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# THE IDENTIFICATION OF EIGHTEEN PRECURSOR miRNA CLUSTERS AND THEIR TARGETS IN BARLEY (*HORDEUM VULGARE* L.)

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**Abstract**

MicroRNAs (miRNAs) are short, endogenous and non-protein coding RNAs that are 18-26 nucleotides (nt) in length. The miRNAs have been shown to play important regulatory roles in almost all plant processes, including responses to various stresses. These regulatory functions of the miRNAs are to negatively control the protein coding sequences at post-transcriptional level. The mature miRNAs (18-26 nt) are generated from long (50-550 nt) precursor miRNAs (pre-miRNAs). Mostly the pre-miRNAs have one mature miRNA sequence in the stem region, but few have been reported with more than one mature miRNAs. Such miRNAs are called pre-miRNA cluster. In current research, various computational tools were used for the identification and characterization of new conserved pre-miRNA clusters and their targets in barley (*Hordeum vulgare* L.). Consequently, a total 18 new pre-miRNA clusters were identified from 17 miRNAs families in barley from total 501,838 express sequence tags (ESTs). These miRNA families were: miR394, 396, 414, 473, 475, 482, 817, 1432, 2118, 2673, 5066, 5070, 5168, 5181, 5201, 5522 and 7757. The miR-5070 was identified as sense and anti-sense cluster and 81 protein targets were identified for pre-miRNA clusters. These protein targets were categorized as: hypothetical protein, metabolism protein, transcription factor, transporter protein, cell signaling protein, growth & development protein and structural proteins. These newly identified pre-miRNA clusters and their 81 targets were reported here for the first time in barley. These results will be a good contribution to fine-tune the regulation of barley for better yields, agronomic traits and stress management.

**Key words:** Cereal grain, Computational tools, ESTs, Small RNA clusters.

**Abbreviations**:
ata = *Aegilops tauschii*
bdi = *Brachypodium distachyon*
BLAST = Basic local Alignment Search Tool
ch_ratio = Core Hairpin Ratio
dbEST = Database of EST
ESTs = Expressed Sequence Tags
GC = Guanine and cytosine
hvu = *Hordeum vulgare*
MFE = Minimum Free Energy (mfe)
miRNA* = Reference miRNA
miRNAs = microRNAs
mtr = *Medicago truncatula*
NCBI = National Center for Biotechnology Information
nt = nucleotid
osa = *Oryza sativa*
Pre-miRNA= Precursor microRNA
ptc = *Populus trichocarpa*
vvi = *Vitis vinifera*

## Introduction

MicroRNAs (miRNAs) belong to non-protein coding RNAs class and constituted from a short length of nucleotides (nt) containing approximately 18 to 26 nt. The mature miRNA sequence is produced by the processing of a long hairpin precursor (Chen *et al.*, 2012) and it has been pointed out that miRNAs are involved in the regulation of gene expression during the messenger RNA translation (Khvorova *et al*., 2007). Zhang *et al*., (2006) has reported that mRNA targeted sites are degraded specifically if they show full complementarity with a miRNA, whereas in partial complementarity, only the translation of mRNA is inhibited. The miRNAs regulate the expression of genes during different metabolic and biological processes. As a result, development of flower, root and leaf, growth from vegetative to reproductive and responses to stresses (biotic and abiotic) are regulated by plant miRNAs (Barozai *et al.*, 2012; Matsui *et al.*, 2013).

MiRNA clusters have been detected in animals and in humans but in plant infrequent miRNA clusters were observed by Yu *et al*., (2006) and Achakzai *et al*., (2019). This study focuses on the identification of mirRNA as the cluster of pre-miRNA. The family of mir-3630 has been described as miRNA clusters in a few plants such as *Capsicum annuum* (Din *et al.*, 2016) and *Vitis vinifera* (Pantaleo *et al.*, 2010).

Barley grain belongs to the poaceae family which is one of the largest monocotyledonous plant family, containing around 785 genera and 10,000 species. This family has great ecological, economical and nutritional importance (Clayton & Renvoize, 1986; Thomasson, 1988; Watson, 1990; Watson & Dallwitz, 1992). Historically, barley was first cultivated as early as 13,000 years ago and since its bread has been used in different cultures of the world as a nutritional source. Traditionally, this grain has been commonly used for malt preparation, and as well as animal feed since ancient era (Trethewey *et al*., 2005; Fincher, 2009). By the way, the low nutritional value of barley for poultry is due to the absence of an intestinal enzyme for efficient depolymerisation of (1,3;1,4)-$\beta$-glucan, which is the major polysaccharide of the endosperm cell walls (Von *et al*., 2000). The improvement of this plant family depends upon genetically resistant varieties, seed productivity, modern cultivation and biotic and abiotic stress tolerance. Plant improvement can be assessed by studying its genetic makeup and sowing it in different locations. Comprehensive identification of barley miRNA clusters is of great importance.

This article reports the characterization and identification of the novel conserved pre-miRNA clusters and their targets in barley (*Hordeum vulgare* L.) by using various computational tools. Thus, totally 18 new pre-miRNA clusters were identified from 17 miRNA families

in barley from total 501,838 express sequence tags (ESTs). These miRNA families were: miR394, 396, 414, 473, 475, 482, 817, 1432, 2118, 2673, 5066, 5070, 5168, 5181, 5201, 5522 and 7757. The miR-5070 was identified as sense and anti-sense cluster. In addition, 81 protein targets were identified for pre-miRNA clusters. These protein targets were categorized as: hypothetical protein, metabolism protein, transcription factor, transporter protein, cell signaling protein, growth & development protein and structural proteins.

