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Genome-wide investigation of regulatory roles of lncRNAs in response to heat and drought stress in *Brassica juncea* (Indian mustard)

Garima Bhatia[a,1], Aman Singh[a,1], Deepika Verma[a], Shailesh Sharma[b], Kashmir Singh[a,*]

[a] Department of Biotechnology, Panjab University, Chandigarh, 160014, India
[b] National Institute of Animal Biotechnology (NIAB), D. No. 1-121/1, 4th and 5th Floors, Axis Clinical Building, Opp. to Talkie Town, Miyapur, Hyderabad, Telangana, 500 049, India

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ABSTRACT

Long non-coding RNAs are in general described as transcripts > 200 nt and lacking potential to code for proteins. In the present study, 7613 putative lncRNAs were identified in *Brassica juncea* genome using *in silico* approaches. Of these, 1614 lncRNAs were found to be differentially expressed in response to heat and drought stress conditions. Further, we characterized these lncRNAs and performed functional annotation based on co-expression analysis strategy and pathways enrichment analysis. On these bases, the identified abiotic stress-responsive lncRNAs were found to be associated with important pathways including both enzymatic and non-enzymatic antioxidants such as glutathione metabolism, phenylpropanoid biosynthesis, cysteine metabolism, etc. Moreover, lncRNAs were also found to co-express with transcription factors associated with abiotic stress response. Finally, those lncRNAs were identified that could act as putative targets and endogenous target mimics of miRNAs involved in response to heat and drought. Further investigation could be carried out for select lncRNA candidates like those for which quantitative polymerase chain reaction analysis was performed in this study or for those which were estimated as promising modulators of stress response based on miRNA-lncRNA interaction studies. Overall, our study highlights the potential role of lncRNAs in *B. juncea* in response to both heat and drought stress and can help elucidate the mechanism of tolerance to these abiotic stress conditions in the oil seed crop.

1. Introduction

Brassica juncea (Indian mustard) is an allopolyploid (AABB, 2n = 36) member Brassicaceae and is an outcome of natural hybridization between *Brassica rapa* (AA, 2n = 20) and *Brassica nigra* (BB, 2n = 16) followed by spontaneous chromosome doubling (Yang et al., 2016). An economically and nutritionally important annual to biennial oilseed crop, *B. juncea* is primarily cultivated in nations such as India, China, and Canada. However, like majority of crops being grown worldwide, its survival, growth, and productivity are affected by biotic and abiotic stress conditions. Particularly, heat/high temperature and drought stresses are emerging as the most potent constraints to crop production owing to shrinking precipitation and drastically changing rainfall patterns (Fahad et al., 2017). Heat stress disturbs plant's cellular homeostasis and affects protein denaturation/synthesis, membrane integrity, etc. often leading to cell injury and even death (Bita and Gerats, 2013; Liu et al., 2014). Drought leads to both oxidative stress-induced cell damage due to overproduction of reactive oxygen species (ROS) and reduced photosynthesis (Hussain et al., 2019). The plant response mechanisms for these stress conditions include dynamic transcriptional reprogramming of genes involved in protection, detoxification, transport, secondary metabolism, etc. (Pandey et al., 2015). In *B. juncea*, till date, different studies have been conducted to understand how enzymatic (such as superoxide dismutase - SOD, glutathione reductase - GR, ascorbate peroxidise - APX, etc.) and non-enzymatic antioxidant systems (such as flavonoids, anthocyanins, ascorbic acid, etc.), transcription factors, and miRNAs modulate the plant response to heat and drought (Bhardwaj et al., 2015, 2014; Sahay et al., 2019; Verma et al., 2019); however, to our knowledge, the role of long non-coding RNAs (lncRNAs) had not yet been explored.

lncRNAs are transcripts with length more than 200 nt but without known coding potential. These transcripts along with other regulatory RNAs help in coordinating biological processes across prokaryotes and eukaryotes. In plants, lncRNAs have been identified in specific tissues,

* Corresponding author at: Department of Biotechnology, BMS Block I, Panjab University, Sector 25, Chandigarh, 160014, India.
E-mail address: kashmirbio@pu.ac.in (K. Singh).
[1] Equal Contribution by both authors.

