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Research article

Why lncRNAs were not conserved? Is it for adaptation?

Elif Karlik[1]

[1] *Istinye University, Faculty of Engineering and Natural Science, Department of Molecular Biology and Genetics, 34010, Istanbul, Turkey*

Abstract

Plants are sessile organisms affected by changing environment, especially biotic and abiotic stress. Long non-coding RNAs (lncRNAs) became prominent as crucial regulators in diverse biological mechanisms, including developmental processes and stress responses such as salinity. In this study, salinity related lncRNAs were sequenced and analyzed according to homology based on rice and maize lncRNA sequences. After sequencing, 72HASATROOT and 72TARMROOT were identified as 568 bp, additionally, 72HASATSHOOT and 72TARMSHOOT were also 568 bp according to reference sequence which are the member of the natural-antisense lncRNA with 565 bp. Besides, 77HASATROOT and 77TARMROOT were identified as 676 and 644 bp, additionally, 77HASATSHOOT and 77TARMSHOOT were 666 bp according to reference sequence alignment that reference sequence was 667 bp and the sno-lncRNA member. Sequencing studies demonstrated sequence alterations resulted in secondary structure changes which may affect the adaptation of varieties in response to stress. As a conclusion, rapid evolution of lncRNAs may be another force for adaptation to changing environment in plants.

Keywords: *Barley, Hordeum vulgare* L., *long non-coding RNAs, sequence analysis*

1. Introduction

Long non-coding RNAs (lncRNAs) are more than 200 nucleotides (nt), opening a new branch for RNA studies, while the eukaryotic genomes were constituted only a small portion of the protein-coding genes. lncRNAs mainly has no coding sequence (CDS) or open reading frame (ORF) (Ulitsky and Bartel, 2013; Chekanova, 2015). lncRNAs are produced approximately from whole genome parts, including sense and antisense strands of a protein-coding genes, intergenic regions, and introns (Mattick and Rinn, 2015).

The functions of lncRNAs are still mystery and under investigation. However, the studies are point to fact that they became prominent as important regulators in gene expression at the transcriptional, post-transcriptional and post-translational levels (Flynn and Chang, 2014; Ren et al., 2021; Statello et al., 2021). Functional analysis of lncRNAs demonstrated they are involved in nuclear structure integrity process by controlling the chromatin remodeling complexes and regulating the expression of either nearby genes or genes elsewhere in cells (Rinn and Chang, 2012; Goff and Rinn, 2015; Song et al., 2019; Grossi et al., 2020).

In recent years, reports on the plant lncRNAs have demonstrated they act as key regulatory elements in nearly all developmental process, including root organogenesis (Ganguly et al., 2021), flowering time (Heo and Sung 2011; Ghorbani et al., 2021), photo morphogenesis (Wang et al., 2014), and reproduction (Zhang et al., 2014; Fang et al., 2019). The studies reported plant lncRNAs with diverse biological mechanisms played in developmental process, biotic and abiotic stress responses (Swiezewski et al., 2009; Heo and Sung, 2011; Wang et al., 2014).

Evolutionary conservation has been considered as useful metric for evaluating the functional importance of genes, although lack of sequence conservation does not imply the opposite (Johnsson et al., 2013; 2014). lncRNAs are found to

* Corresponding author.
E-mail address: elif.karlik@istinye.edu.tr (E. Karlik).
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evolve rapidly based on the comparison of lncRNA exons or mRNA untranslated regions or protein-coding sequences or (Marques and Ponting, 2009; Young et al., 2012; Ramírez-Colmenero et al., 2020).

Interestingly, processing and localization of conserved lncRNAs are not conserved, indicating that these processes contribute the rapid evolution of function (Ulitsky and Bartel, 2013; Guo et al., 2020). While some lncRNAs are sytenically conserved across multiple species, some of them only share low level of sequence homology (Tsagakis et al., 2020). Additionally, tissue specificity of lncRNAs is found to be conserved compared to mRNAs (Hezroni et al., 2015). However, expression levels of the same lncRNAs are distinctly regulated in different tissues (Karlik and Gozukirmizi, 2018). Additionally, lncRNA promoters are strongly conserved, indicating that selective limitations may act at the transcriptional level (Necsulea et al., 2014; Tsagakis et al., 2020).