## Materials and Methods

**Reference miRNAs and prediction of the candidate's pre-miRNAs:** To identify the miRNAs in the barley plant, reference miRNAs (miRNAs*) and prediction of the candidate's pre-miRNAs are used in the study according to a procedure reported by Barozai *et al*., (2008). We subjected 6,394 plant precursors miRNAs of both monocots and dicots (pre-miRNA) sequences, obtained from the microRNA Registry Database, available at (http://www.mirbase.org/) and reported by Kozomara & Griffiths-Jones (2014), Version (Rfam released 21 June, 2014). To search for potential conserved miRNAs in the barley plant, the miRNAs* of the total known plant miRNA sequences, both precursors and matures were downloaded from the microRNA Registry Database (Version Rfam 21 released June, 2014).

**Identification of candidate sequences:** MiRNA* sequences both mature and precursor sequences were subjected to Basic Local Alignment Search Tool (BLAST) against barley ESTs using BLASTn program http://blast.ncbi.nlm.nih.gov/Blast.cgi according to reported procedures (Altschul *et al.,* 1990; Achakzai *et al*., 2018) following 0-4 mismatches with miRNAs*.

**The prediction of barley secondary structures:** Hairpin structure generation for sequences related to initial potential candidates is a key condition (Ambros *et al.,* 2003). The initial potential hairpin structure sequences were predicted by using secondary structures/hairpin sequences generation search tool algorithm MFOLD, version 3.6 (Zuker, 2003) and available at http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi. The MFOLD parameters were set as reported previously for identification of miRNAs in various plants and animals (Barozai, 2012). Minimum free energy (MFE) of the self-folded stem loop structure ≤−18 Kcal/mol was preferred as pointed out by Ambros *et al*., (2003).

**Minimizing of false positive data:** During the novel miRNAs identification in bioinformatics, false positive reduction is a very important step. In this regard, Barozai *et al*., (2008) has taken various steps. For orthologous discovery, various steps have been taken. For the identification of novel miRNA orthologs on the basis of conservation in a range of 0-4 mismatches in mature sequences, pre-miRNA length makes them very appropriate. The same gene's repeated ESTs were filtered to produce a single representation from a gene. Barley pre-miRNAs were passed through structure and sequence filtration for their authentication. After that, miRNAs validation was achieved by employing parameters concerned to stem-loop structures. For the false positive removal and the novel miRNAs real structures confirmation of the crop, all these steps were performed.

**Strand orientation of microRNA clusters:** Precursor miRNA clusters obtained from the NCBI which openly available at (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Hence in this step, blast results revealed the existence of miRNA clusters in sense or anti-sense strand. In this regard, one column in the characterisation table shows the strand orientation either in plus or minus strand.

**The organ expression of microRNA clusters:** By the way, organ of expression for miRNA clusters in the crop obtained from readily available EST at (https://www.ncbi.nlm.nih.gov/nucest/). Various screening techniques are being used to classify the available tissue type and their organ of expression. Likewise, the current study introduces one more column in the characterization table for screened tissues and their expression parts.

**Ancestral conservation and analyses of putative pre-miRNAs clusters:** For conservation analysis, sequence logo generator studies were performed by using web logo software (http://weblogo.berkeley.edu/logo.cgi, version 2.8) of different plant species precursors such as: *A. tauschii* and *Brachypodium distachyon* (bdi) according to a procedure previously reported (Crooks *et al*., 2004). Phylogenetic analysis of miR-396 was performed by comparing with different plant precursors related to *O. sativa, Glycine max* and *A. tauschii* by using a software freely available at (http://www.genome.jp/tools-bin/clustalw) and following a reported procedure (Larkin *et al*., 2007).

**Targets prediction of innovative miRNAs:** miRNA possible targets were achieved according to a reported method (Dai & Zhao, 2011) employing psRNAtarget software, with a few modification parameters, freely available at (http://plantgrn.noble.org/v1_psRNATarget/) and the targets were further classified in different categories as demonstrated previously (Zhang *et al*., 2006).

## Results and Discussion

**New conserved barley miRNA clusters:** MiRNA clusters were identified by using the available source, namely barley ESTs 501,838, for screening. As a result, 18 novel barley pre-miRNA clusters were identified after filtration and compilation process by homology searches. These pre-miRNA clusters belonged to 17 miRNA families, which are as: miR-394, 396, 414, 473, 475, 482, 817, 1432, 2118, 2673, 5066, 5070, 5168, 5181, 5201, 5522 and 7757. The miR-5070 was identified as sense and anti-sense orientation. The transcription of miRNAs sense/antisense has been reported to achieve from both stands (sense/antisense) of genomic loci (Stark *et al*., 2008). In this study, a new barley conserved miRNA cluster (hvu-mir5070) was observed to be transcribed both in plus and minus strand as supported by Xia *et al*., (2012) in apple. The Table 1 shows and reports the newly discovered 18 miRNA clusters. Using the same procedure, Din *et al*., (2016) discovered different miRNA clusters in *Capsicum annuum* (Chili). Ambros *et al*., (2003) explained the criteria B, C and D for homology searches for profiling of miRNAs in various plants and