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at distinct developmental stages, or in response to particular stress conditions (Bhatia et al., 2017; Nejat and Mantri, 2018; Sun et al., 2018).Of the identified lncRNAs expressing under abiotic stress conditions in plants, *INDUCED BY PHOSPHATE STARVATION 1 (IPS1)* is a classic example of a functionally characterized cytoplasmic lncRNA that acts via target mimicry to sequester miR-399, which originally targets *PHOSPHATE2(PHO2)* mRNA (Franco-Zorrilla et al., 2007). Another interesting example is *DROUGHT INDUCED lncRNA (DRIR)*, which enhances drought and salt stress tolerance in *Arabidopsis thaliana* by regulating the expression of genes involved in abscisic acid (ABA) signaling, water transport, and stress relief processes (Qin et al., 2017). However, via *in situ* hybridization, it has been demonstrated to be nucleus-localized, which suggests the possibility of underlying mechanisms other than target mimicry. Besides, drought-responsive lncRNAs have been identified in plants such as *Setaria italica* (foxtail millet), *Zea mays* (maize), *Populus trichocarpa* (California poplar), *Hordeum vulgare* L. ssp. *Spontaneum* (Tibetan wild barley) and *Manihot esculenta* Crantz (cassava) (Qi et al., 2013; Qiu et al., 2019; Shuai et al., 2014; Xiao et al., 2019; Zhang et al., 2014), while heat stress-responsive lncRNAs have been reported in plants like *B. rapa* ssp. chinensis (non-heading Chinese cabbage), *Raphanus sativus* (radish), and *Cucumis sativus* (cucumber) (He et al., 2019; Song et al., 2016; Wang et al., 2019; Yang et al., 2019). Interestingly, these independent drought and heat stress-responsive lncRNA identification studies were based on RNA-seq data.

With this background, we harnessed the RNA-seq data available for *B. juncea* under heat and drought stress and identified 1614 lncRNAs responsive to these abiotic stress conditions. It is important to analyze huge volumes of data being submitted in the public domain to add meaning and new dimensions to our pre-existing knowledge. This study was an attempt in this direction and we observed the potential of lncRNAs in regulation of *B. juncea* response to drought and heat via interaction with transcription factors (TFs) and miRNAs. Together, these transcripts and their putative interactions can further be explored to enhance the abiotic-stress tolerance levels in this oilseed crop.

2. Materials and methods

2.1. RNA-seq data collection

To obtain the transcriptomic data corresponding to heat and drought stress, National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database was screened thoroughly (https://www.ncbi.nlm.nih.gov/sra). Details of the selected SRA data for this study in *B. juncea* are summarized in the Supplementary Table 1. The downloaded transcriptomic data were filtered to remove low quality reads and adaptor sequences using NGS-QC toolkit (Patel and Jain, 2012). The cut-off value for PHRED quality score was 20 (default).

2.2. Computational prediction of B. juncea lncRNAs using the collected transcriptomic data

The computational pipeline used to identify lncRNAs is as shown in Fig. 1. Prior to assembly, reads were aligned to an indexed genomic file using STAR aligner (v2.4.0.1) (Dobin et al., 2013). Next, genome-guided transcriptome assembly was carried out using Cufflinks (v2.2.1) (Trapnell et al., 2010). The reference genome for *B. juncea* was downloaded from BRAD database (Cheng et al., 2011). The independent assemblies were merged using Cuffmerge. The finally assembled transcripts were filtered on the basis of their length and sequences < 200 nt long were discarded. Next, Coding Potential Calculator (CPC) (Kong et al., 2007) was used to eliminate the transcripts with coding potential > 0. For the resulting potentially non-coding transcripts, BLASTX analysis (Altschul et al., 1997) was conducted against NCBI non-redundant (NR) protein database with an e-value of 1e-5 to eliminate sequences with significant homology to the known protein sequences. For the verification of our approach, a standalone BLASTN analysis was performed to compare our results with the previously reported *B. rapa* lncRNAs in CANTATAdb (Szcześniak et al., 2016).