In our previous study, the expression levels of maize (CNT0018772) and rice (CNT0031477) lncRNAs were evaluated in four different barley cultivars (Beyşehir 99, Hasat, Konevi 98 and Tarm 92) under salt stress conditions. Our study showed the expression levels of both lncRNAs were down-regulated compared to control. However, one of these barley cultivars, Tarm92, showed up-regulation for rice (CNT0031477) lncRNA (Karlik and Gozukirmizi, 2018). In this study, both lncRNAs sequences were retrieved from barley cultivars (Hasat and Tarm 92) according to sequence homology. Sequences from both root and shoots of lncRNAs were analyzed for secondary structure and splicing events to understand the processing of RNA.

2. Materials and methods

The PCR products of CNT0018772 and CNT0031477 were used for sequence analysis and then re-sequenced. By using BLASTN, the sequence homology search was conducted in barley genome retrieved from the Ensembl website (Ensembl Plants, 2021). The nucleotide sequences of lncRNAs of barley were submitted to GenBank (accession numbers CNT0018772 for 72HASATROOT MK369941, 72HASATSHOOT MK369942, 72TARMROOT MK369943, 72TARMSHOOT MK369944, and CNT0031477 for 72HASATROOT MK369945, 72HASATSHOOT MK369946, 72TARMROOT MK369947, 72TARMSHOOT MK369948).

To validate lncRNAs, the sequences were ≥ 200 bp were selected as lncRNAs. The NCBI ORF Finder was utilized to ensure that transcripts encode no ORFs or incomplete ORFs were considered as lncRNA candidates (NCBI, 2021). For coding potential evaluation, the Coding Potential Calculator 2 (CPC2) (Kang et al., 2017) and Coding-Potential Assessment Tool (CPAT) (Wang et al., 2013) were used that they are relied on the detection of quality, completeness, and sequence similarity of the ORF to protein in current protein databases.

The excision of introns or alternative splicing events (AS) were analyzed by ACESCAN2 (Yeo et al., 2004) and NetGene2 databases (Hebsgaard, 1996). The bioinformatics tools were used to identify to exonic/intronic splicing enhancers. ESE Finder 3 was used to define (ESE Finder, 2021) putative ESEs associated with common serine/arginine-rich splicing factors (Cartegni, 2003; Smith et al., 2006). The secondary structure of lncRNAs were predicted by using Vienna RNA package RNAfold bioinformatics tool (Lorenz et al., 2011; RNAfold, 2021).

3. Results

To obtain barley lncRNA sequences, PCR analysis was performed using probe-primers of CNT0018772 and CNT0031477 indicated in probe design and used Hasat and Tarm cDNA as a template. To validate the two new putative barley lncRNA, PCR products were re-sequenced, which were ~120 bp and ~95 bp for CNT0018772 (maize) and CNT0031477 (rice), respectively. According to sequencing results, the sequence homology search was conducted to isolate new lncRNAs in barley genome retrieved from the Ensembl website by using BLASTN (Altschul et al., 1990; Ensembl Plants, 2021). After re-sequencing, homolog sequences of maize CNT0018772 lncRNAs were obtained as 1370 and 1393 bp in. Hasat for roots and shoots respectively, additionally, as 1303 and 1350 bp in. Tarm for roots and shoots, respectively. However, re-sequencing results showed that homolog sequence of rice CNT0031477 lncRNAs were found to be as 2496 and 1451 bp in. Hasat for roots and shoots respectively, additionally, as 1378 and 1345 bp in. Tarm for roots and shoots, respectively. Also, coding potential of the re-sequenced regions of Hasat and Tarm barley lncRNas were analyzed by using CPC2 (Kang et al., 2017) and CPAT (Wang et al., 2013) programs, resulting in no coding potential.

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4. Discussion

The evolutionary history of lncRNAs may contribute to understand their functionality. Plenty of lncRNAs exhibited rapid evolution process in terms of sequence and expression levels, while tissue specificities were often conserved. However, evolutionarily conserved lncRNAs tend to demonstrate stable and critical functions across species, despite their low number (Necsulea et al., 2014; Andergassen et al., 2019). Studying with lncRNAs includes serious research challenges, such as the lack of the strong statistical signals associated with protein coding genes, ORF, G+C content and codon-usage biases, also the lack of the 2D or 3D structure information (Rivas and Eddy, 2000).