animals. After applying this formula, it was concluded that all these 18 novel barley miRNA clusters fulfilled the imperial formula. Ambros *et al*., (2003) have also supposed that the criteria D is enough for the profiling of a miRNA as a novel candidate. Consequently, eight (8) pre-miRNA clusters were reported from miRNA* of *A. tauschii*, three (3) pre-miRNA clusters from miRNA* of *B. distachyon*, three (3) from miRNA* of *O. sativa*, two (2) from miRNA* of *Populus trichocarpa*, one (1) from miRNA* of *Vitis vinifera* and the last one (1) from miRNA* of *Medicago truncatula.* Totally, 39 matures were obtained as a result. The miR396 has also been reported by Pacak *et al*., (2017) as a miRNA, while in the research conducted by us, the miR396 was predicted to be a cluster. The second difference between this miRNA and cluster is in the accession number. The accession number for the miRNA has been reported as MLOC_67201.2, while for the cluster has been predicted as AV925436.1.

**Barley pre-miRNA clusters characterization:** Table 1 shows the characterization of barley pre-miRNA clusters in terms of precursor length, MFE, mature sequence, number of mismatches, mature sequence length, source EST, mature sequence arm, GC content, strand orientation and organ of expression.

**Barley pre-miRNA clusters length:** The new barley conserved pre-miRNA clusters lengths were summarized in Table 1, ranged from 84 to 480 nt with an average of 217 nt. Among them, only two precursors were below 100 nt, eight precursors were observed between 100 to 200 nt, three were in the range of 201 to 300 nt, three were found in the range of 301 to 400 and at the last two precursors were in the range of 401 to 500 nt. The procedure of obtaining these outcomes was like the previous methods adopted by different researcher (Sunker & Jagdeeswaran, 2008; Fuliang *et al*., 2010).

**MFE:** The next parameter shown in the Table 1; is the minimum free energy (MFE), which is an indicator for pre-miRNA cluster hairpin structure's stability and constancy (Fig. 1). There is an indirect correlation between the secondary structure of miRNA cluster's stability and the amount of MFE. The average MFE of the barley new conserved pre-miRNA clusters was obtained as of about -84.42 kcal $mol^{-1}$ with a minimum of -192.21 kcal $mol^{-1}$ and maximum of -13.70 kcal $mol^{-1}$. The MFEs observed for *Oryza sativa* plant have been explained by Barozai *et al*., (2012) using the same procedure.