2.3. In silico characterization and classification

The chromosome sequences of *B. juncea* were downloaded from BRAD and standalone BLASTN analysis was performed to investigate the chromosomal distribution of lncRNAs with the following parameters: percent identity > 90 and e-value $<$ 1e-5. Similar analysis was performed for the coding sequences (CDS) or mature mRNAs of the plant to draw comparisons between the two transcript classes. In order to classify the lncRNAs based on different genomic positions with respect to protein-coding transcripts, Cuffcompare was used and classification was done according to the “class codes”.

2.4. Differential expression analysis

The collected SRA data (as mentioned above) corresponding to control, heat and drought stress conditions were used to determine the expression levels of both the identified lncRNAs and mRNAs. Differential expression analysis was performed for the transcripts using Cuffdiff and the expression values were quantified as FPKM (Fragments per kilobase of transcript per million mapped reads). Two-fold change values were calculated as: log2 (FPKM stress/FPKM control). Transcripts with FPKM < 1 were not considered. Heat maps were developed to study the trends of expression for both lncRNAs and mRNAs using Hierarchical Clustering Explorer v3.5 (http://www.cs.umd.edu/hcil/hce/).

2.5. Functional annotation of the identified lncRNAs

Functional annotation of the identified lncRNAs was carried out based on co-expression analysis with respect to mRNAs. The co-expressing lncRNA and mRNA pairs were identified using CoExpress v1.5 tool (Nazarov et al., 2013) based on FPKM values and Pearson-correlation coefficient ≥ 0.9. Next, gene ontology (GO) enrichment analysis was conducted for the mRNAs co-expressing with lncRNAs using Blast2GOsoftware (Conesa and Götz, 2008). Additionally, pathway enrichment analysis was conducted using Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways database (Kanehisa et al., 2017).

2.6. Association of the identified lncRNAs with transcription factors (TFs)

In order to investigate the association of TFs with the identified abiotic stress-responsive lncRNAs, CDS of the known TFs for *B. rapa* available in the Plant TF database v4.0 (PlantTFDB) (Jin et al., 2017) were downloaded, and co-expression analysis (as described above) was conducted to identify the co-expressing TF-lncRNA pairs with similar parameters.

2.7. Interaction of lncRNAs with miRNAs

We explored the interaction between miRNAs and the identified abiotic stress-responsive lncRNAs in *B. juncea* using the following: *B. rapa*-specific 157 mature miRNAs available at miRNA database (miRBase) (Kozomara et al., 2019) and 51 conserved and 37 true novel *B. juncea* miRNAs reported by Bhardwaj et al., 2014. Target sites for these miRNAs in lncRNAs were predicted using plant small RNA target analysis server (psRNATarget) (Dai et al., 2018) with default parameters. Further, endogenous target mimics (eTMs) were predicted using TAPIR (http://bioinformatics.psb.ugent.be/webtools/tapir/) (Bonnet et al., 2010) with mfe_ratio $> = 0.7$ for *B. rapa*-specific and conserved *B. juncea* miRNAs and mfe_ratio $> = 0.6$ for true novel *B. juncea* miRNAs.