Recently, LncRNA:DNA triplex formation was used to reveal the general mechanism of lncRNAs directing gene regulation and mediating 3D chromatin organization (Soibam and Zhamangaraeva, 2021). In this study, two barley lncRNAs were re-sequenced which were homologous with maize and rice lncRNAs. Barley lncRNAs CNT0018772 for—MK369941, MK369942, MK369943 and MK369944— revealed that these sequences were associated with barley CNT20168342 lncRNA according to CANTATAdb (Szcześniak et al., 2016). Obtained re-sequencing data CNT20168342 were resulted with 1370 (MK369941), 1393 (MK369942), 1303 (MK369943) and 1350 (MK369944) bp, while CNT20168342 lncRNA was 443 bp. Additionally, re-sequencing studies demonstrated MK369945, MK369946, MK369947 and MK369948, which are homolog with rice lncRNA CNT0031477 were found to be 2496, 1451, 1378 and 1345 bp, respectively. Homology and RNA processing analysis showed that both these two lncRNAs were possibly under processing, indicating that these lncRNAs undergo the excision of introns or AS due to ACESCAN2 (Yeo et al., 2004) and NetGene2 databases (Hebsgaard, 1996). Characterization of splicing enhancers of these two lncRNAs were exhibited in Fig. 1. Studies indicated that AS is a mechanism which is now not only creating multiple protein isoforms. Moreover, AS produces and regulates small and long non-coding RNAs (Ulitsky and Bartel, 2013). After analyzing of sequences 72HASATROOT

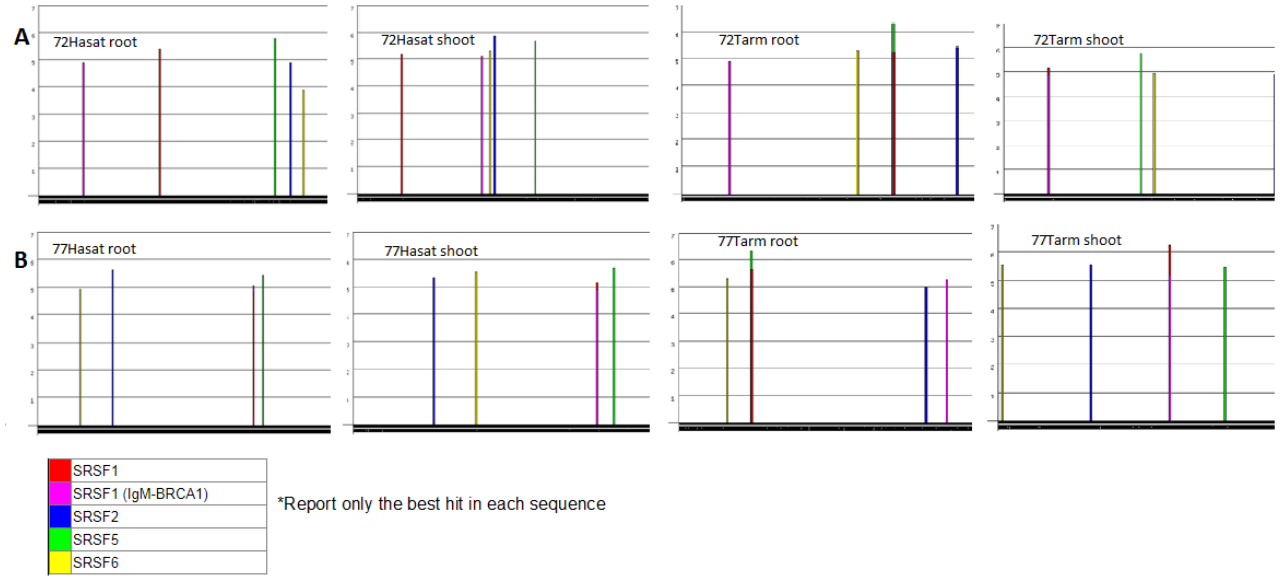

Fig. 1. Characterization of splicing enhancers of re-sequenced barley lncRNAs. (A) Representative display of ESE elements in 72 barley lncRNAs sequences, (B) Representative display of ESE elements in 77 barley lncRNAs sequences.