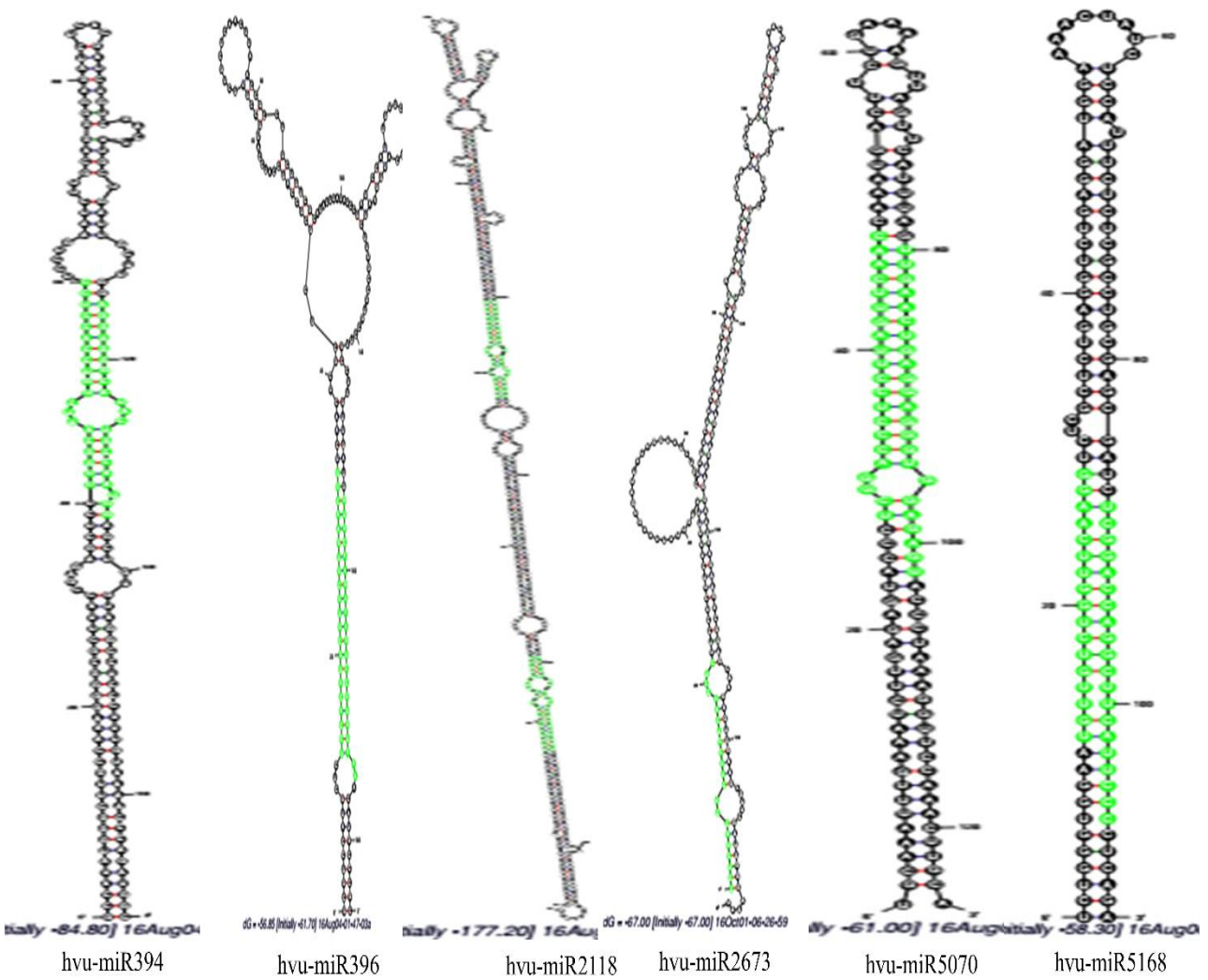


Fig. 1. The novel identified barley miRNA clusters' secondary structures. These structures were developed through M. fold. These structures clearly were showed the mature miRNAs in the stem region of the stem-loop structures.

**Table 1. The novel identified miRNAs cluster were characterized in terms of PL precursor miRNA length, MFE minimum free energy, MS mature sequence, NM number of mismatches (represented in bold), ML mature sequence length, MSA mature sequence arm, SE source of EST, GC% GC percentage, Strand of orientation and Organ of expression.**

| *Hordeum vulgare* miRNAs | Source miRNAs | PL | MFE | MS | NM | ML | Acc. No. | MSA | GC% | Strand orientation | Organ of expression |
|---|---|---|---|---|---|---|---|---|---|---|---|
| hvu-miR394 | ata-miR394 | 171 | -84.80 | UUGGCAUUCUGUCCACCUCC | 0 | 20 | DK624890.1 | 3′ | 55.00 | Sense strand | Shoot |
| | | | | AGGUGGGCAUACUGCCAAUGG | 0 | 21 | | 5′ | 57.14 | | |
| hvu-miR396 | ata-miR396a | 185 | -56.85 | UCCACAGGCUUUCUUGAACUG | 0 | 21 | AV925436.1 | 5′ | 47.61 | Sense strand | Seedling shoot |
| | | | | GUUCAAGAAAGUCCUUGGAAA | 0 | 21 | | 3′ | 38.10 | | |
| hvu-miR414 | osa-miR414 | 350 | -111.41 | UC**C**UCCUCAUCAUCAUC**A**UCC | 2 | 21 | BF621429.2 | 5′ | 47.62 | Anti-sense strand | Seedling shoot |
| | | | | UCAUCCUCAUCAUCAUC**A**UCC | 1 | 21 | | 5′ | 42.86 | | |
| hvu-miR473 | ptc-miR473a | 119 | -57.10 | ACUCUCCCUCAAGGCUUCCA | 0 | 20 | DB903445.1 | 5′ | 55.00 | Anti-sense strand | Mixture of leaf |
| | | | | UGAGGCCUUUGGGGGAGAGUGG | 0 | 22 | | 5′ | 63.64 | | |
| hvu-miR475 | ptc-miR475a | 138 | -54.70 | AAUGGCCAUUGUAAGAGUAGA | 0 | 21 | CF230537.1 | 5′ | 38.10 | Sense strand | Cambial zone |
| | | | | UUACAGUGCCCAUUGAUUAAG | 0 | 21 | | 3′ | 38.10 | | |
| hvu-miR482 | vvi-miR482 | 480 | -192.21 | U**G**UUUCCU**GU**U**G**CCUCCCAUUCC | 4 | 23 | DK695156.1 | 5′ | 52.17 | sense strand | Flower |
| | | | | **A**CUUUCCU**GA**U**G**CCUCCCAUUC | 4 | 22 | | 3′ | 50.00 | | |
| hvu-miR817 | osa-miR817 | 254 | -98.43 | UA**G**-UUGAGACCCGAUUGA | 4 | 19 | BI960757.1 | 5′ | 42.11 | Sense strand | 20 DAP spike |
| | | | | UCCAACU**A**GAGGCCC**AU**UUGA | 3 | 21 | | 3′ | 47.62 | | |
| hvu-miR1432 | ata-miR1432 | 210 | -96.97 | UCAGGAGAGAUGACACCGAC**A** | 1 | 21 | DK677605.1 | 5′ | 52.38 | Sense strand | Shoot and root |
| | | | | U**A**GGUGUCA**UC**C**C**GCCUGAAC | 3 | 21 | | 3′ | 57.14 | | |
| hvu-miR2118 | ata-miR2118a | 420 | -174.80 | UUCCU**G**AUG**C**CUCCCAUUCCU | 2 | 21 | DK695156.1 | 5′ | 52.38 | Sense strand | Flower |
| | | | | GGGA**G**UGGGAACAUGGAGG**G**A | 2 | 21 | | 5′ | 61.90 | | |
| | | | | UUCCU**GU**UG**C**CUCCCAUUCCU | 3 | 21 | | 3′ | 52.38 | | |
| | | | | GGGAAUGGGAACAUGGAGGAA | 0 | 21 | | 3′ | 52.38 | | |
| hvu-miR2673 | mtr-miR2673a | 200 | -67.00 | CCUCUUCCUCUUCCUCUUCCUC | 1 | 22 | BI778853.1 | 5′ | 54.54 | Anti-sense strand | Root |
| | | | | CCUCUUCCUCUUCCUCUUCCUC | 2 | 22 | | 5′ | 54.54 | | |
| | | | | CCUCUUCCUCUUCCUCUUCCUC | 3 | 22 | | 5′ | 54.54 | | |
| hvu-miR5066 | bdi-miR5066 | 321 | -105.40 | AAGUGUAUA**U**GUGGAGUGUCU | 1 | 21 | DK864765.1 | 5′ | 38.10 | Anti-sense strand | Flower |
| | | | | A**G**G**C**GUAUA**U**GUGGA**U**UGCCU | 4 | 21 | | 3′ | 47.62 | | |
| hvu-miR5070 | ata-miR5070 | 137 | -75.10 | UC**C**-ACCCU**C**CU**C**AGUUCAAC | 2 | 21 | DK813913.1 | 5′ | 52.38 | Sense strand | Shoot and root |
| | | | | UUGAACUAAG**G**AGGGUC**C**GAG | 2 | 21 | | 3′ | 52.38 | | |
| hvu-miR5070 | ata-miR5070 | 125 | -61.00 | UC**G**GACCCU**C**CUUAGUUCAAC | 1 | 21 | DK813913.1 | 5′ | 52.38 | Anti-sense strand | Shoot and root |
| | | | | UUGAACU**G**AG**G**AGGGU-GGAG**A**GU | 4 | 24 | | 3′ | 50.00 | | |
| hvu-miR5168 | ata-miR5168 | 113 | -58.80 | GGAAUGUUGUCUGGUUCAAGG | 0 | 21 | DK659519.1 | 5′ | 47.61 | Sense strand | Shoot and root |
| | | | | UCGGACCAGGCUUCA**U**UCCCC | 1 | 21 | | 3′ | 61.90 | | |
| hvu-miR5181 | ata-miR5181 | 142 | -65.10 | UC**U**GAUCCA**U**AA**C**AAGUGUCG | 3 | 21 | DK621148.1 | 5′ | 42.85 | Sense strand | Shoot |
| | | | | **G**ACUUAUUUUGGA**U**C**A**GAGGG | 3 | 21 | | 3′ | 42.85 | | |
| hvu-miR5201 | bdi-miR5201 | 85 | -35.50 | **G**GGG**AG**G**G**GCAAAUGAUCAAA | 2 | 21 | DK672668.1 | 5′ | 52.38 | Sense strand | Shoot and root |
| | | | | UGAU**G**AUUUGCCCC**AG**G**A**UUGU | 4 | 22 | | 3′ | 45.45 | | |
| hvu-miR5522 | osa-miR5522 | 380 | -110.66 | AACAAUAGGAAUGGGAGGCA**A** | 1 | 21 | DK695156.1 | 5′ | 42.85 | Anti-sense strand | Flower |
| | | | | AACA**G**UAGGAAUGGGAGGCAU | 1 | 21 | | 3′ | 47.61 | | |
| hvu-miR7757-3p 2-1 | bdi-miR7757 | 84 | -13.70 | AG**GU**UACUUGAUAUGUAAA**U**G | 3 | 21 | BU996533.1 | 5′ | 28.57 | Sense strand | Male inflorescences |
| | | | | GA**U**AG**C**UG**G**AUGUUUUGUUUA | 3 | 21 | | 5′ | 33.33 | | |