LncRNA secondary structure was determined using Vienna RNAfold

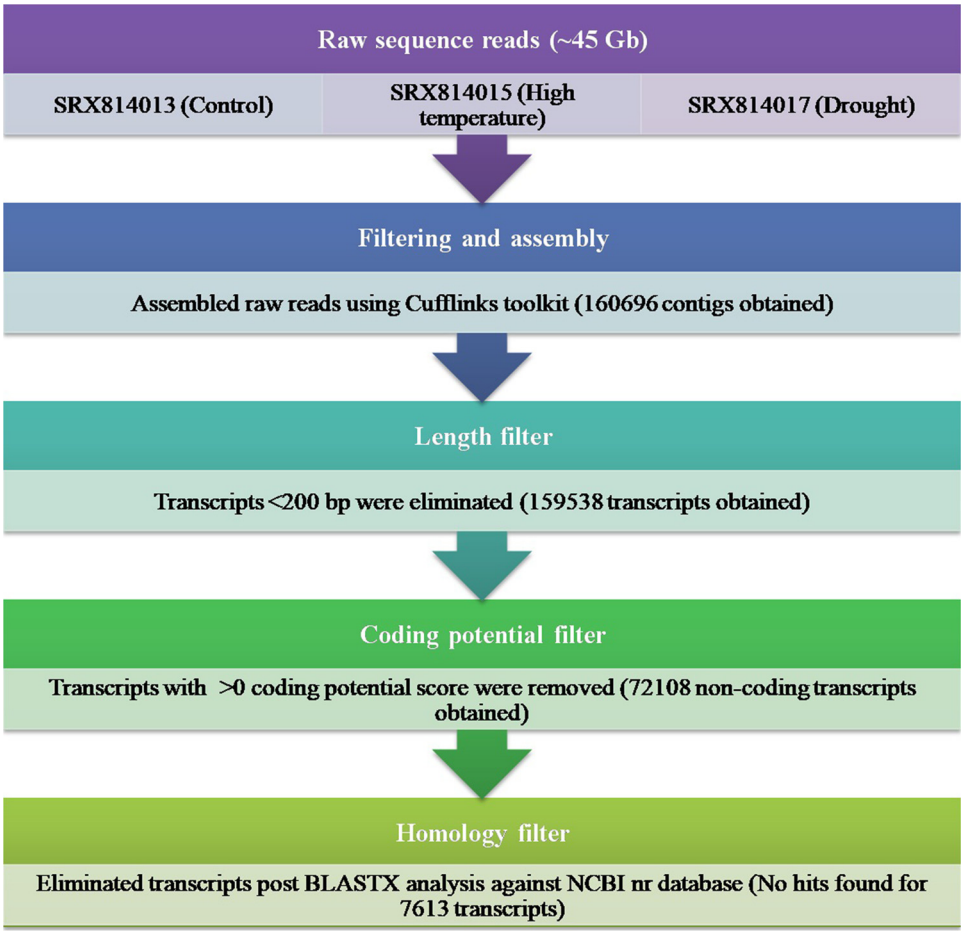

Fig. 1. Computational pipeline followed for genome assembly and lncRNA identification. With each step, the transcripts were narrowed down to putative lncRNAs in *Brassica juncea*.

web server (http://rna.tbi.univie.ac.at/) (Hofacker, 2003) based on minimum free energy.

Finally, interaction network analysis of the miRNAs and lncRNAs was conducted using Gephi (https://gephi.org/) (Bastian et al., 2009).

2.8. qRT-PCR based expression analysis of lncRNAs

2.8.1. Plant materials and RNA extraction

The expression of selected abiotic-stress responsive lncRNAs was studied in *B. juncea* plants, which were grown under following conditions: soil:soil rite = 2:1, temperature 20–24 °C, and 16/8 h (light/dark) in the plant growth chamber at the Department of Biotechnology, Panjab University, Chandigarh. These plants (20 days old) were exposed to stress conditions in triplicates as follows: for drought stress, 20% (w/v) PEG 8000 solution for 4 and 8 h; for heat stress, exposure to 40 °C for 4 and 8 h. The leaves of the plants under respective stress and control conditions were harvested in liquid nitrogen. Total RNA was isolated using the protocol by Ghawana et al. (2011). Further, the quantity and integrity of RNA samples were analyzed by measuring 260/280 nm ratios using Nanodrop spectrophotometer and by 1.2% agarose gel electrophoresis, respectively.

2.8.2. qRT-PCR analysis

cDNA was prepared using Superscript III first strand cDNA synthesis kit (Invitrogen USA) and 4 and 8 h samples were pooled for each of the two stress conditions independently. The primers for qRT-PCR were designed using Primer3 Input software (Supplementary Table 2). PCR amplifications were conducted using Bio-Rad CFX96™ Real-Time PCR system. *Tonoplastic Intrinsic Proteins-41 (TIPS-41)* was used as internal control gene for normalization of gene expression. The experimental conditions were as follows: 95 °C for 7 min, 40 cycles of 95 °C for 20 s, Tm for 20 s and 72 °C for 20 s. $2^{-\Delta\Delta CT}$ method was used to estimate the relative gene expression (Livak and Schmittgen, 2001). All the experiments were conducted in triplicates.