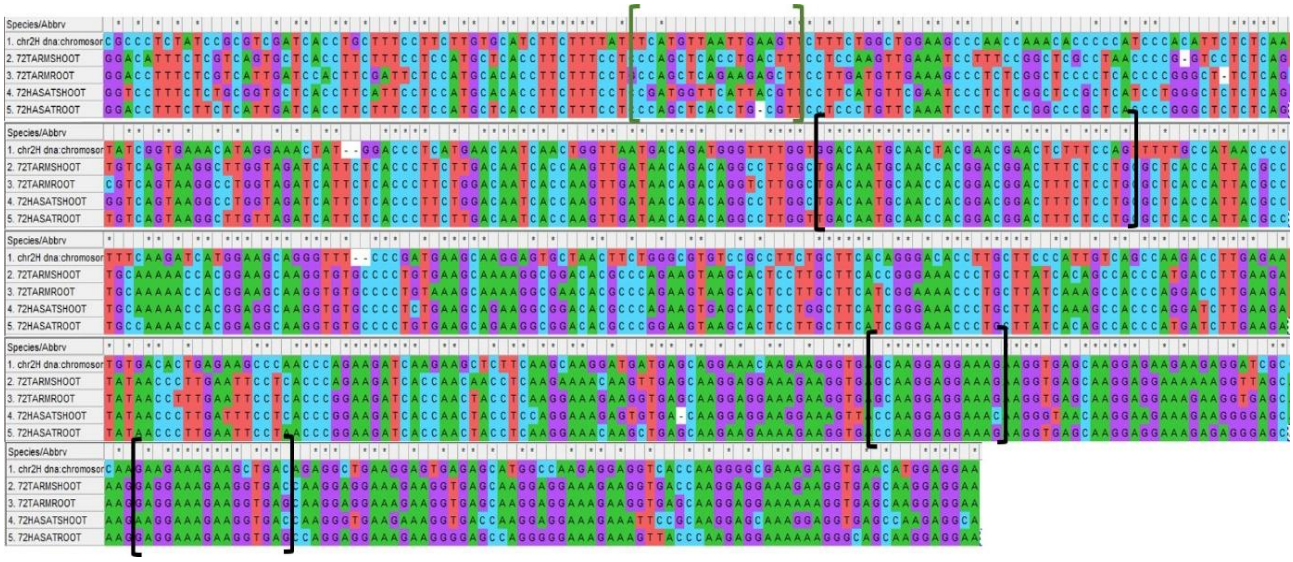

Fig. 2. The re-sequencing analysis of *CNT0018772* comparing with reference barley genome (The letters in dark rectangles are highly conserved, while the letters in green rectangles are not conserved).

