR166a, and miR399 (Table 1 and Table S4). In the GO enrichment analysis of miRNA target genes using the agriGO tool, we found 102 significant (FDR<0.05) different GO terms in three domains, consisting of molecular function, biological process, and cellular component (Table S5). Among the terms, the most significant one was “protein amino acid phosphorylation” term (GO:0006468, FDR= 4.70E-58). Our findings in eTM analysis suggest that barley lncRNAs might regulate several distinct cellular and molecular processes via mimicking specific miRNA target transcripts. As it was previously reported by recent studies, lncRNAs might behave as a mimicry-transcript that targeted by miRNAs and fate it to degradation (Juan et al., 2013). It was firstly reported in Arabidopsis, over-expression of non-coding gene *IPS1* suppressed the miR399 expression that resulted in elevated expression of the miR399 target (Franco-Zorrilla et al., 2007). On the other hand, we determined and measured the boron-responsive barley miRNAs (Ozhuner et al., 2013). Accordingly, miR5049 was down-regulated in B-stressed leaf (three times than control leaf). Also, miR399 was over-

1 expressed in leaf and suppressed in root tissue upon B-exposure (three times of each
2 tissue than that of control). In this study, miR5049 and miR399 were also found to be as
3 regulated miRNAs upon B-treatment by eTM analysis (Table S4) where
4 TCONS_00032652 and TCONS_00043651 have predicted as the target mimic
5 sequences, which are able to decoy the miRNA activities, respectively. Thus, expression
6 of transcripts targeted by these miRNAs might be altered due to differential expressions
7 of lncRNAs. *Phosphate transporter 2 (PHO2)* and *putative ubiquitin-conjugating*
8 *enzyme (UBC)* were found to be the target genes of miR399. Also, *tubby protein-like*
9 transcript was determined as the miR5049 target. In the transcriptome analysis,
10 ubiquitin carboxyl-terminal hydrolase gene was highly up-regulated in leaf tissue upon
11 excess B treatment (Tombuloglu et al., 2015). Interestingly, expression profiles of
12 miRNA and its corresponding lncRNA target mimic transcript provide insights into the
13 regulation of B-stress in plants. For instance, lncRNA TCONS_00043651, a potential
14 target mimic sequence of miR399, up-regulated in roots (three times than that of
15 control) upon B-exposure. Oppositely, miR399 expression was reflected with the same
16 pattern in a negative direction (three times down-regulated). Similarly, five times
17 increase of lncRNA TCONS_00043651 in leaf tissues may prevent the expression of
18 miR399, which was up-regulated only three times (Table S2). These preliminary results
19 suggest that boron regulation can be cooperatively controlled by the interaction of
20 miRNA-eTM (lncRNA)- coding target transcript modules.

21

4. Conclusion

With the development of next-generation sequencing technologies and advancement in bioinformatics, more transcriptional datasets were generated including the units with little or no protein-coding potential. In recent years, the lncRNAs considered as regulatory molecules in several bioprocesses. Though a large number of lncRNA transcripts were identified in plants, no such genome-wide study was conducted for barley as an important crop. Another missing biological hypothesis is that the possible involvement of lncRNAs in boron-response mechanism. Here, we reported the genome-wide discovery of ~8,000 barley lncRNAs and measured their expression pattern upon excessive boron (B) treatment. Furthermore, we functionally annotated the coding transcripts, which are co-expressed with lncRNAs and showed that cooperative interaction of miRNA-eTM (lncRNA)- coding target transcript modules might regulate the boron-response in barley.

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3

4 **Author Contributions**

5 GK and TU organized the study. GK performed analyses and TU interpreted the data.

6 GK and TU wrote the manuscript.

7

8 **Conflicts of Interest**

9 The authors declare no conflict of interest.

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9

10 **Figure legends**

11

12 **Figure 1. Chromosomal distribution of barley lncRNAs and their expression in**
13 **normal and stress conditions.** A. Total number of lncRNAs localized to each barley
14 chromosome (from chromosome 1 to 7). B. Box plot representation of expression levels
15 of lncRNAs before and after boron exposure in root and leaves. C. Expressional
16 changes of lncRNAs across samples are being illustrated as heat map graph.

17

18 **Figure 2. Differentially expressed transcripts and GO term enrichment analysis.**
19 A-B. Venn diagram of differentially expressed coding (A) and lncRNA (B) transcripts
20 in leaf and root. C-D. Significant biological process (C) and molecular function (D) GO
21 terms associated with differentially expressed coding transcripts.

22

23 **Figure 3. Ion transport and response to stimulus associated lncRNAs.** A-C.
24 lncRNAs and coding transcripts related to ion transport (A) and response to stimulus
25 (C) GO terms. lncRNAs and coding transcripts are represented as circles and round
26 rectangles, respectively. Differentially expressed lncRNAs before and after boron
27 exposure are shown as colored. Green colored lncRNAs are differentially expressed
28 only in leaves. However, lncRNAs are colored as red if they are differentially regulated

in both root and leaves. LncRNAs and associated coding transcripts (co-expressed and co-localized) are linked to each other with network edges. B-D. Heat map representation of expression levels both lncRNAs and coding transcripts associated with ion transport (B) and response to stimulus (D) GO terms.

Figure 4. Establishment of localization associated lncRNAs. A. Network representation of establishment of localization related coding transcripts and lncRNAs (the same color scheme as in Figure 3 is used). B. Expression levels of establishment of localization GO term associated coding and lncRNAs.

Table:

Table 1. Computationally identified putative eTM sequences having potential to act as miRNA sponge.

Supplementary Materials:

Table S1. Barley previously unannotated.transcripts.bed

Table S2. Expression of lncRNAs and coding transcripts

Table S3. GO term enrichment analysis

Table S4. eTM sequences

Table S5. GO term enrichment analysis of eTM related miRNA target genes

Figure 1

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