3. Results

3.1. Genome-wide identification of B. juncea lncRNAs

After careful scrutiny of NCBI SRA database, around 45 Gb of raw RNA-seq data for control, heat, and drought stress conditions in *B. juncea* were downloaded. Post the quality check and filtering using NGS-QC toolkit, low-quality reads were eliminated. The high-quality filtered raw reads were mapped to *B. juncea* genome and assembled using Cufflinks. Next, independent assemblies were merged using Cuffmerge and 160696 contigs were obtained. Out of these, transcripts longer than 200 nucleotides, that is, 159538 were chosen. Coding potential of these sequences was calculated, and transcripts with score > 1 were eliminated. For the resulting 72108 potentially non-coding transcripts, BLASTX analysis against NCBI non-redundant database was conducted, and 7613 transcripts were obtained for which no hits were found. These were designated as putative lncRNAs in *B. juncea*. For verifying the results of our pipeline, a standalone BLASTN analysis was performed for the identified lncRNAs with respect to the previously reported lncRNAs in *B. rapa* available in the CANTATA database. We observed 1198 hits that confirmed correctness of our prediction pipeline.

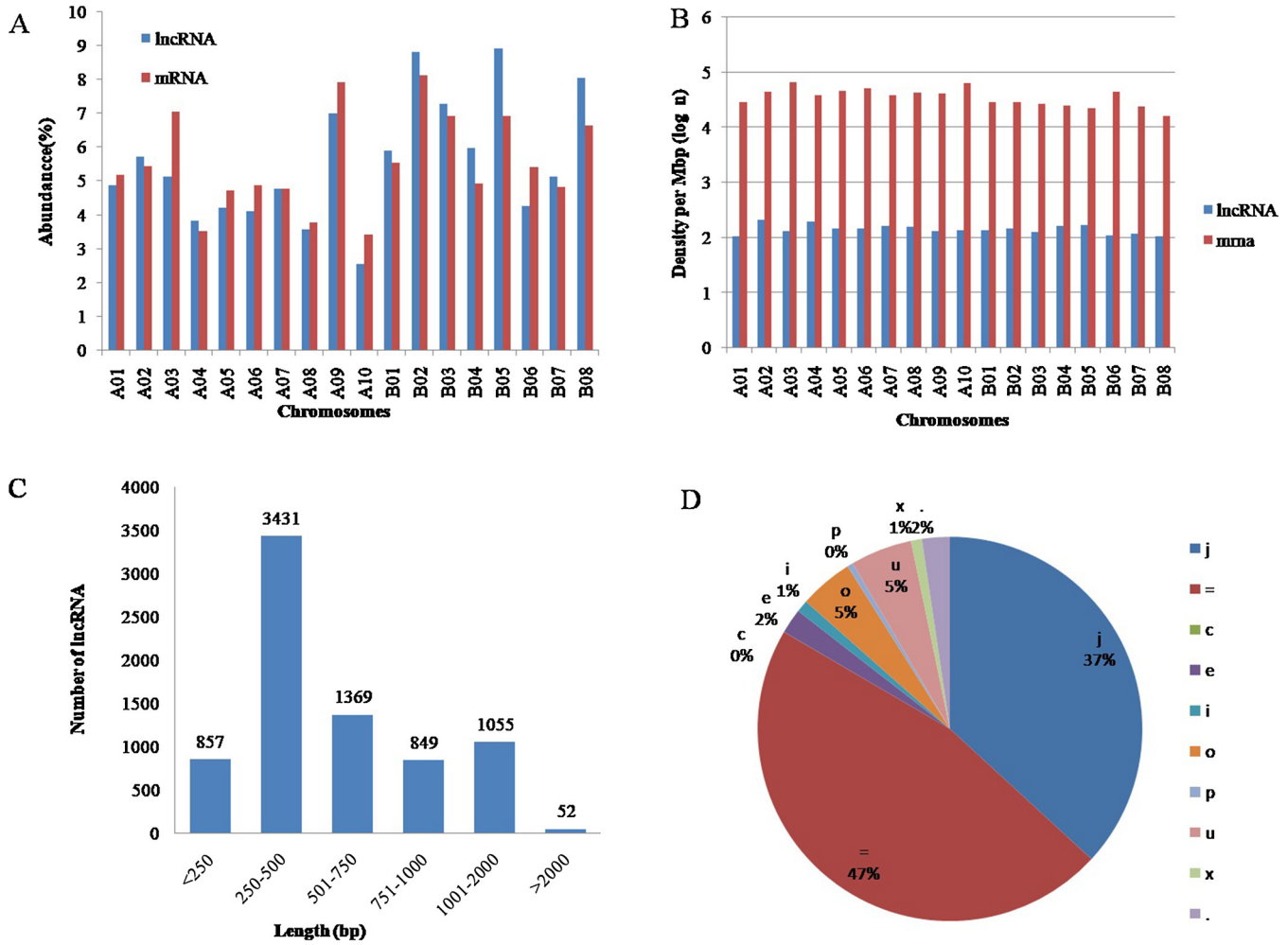

Fig. 2. Features of the identified *B. juncea* lncRNAs (A) Chromosome-wise distribution of lncRNAs and mRNAs. The results are depicted as abundance (percentage) of lncRNAs and mRNAs per chromosome (B) Chromosomal density distribution of lncRNAs and mRNAs. (C) Length distribution of lncRNAs. (D) Classification of lncRNAs on the basis of their genomic locations with respect to adjacent protein coding genes. The pie chart depicts the percentage of lncRNAs falling into different categories (that is, class codes as per Cuffcompare-based analysis). The class codes correspond to the following: "=", complete match with intron chain; "c", contained; "j", potentially novel isoform; "e", single exon transcript overlapping a reference exon that could be a pre-mRNA fragment; "i", lncRNA falling entirely within a reference intron; "o", generic exonic overlapping with the reference transcript; "p", possible polymerase run-on fragment; "u", unknown intergenic transcript; "x", exonic overlap with the reference transcript on the opposite strand; "s", intronic overlap with the reference transcripts possibly due to mapping errors. Chr-chromosome.