**Barley mature sequences:** The mature sequences of pre-miRNA clusters were examined in barley. More than one mature were found in the cluster. In tae-miR2118; four matures were observed followed by tae-miR2673 with three matures as obtained by Din *et al*., (2016) in *Capsicum annuum* (chilli).

**Mismatches in barley mature:** The characterization of the barley novel conserved miRNA was achieved for the acceptable range of mismatches between the miRNA* and potential barley miRNAs. The mature sequences were selected from 0 to 4 nt mismatches with an average of 2 nt mismatch. According to the data shown in Table 1, the number of mismatches found in the mature were classified in different categories. Therefore, 25.64% of the total matures were found without any mismatch, 20.51% with 1 mismatch, 17.94% with 2 mismatches, 20.51% with 3 mismatches and 15.38% with 4 mismatches. The number of mismatches in miRNAs of other plants have been previously detected by various researchers (Baloch *et al*., 2015a; 2015b).

**Barley mature length:** Lengths of all the mature miRNA sequences were also counted. As a result, the number of nts for the sequences were obtained over the range of 19 – 24 with an average of 18 nt. The lengths of mature miRNA sequences and their number of nts in many plants have been measured and counted in the same manner as reported previously (Wang *et al*., 2012; Barozai, 2012; Ghani *et al*., 2013).

**Barley mature arm:** Out of the total 39 matures, 61.53% of the total matures were located at 5' arm of the hairpin structure and 38.46% of the total matures were existed at 3' arm as shown in Fig. 1.