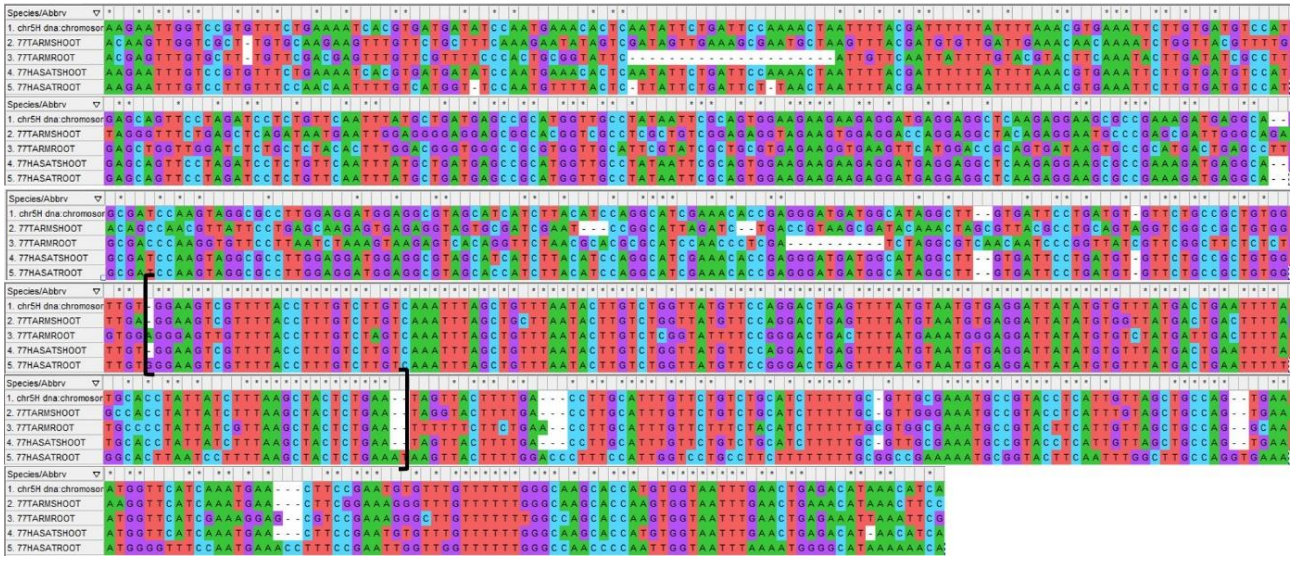

Fig. 3. The re-sequencing analysis of *CNT0031477* comparing with reference barley genome (The letters in dark rectangles are highly conserved).

and 72TARMROOT were identified as 568 bp, additionally, 72HASATSHOOT and 72TARMSHOOT were also 568 bp according to reference sequence alignment that reference sequence (chr2H:21427685:21430889:-1) was 565 bp. Also, sequence alignment with reference barley genome demonstrated that these lncRNA was not conserved and may have rapid evolutionary turnover in barley genotypes (Fig. 2). However, the majority of lncRNAs can be transcribed from different part of the genome, including intergenic regions, promoters, enhancers or even introns (Guttman et al., 2009; Khalil et al., 2009; Yang et al., 2011; Salzman et al., 2012), indicating that this event may be regulated by alternative forms of lncRNAs or different lncRNA interactions which needs further studies to validate. Regulation of CNT0018772 lncRNA by AS may influence gene expression under salinity stress conditions according to our previous gene expression study results. Our previous expression studies demonstrated that expression levels of CNT0018772 lncRNA were down- regulated. After sequence analysis, 77HASATROOT and 77TARMROOT were identified as 676 and 644 bp, additionally, 77HASATSHOOT and 77TARMSHOOT were 666 bp according to reference sequence alignment that reference sequence (chr5H:612797513:612800868:-1) was 667 bp. Also, sequence alignment with reference barley genome demonstrated that only small part of this lncRNA was conserved (Fig. 3), suggesting these lncRNA was not conserved, and may have rapid evolutionary turnover in barley genotypes. Besides in our previous expression studies, expression analysis of CNT0031477 lncRNA indicated these lncRNA could be expressed both shoot and root tissues under 150 mM salt treatment and control condition, suggesting that these lncRNA may not be tissue specific (Karlik and Gozukirmizi, 2018).

In plants, as more forms and functions of AS are considered to modulate diverse biological mechanisms, including flowering time, circadian rhythms, and response to stress (Simpson et al., 2016; Ling et al., 2017; Verhage et al., 2017; Zhang and Xiao, 2018; Dikaya et al., 2021). lncRNAs in plants influence the gene expression and regulation both in direct and indirect ways. For example, COLDAIR effects flowering time based on chromatin state and structure or APOLO effects on auxin action by chromatin looping (Heo and Sung, 2011; Ariel et al., 2014).

In our previous study, expression levels of maize lncRNA CNT0018772 interacting with maize 40S ribosomal protein S6 (RPS6) (Szcześniak et al., 2016; Karlik and Gozukirmizi, 2018), which phosphorylates eukaryotic ribosomes, initiating the translation was observed. Sequence analysis of re-sequenced these barley lncRNA was located on chr2H:108632412-108644592 (-), while this barley genome region comprises HORVU2Hr1G010890 and HORVU2Hr1G010870 protein-coding genes homolog with 40S ribosomal protein S6 (RPS6) in barley, suggesting these barley lncRNAs may be antisense lncRNA which may also be paired with RPS6 as a natural antisense- coding protein pairs lncRNA (NAT-lncRNA). This study suggests that CNT0018772 lncRNA may regulate RPS6 expression by AS based on RNA-RNA interaction database (Szcześniak et al., 2016). Moreover, homology analysis among two —Hasat and Tarm— and Morex revealed that these barley lncRNA exhibited low sequence conservation. Diederichs (2014) evaluated the lncRNA evolution at four dimensions, including sequence, structure, function and syntenic expression adds up to lncRNA conservation. Although there are a few experimental cases showed sequence conservation levels of lncRNAs, most lncRNAs demonstrated weak or untraceable primary sequence conservation (Nitsche and Stadler, 2017; Tavares et al., 2019), suggesting that secondary structures among lncRNA homolog may be more conserved than the sequences.