3.2. *In silico characterization of the identified lncRNAs*

The predicted lncRNAs were characterized *in silico* to understand how they occur in the *B. juncea* genome. These characteristics were also analyzed for the mature mRNAs in order to gain insight into the two different transcript categories (Fig. 2).

3.2.1. *Chromosomal distribution*

The entire genome of *B. juncea* is divided into 18 chromosomes from A01 to A10 and B01 to B08. The distribution of 6639 lncRNAs across the 18 chromosomes could be studied based on BLASTN analysis (Fig. 2A). The lncRNAs were distributed unevenly with the highest and lowest percentage of abundance observed on chromosomes B05 (8.9 %) and A10 (2.5 %), respectively. However, distribution pattern of mature mRNAs revealed the highest and lowest percentage of abundance with respect to chromosomes B02 (8.1 %) and A10 (3.4 %), respectively. Further, it was observed that more than half of the lncRNAs (that is, 3605) belonged to the B subset of *B. juncea* genome despite having lesser number of chromosomes. An opposite trend was observed for the mature mRNAs, where more transcripts (that is, 36203) were derived from A subset of the genome.

3.2.2. *Chromosomal density*

Almost an even density distribution was observed for the predicted lncRNAs in *B. juncea* with very little differences as shown in Fig. 2B. lncRNAs were more densely present on chromosome A02 (10.14 lncRNAs per Mbp of nucleotides) and least densely present on chromosome A01 (7.57 lncRNAs per Mbp of nucleotides). The chromosomal density trend of mature mRNAs revealed that they are more densely present on the chromosomes than the lncRNAs. The highest density was reported on chromosome A03 (123.8 mRNAs per Mbp of nucleotides) and least was reported on chromosome B08 (67.77 mRNAs per Mbp of nucleotides).

3.2.3. *Length distribution*

The length of the identified lncRNAs ranged from 200 to 7617 bp; however, length of around 45% of lncRNAs ranged between 250 and 500 nucleotides (Fig. 2C).