**Barley GC%:** Characterization of the barley novel miRNA clusters secondary structures is also reflected by their stability, which was performed by the engagement of nt in guanine (G)/uracil (U) base pairing or Watson - Crick Model. Most of nts of mature miRNAs showed hydrogen bonding with its counterparts in the opposite arm as illustrated in Fig. 1. Stability of the barley miRNA cluster matures in hairpin structures could be evaluated by the percentage of the bonded of G/U base pairings with the opposite arm of the mature to the un-bond. As the number of GC content increases, the stability of the precursor also increases. According to the result, the study revealed that the range of the % age of the bonding was from 29% to 64% with an average of 49%. Due to high bonding order, small bulges appeared in clusters which increased their stability and thus hairpins exhibited almost linear structures. These matures were found to be located on the stem area of hairpin structures.

**Strand orientation of barley:** Among the new conserved pre-miRNA clusters 6 out of 18 were on the anti-sense strand which become 33% of the total and 12 out of 18 were found in the sense strand which constitute 67% of the total miRNA clusters.

**Organ of expression for miRNAs:** Determination of the organ of expression for these precursors is an important parameter. Therefore, the barley precursor miRNA clusters were found in different organs of barley plant as: 1 out of 18 in inflorescences (5.55% of the total), 1 out of 18 in days after pollination spike (5.55% of the total), 1 out of 18 in the cambial zone (5.55% of the total), 1 out of 18 in cambial zone (5.55% of the total), 1 out of 18 in the mixture of leaves (5.55% of the total), 1 out of 18 in roots (5.55% of the total), 4 out of 18 in shoot regions (22.22% of the total), 4 out of 18 in flowers (22.22% of the total) and 5 out of 18 in root and shoot regions (27.77% of the total). Using the same procedure, various researchers have determined precursors in different organs of different plants previously (Barozai *et al*., 2014; Baloch *et al*., 2015a; 2015b).

**Conservation study of barley pre-miRNAs:** For conversation studies, barley pre-miRNA cluster (mir-396) was selected with two matures and showed conserved nature with *A. tauschii* and *B. distachyon* as shown in the highlighted red box of Fig. 2. For phylogenetic tree analysis, one of the newly identified pre-miRNAs i.e. hvu-mir396 precursor cluster was selected with the same pre-miRNA family from different plants. So, phylogenetic analysis suggested that based on pre-miRNA sequences, barley is more closed to monocot *A. tauschii (ata)* as compared to *O. sativa (osa)* and *G. max* as shown in Fig. 3. Using the same procedures, various researchers have determined precursor conservation and phylogenetic tree in different plants previously (Barozai *et al*., 2014; Baloch *et al*., 2015a; 2015b).

**Targets prediction of barley miRNA clusters:** The validation and profiling of barley miRNA clusters are very important steps for targeted genes and their significant functions. For that reason, we implemented strict criteria to explore their targets. Bartel (2009) has reported that one miRNA can target a group of proteins. Table 2 shows the targets prediction of miRNA matures. According to the obtained results, single miRNA can target different multiple barley genes. The number of total targeted proteins were obtained as 81 and divided into different classes based on their functions and in the descending order of the number of targeted proteins in each class. These related functions include hypothetical, metabolism, transcription factor, transportation, cell signalling, growth & development and structural.

The largest number of targeted proteins were obtained in hypothetical class of proteins. They were 37 off 81 which make 46% of the total profiled miRNA clusters targeted proteins. The list of targeted hypothetical proteins includes os02g0823000 protein, chromosome chr12 scaffold_36, os05g0429500 protein, chromosome chr18 scaffold_1, chromosome undetermined scaffold_9, tobacco fibrillarin homolog, ubiquitin specific protease 10, alpha-hordothionin precursor (purothionin II), chromosome chr18 scaffold_59, tRNA wybutosine-synthesizing protein 1 homolog, chromosome undetermined scaffold_310 etc. Many researchers have previously reported various targeted hypothetical proteins for different plants miRNAs using the same procedure (Wang *et al*., 2012; Farzana *et al*., 2017).

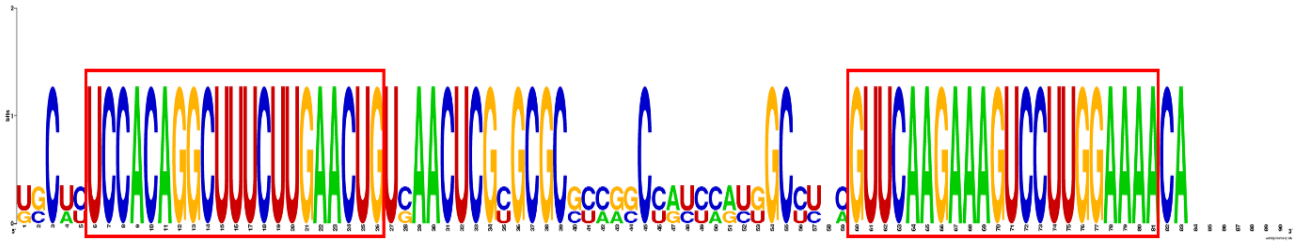

Fig. 2. Conservation of barley miRNAs. The barley miRNA conservation was studied by aligning of pre-miRNAs with *A. tauschii* and *B. distachyon* using weblogo software (a sequence logo generator). The cluster mature sequences showed conserved nature as represented in a box.