Chen and Carmichael groups (2009; 2010) identified a class of lncRNAs named as small nucleolar RNA-related long noncoding RNAs (sno-lncRNAs) which are produced from introns and have a unique structure (Yin et al., 2012). snoRNAs are conserved nuclear RNAs (about 70-200 nt), modifying small nuclear (snRNAs) or ribosomal RNA (rRNA) or involving in the processing of rRNA during ribosome subunit maturation (Kiss, 2001; Boisvert et al., 2007; Matera et al., 2007). snoRNAs are classified into two main groups: box C/D and box H/ACA snoRNAs. The processing of snoRNAs differs based on the sequence content among introns (the number of encoded snoRNA genes) that some introns are used to produce lncRNAs, whereas others are used to generate snoRNAs (Wilusz, 2016). Re-sequencing analysis of CNT0031477 lncRNA revealed that these lncRNAs were at chr5H:612799183- 612799474 (-) and chr5H:612921653-612921944 (+). Additionally, according to Ensemble data, chr5H:612799183-612799474 (-) genome region contains one transcript named as HORVU5Hr1G101570.1 which is no protein coding gene, and there snoRNAs (ENSRNA050017778, ENSRNA050017798 and ENSRNA050017792). Chr5H:612921653-612921944 (+) genome region was also found to be contained one transcript named as HORVU5Hr1G101650.2 and three snoRNAs (ENSRNA049476433, ENSRNA049476461 and ENSRNA049476383), indicating these lncRNA might be a member of sno-lncRNA class. To date, at least 19 tissue- and species-specific sno-lncRNAs have been determined in different studies (Zhang et al., 2014). In human chromosome 15, most known sno-lncRNA, which regulate AS and many other post-transcriptional events, is produced from the 15q11–q13 region is exposed to genomic imprinting and involved in Prader–Willi Syndrome (PWS) (Sahoo et al. 2008; Yin et al., 2012). However, sno-lncRNAs have not been identified yet and, their functions are still unknown. Moreover, sequence analysis among other lncRNAs have also uncovered that these sno-lnRNAs are semi-conserved on the contrary the idea of most lncRNAs undergo rapid sequence evolution and may play important roles (Ulitsky et al., 2011; Ramírez-Colmenero et al., 2020). Due to their tissue specific expression nature, products of sno-lncRNAs, which could be snoRNA or lncRNA, should be investigated in further studies, also depending on condition matter.

To date, there has a passing acquaintance with lncRNA's secondary structure and the interaction between structure and function. In mammals, conservation of lncRNAs in primary sequence, and gene structure are rare at orthologous, indicating lncRNAs undergone rapid turnover during evolution (Kutter et al., 2012; Wood et al., 2013; Ramírez-Colmenero et al., 2020). Some mutations could favor evolutionarily positive selection by stabilizing RNA structures within lncRNAs. However, Parallel Analysis of RNA Structures (PARS) study showed that physiological stimuli mostly altered RNA structures in yeast (Wan et al., 2012). While they compared the RNA structure stability, ncRNAs including, rRNA, tRNA, snoRNA and snRNA exhibited more stability than protein coding mRNAs. In this study, Vienna RNA package RNAfold bioinformatics tool was used to create the predicted secondary structure of eight lncRNA transcripts sequences (Fig. 4). The results demonstrated primary sequence alterations resulted in secondary structure