3.2.4. *Classification*

The lncRNAs were classified according to their genomic locations with respect to those of the neighbouring protein coding genes. Nearly half of the lncRNAs (47 %) showed a complete match with an intron

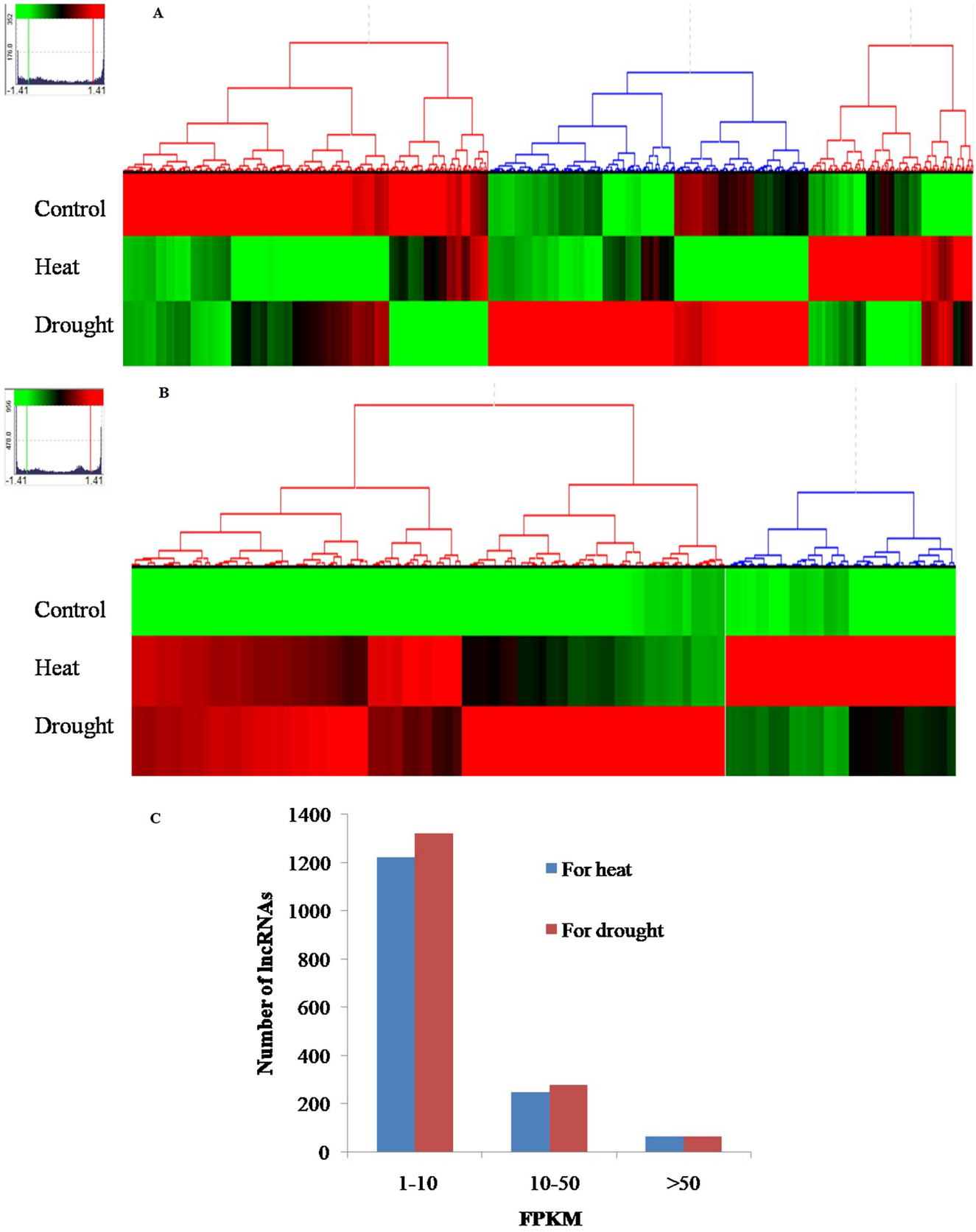

Fig. 3. Expression profile of lncRNAs (A) Differential expression analysis for heat and drought responsive lncRNAs (FPKM > 1). (B) Fold change expression analysis for heat and drought responsive lncRNAs. Arrangement is according to similar expression levels and hot spots are in red. (C) Distribution of lncRNAs based on their expression levels during heat and drought stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chain (class code "="). Of the total lncRNAs, 37% were potentially novel transcripts (at least one splice junction is shared with a reference transcript, class code "j"). The remaining lncRNAs were contained in different categories such as unknown, intergenic (class code "u") and generic exonic overlap with a reference transcript (class code "o"), besides others as shown in Fig. 2D.