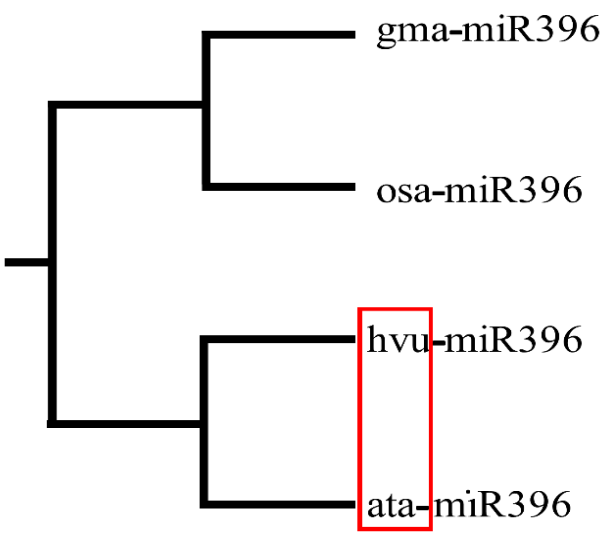


Fig. 3. Phylogenetic analysis of barley miRNA396. The Phylogenetic analysis of the barley pre-miRNAs (hvu-miR396) with *A, tauschii*, *O. sativa*, *G. max* miRNAs, was done with the help of ClustalW and cladogram tree was generated using neighbor joining clustering method. The phylogenetic tree shows that on the basis of pre-miRNA sequences, the barley is more closed to *A, tauschii* as compared to *O. sativa*, *G. max*.

The second class presents targeted metabolism proteins. Totally, 20 off 81 targeted metabolism proteins were predicted, which make 25% of the total targets. A few of these targeted metabolism proteins are as: glutathione transferase F6, ketol-acid reductoisomerase, hydrolase-like, alpha-L-arabinofuranosidase/beta-D-xylosidase, isoenzyme ARA-I, ATP-dependent metalloprotease FtsH precursor, isoflavone reductase-like protein, sucrase-like protein, AKIN gamma, anionic peroxidase precursor, early nodulin 75-like protein, fertility restorer B-like, cellulose synthase-4, 3-hydroxy-3-methylglutaryl-coenzyme A reductase 3 etc. The main function of these targeted proteins is metabolism, which may involve in a cell cycle, meristem formation etc (Jin & Martin, 1999; Din *et al*., 2016).

The third class of targeted proteins include all those proteins which are characterized as transcription factors. They assist in the development of plants and are present in almost all plants (Frazier *et al*., 2010; Xie *et al*., 2010; Din & Barozai, 2014a; 2014b). According to the results, 13 off 81 transcription factors were obtained, which make 16% of all the targets. These obtained factors include transcription activator, transducin family protein-like, DNA-binding protein-like, eukaryotic translation initiation factor 5B, single-stranded nucleic acid binding protein, tRNA wybutosine-synthesizing protein 1 homolog, Myb-like DNA-binding domain, SHAQKYF class family protein, expressed protein etc.

In the fourth class, a number of barley targeted transport proteins by barley miRNA clusters have been given. These proteins are commonly involved in the transport of many life sustaining materials inside a cell. The examples of targeted transport proteins found in the barley plant include monosaccharide transporter 4, membrane protein-like, StAR-related lipid transfer protein 7, (StARD7) (START domain- containing protein 7) (Protein GTT1) and MDR-like ABC transporter. In this class, 4 off 81 transport proteins were obtained, which make 5% of the total targeted proteins. Many targeted transport proteins, obtained by the same procedure, in different plants have been previously reported (Din *et al*., 2016).

The fifth class of targeted proteins were predicted as signalling proteins, which are involved in signal transduction in a cell. Therefore, 4 off 81 signalling proteins were predicted, which make 5% of the total targets. The examples of these targeted obtained proteins include Nup133 nucleoporin family protein, phosphate/ phosphoenolpyruvate translocator, Pto-like serine/threonine kinase etc. Many researchers have predicted many cell signalling targets previously in different plants (Din *et al*., 2016; Farzana *et al*., 2017). According to Curaba *et al*., (2014), miRNAs are involved in signalling pathways of phytohormones through the regulation of their metabolic activities in a cell.

The sixth and seventh classes of targeted proteins are related to growth & development and structural proteins respectively. The former class contains 2 out of 81 targeted proteins, which constitutes 2.5% of the total and the later class contains 1 out of 81 targeted proteins, which makes above 1% of the total. Growth and development proteins were as: growth-regulating factor and growth-regulating factor 3 and an example of the structural protein is beta tubulin 6. Following the same procedure, Din *et al*., (2016) and Farzana *et al*., (2017), have predicted these proteins in different plants. Pacak *et al*., (2017) have identified various miRNAs such as miR393-5P, miR396, miR156-5P and miR159-3P, to be involved in various regulatory functions during different developmental stages of barley.