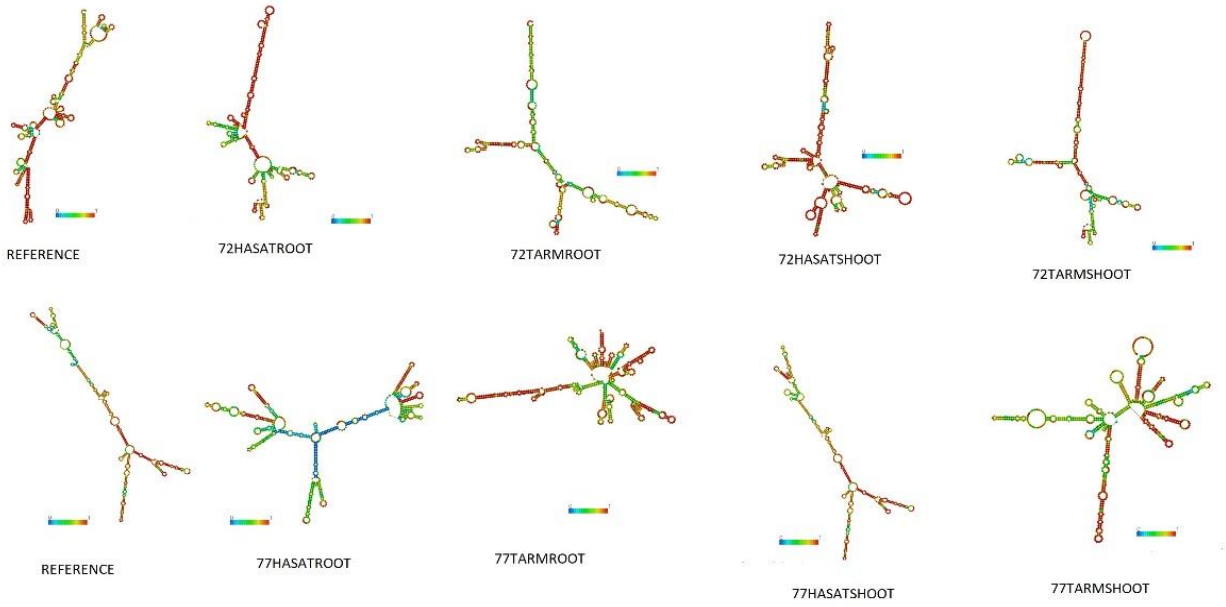

Fig. 4. The re-sequencing analysis of *CNT0031477* comparing with reference barley genome. The letters in dark rectangles are highly conserved.

changes. As known, these secondary structures can affect the higher- order of tertiary structures which can be associated with the interaction of RNA-binding proteins (RBPs), direct catalysis functions, formation of scaffolds, and regulation of posttranscriptional modifications (Cruz and Westhof, 2009; Qi et al., 2021). Interestingly, RNA secondary structure may be a significant mark for sensing a signal. Specifically, RNA folding can be conformationally altered in response to fluctuations in temperature, covalently modified nucleotides, cellular osmolarity or other signals (Narberhaus, 2010; Kortmann and Narberhaus, 2012; Qi et al., 2021), indicating that secondary structure of these lncRNAs studied in this study may be altered by sensing of osmatic stress. However, I determined the expression levels of CNT0018772 and CNT0031477 were found to be down-regulated in our previous study, suggesting that secondary structure alterations may not influence functions of these lncRNAs.

Understanding the structure of lncRNAs which are considered as emerging regulatory elements will be crucial to fully comment the evolution, form, and function (Johnsson et al., 2014; Fanucchi et al., 2019; Soibam and Zhamangaraeva, 2021).

Identification of conserved lncRNAs is one of the problems for evaluating the origin of the lncRNAs which is still unknown. According to low level of sequence conservation, lncRNAs may not evolve by using the gene duplication event in comparison with protein-coding genes. However, it is assumed that protein-coding genes lose their original function and become lncRNAs (Hezroni et al., 2017; Tsagakis et al., 2020). Another possibility of emerging lncRNAs is non-coding parts of the genome such as promoters might eventually gain function as a lncRNA (Tsagakis et al., 2020). However, plants are sessile organisms and need to cope with changing environmental conditions throughout their life cycles. Genotypic and phenotypic plasticity are the main components for coping with this variability and unpredictability of stress occurrence (Hilker and Schmülling, 2019; Ramírez-Colmenero et al., 2020). Rapid evolution of lncRNAs may be another force for adaptation to changing environment in plants. Changing the regulation of themselves and playing important roles in gene regulation may contribute the adaptation of plants in response stress.

4. Conclusion

RNA processing analysis in this study indicated lncRNAs undergone rapid evolution, however, similarly the function of most lncRNAs remains largely mystery. Today, the functional characterization of lncRNAs is still challenging. Studies indicated lncRNA functions basically depend on structure and protein interaction repertoire of lncRNAs. Our findings suggest that RNA processing of lncRNAs is important for determining the functions of lncRNAs under control and stress conditions.

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