**Table 2. Barley miRNA targets. The Barley (*Hordeum vulgare* L.) miRNA families and their putative targeted proteins function.**

| miRNA | Target Acc. | Target description | Function |
|---|---|---|---|
| hvu-miR394, hvu-miR396, hvu-miR414, hvu-miR473, hvu-miR475, hvu-miR817, hvu-miR1432, hvu-miR5066, hvu-miR5070, hvu-miR5168, hvu-miR5181, hvu-miR5201, hvu-miR5522, hvu-miR7753. | TC241454, AJ475774, TC263405, TC239020, BM378047, TC255113, TC255113, AV833458, CV061708, TC257444, BJ479164, TC245181, TC244453, TC263680, BU993452, TC262469, BI951103, CD663087, DN157385, BI955376, TC243958, BE437802, TC265188, TC253280, TC256085, TC255128, TC241706, CV063912, TC264752, TC240048, BM097988, TC259374, TC261743, TC281193, TC274883, DN178867, EX601047, EX585334. | Chromosome undetermined scaffold_91, Chromosome chr12 scaffold_36, Alpha-hordothionin precursor (Purothionin II) [Contains: Alpha- hordothionin; Acidic protein], Tobacco fibrillarin homolog, ubiquitin specific protease 10, Chromosome chr18 scaffold_59, Os12g0160400 protein, Os02g0823000 protein, Chromosome chr1 scaffold_5, Os05g0373700 protein, Os09g0563300 protein, Chromosome chr17 scaffold_12, Os04g0521900 protein, Os04g0634400 protein, Chromosome undetermined scaffold_310, Os07g0275300 protein, Ssu72-like protein, expressed, Os05g0406000 protein, Os09g0244600 protein, Expressed protein, Os08g0551200 protein, Os10g0124300 protein, Os04g0483600 protein, Os12g0235700 protein, Os05g0429500 protein, Os05g0429500 protein, Os03g0165100 protein, Os03g0758600 protein, Os08g0162100 protein, Os08g0162100 protein, Os12g0576600 protein, Os01g0149500 protein, Os12g0104800 protein, Os10g0547000 protein, Os01g0529800 protein, Os08g0137800 protein, Chromosome chr18 scaffold_1, Os07g0121200 protein. | Hypothetical protein |
| hvu-miR473, hvu-miR482, hvu-miR817, hvu-miR1432, hvu-miR5181, hvu-miR5168, hvu-miR5201, hvu-miR5522, hvu-miR7753. | BE215166, TC281553, CA019025, TC272844, TC264251, BI960123, TC253226, TC247961, BM099106, TC273290, TC261486, TC240557, TC245622, TC239568, TC259400, BM443289, TC244123, TC274430, BQ471200, TC250019. | Glutathione transferase F6, Ketol-acid reductoisomerase, mitochondrial, tRNA synthetase class II (D, K and N) family protein, Hydrolase-like, RNA polymerase Rpb3/Rpb11 dimerisation domain containing protein, Alpha-L-arabinofuranosidase/beta -D-xylosidase isoenzyme ARA-I, Alpha-L-arabinofuranosidase/beta-D-xylosidase isoenzyme ARA-I, Alpha-L-arabinofuranosidase/beta-D-xylosidase isoenzyme ARA-I, ATP-dependent metalloprotease FtsH precursor, Isoflavone reductase-like protein, Isoflavone reductase-like protein, Isoflavone reductase-like protein, Sucrase-like protein, AKIN gamma, Anionic peroxidase precursor, Early nodulin 75-like protein, Fertility restorer B-like, Cellulose synthase-4, 3-hydroxy-3-methylglutaryl-coenzyme A reductase 3, Os10g0188000 protein. | Metabolism |
| hvu-miR396, hvu-miR414, hvu-miR475, hvu-miR1432, hvu-miR5181, hvu-miR5201, hvu-miR5522. | CA026441, BU998119, BI957305, CV062681, TC272192, TC274422, CB882473, EX574164, TC264183, TC243360, TC250740. | Transcription activator, DNA-binding protein-like, Eukaryotic translation initiation factor 5B, Single-stranded nucleic acid binding protein, tRNA wybutosine-synthesizing protein 1 homolog, Os04g0508900 protein, Os04g0508900 protein, Myb-like DNA-binding domain, SHAQKYF class family protein, Expressed protein, SCAR-like protein 2, Expressed protein, Transducin family protein-like. | Transcription factor |
| hvu-miR396. | CB860915, TC279628. | Growth-regulating factor 1, Growth-regulating factor 3. | Growth & Development |
| hvu-miR473, hvu-miR475, hvu-miR5522. | TC257839, TC265736, TC272532, TC270005. | Pto-like serine/threonine kinase, Nup133 nucleoporin family protein, Nup133 nucleoporin family protein, Phosphate/ phosphoenolpyruvate translocator. | Cell signalling |
| hvu-miR1432, hvu-miR5070, hvu-miR5201, hvu-miR7753. | BY847007, TC269416, TC262474, CA019624. | Monosaccharide transporter 4, Membrane protein-like, StAR-related lipid transfer protein 7 (StARD7) (START domain-containing protein 7) (Protein GTT1), MDR-like ABC transporter. | Transporter |
| hvu-miR1432. | TC238726. | Beta tubulin 6. | Structural proteins |

## Conclusions

We have identified novel 18 new conserved miRNA clusters belonging to 17 miRNA families and one miRNA cluster in both sense and anti-sense strand from barley EST sequences based on bioinformatics tools. Seventeen families were reported for the first time. These findings will be helpful to clarify the functions and processing of miRNAs in barley. It has been also proved that bioinformatics approaches are efficient tools for the identification of new pre-miRNA clusters in different plant species. 81 total targeted proteins by mature miRNAs in barley related to different protein functional classes such as hypothetical, metabolism, transcription factors, transport, signalling, growth & development and structural were obtained.

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