3.3. Expression profile of the identified B. juncea lncRNAs

In order to identify heat- and drought-responsive coding sequences and long non-coding RNAs, FPKM values were calculated using Cuffdiff and those with FPKM > 1 were considered. We found 1614 lncRNAs that were differentially expressed in response to heat and drought stress in *B. juncea* (Fig. 3A). Likewise, 25,665 differentially expressed mature mRNAs were observed (Supplementary Fig. 1A). Furthermore, log 2 fold change was estimated for the abiotic stress conditions compared to the control and 195 lncRNAs (Fig. 3B) and 3828 mature mRNAs were observed (Supplementary Fig. 1B). Next, based on the expression levels or FPKM values, the abiotic stress responsive lncRNAs were divided into 3 categories, that is, low (> = 1 and < 10), moderate (> = 10 and < 50) and high (> 50) expressing. It was observed that majority of the differentially expressed lncRNAs fell into low and moderate expression categories (Fig. 3C).

3.4. Functional annotation of the abiotic stress-responsive lncRNAs in B. juncea

In order to understand the possible functions of the identified stress-responsive lncRNAs, co-expression analysis was conducted. The rationale behind this approach was that lncRNAs expressing along with coding sequences or mature mRNAs, are likely to be involved in similar functions. Co-expression correlation was estimated using Pearson correlation coefficient with $R^2 \geq 0.9$ for 1614 lncRNAs and 25665 mature mRNAs. The highly correlated pairs were identified and it was found that 842 lncRNAs co-expressed with 469 mature mRNAs (Supplementary File 1). Further, GO enrichment analysis was conducted for these 469 mature mRNAs using Blast2GO. About 83% of these could be annotated functionally and were divided into three categories as per the assigned GO terms (Fig. 4A–C). The top 20 terms in each category included GO terms that indicate the involvement of lncRNAs in response to abiotic stress conditions (heat and drought) (Supplementary File 2); for instance, (i) the biological processes category included terms such as GO:0006950 'response to stress' and GO:0009628 'response to abiotic stimulus'. (ii)The molecular functions category included terms such as GO:0043167 'ion binding majority' and GO:0016491 'oxidoreductase activity'. (iii) The cellular components category included terms like GO:0005622 'intracellular' and 'GO:0043229 intracellular organelle'. Interestingly, 29, 18, 18, 14, and 12 coding sequences co-expressing with lncRNAs were found to be involved in biological processes: 'response to abscisic acid', 'response to oxidative stress', 'response to water deprivation', 'response to heat', and 'cellular oxidant detoxification', respectively (Supplementary File 2).

To further deepen the annotation of the co-expressing sequences, domain analysis using InterProScan was conducted, which helped refine the results with respect to domain architecture, families, and repeats (Supplementary Fig. 2). Fig. 4D shows the different domains along with their InterPro IDs. Domains such as thioredoxin domain (IPR013766), glutathione S-transferase C-terminal (IPR004046), protein kinase domain(IPR000719), histidine kinase/HSP90-like ATPase (IPR003594), heme peroxidise (IPR002016), DnaJ domain (IPR001623), heat shock protein Hsp90 N-terminal (IPR020575) and manganese/iron superoxide dismutase N-terminal (IPR019831) indicate the plausible involvement of co-expressing-lncRNAs in regulating the plant response to heat and drought stress.

Further enzyme codes (EC) based classification was conducted for the annotated sequences (Fig. 4E). Based on EC distribution pattern, it was observed that out of the six major EC classes, the maximum co-expressing lncRNA-mRNA pairs belonged to oxidoreductases, transferases, and hydrolases classes. Next, pathways enrichment analysis was conducted based on KEGG pathways database, which showed that the heat- and drought-responsive *B. juncea* lncRNAs could possibly be involved in representatives of 68 pathways (Supplementary File 3). Among these, based on the number of highly enriched enzymes, lncRNAs are likely to be involved in regulation of the following pathways: 'glycolysis/gluconeogenesis', 'carbon fixation in photosynthetic organisms', 'cysteine and methionine metabolism', 'glyoxylate and dicarboxylate metabolism', 'glutathione metabolism', 'phenylpropanoid biosynthesis', 'arginine and proline metabolism', 'ascorbate and aldarate metabolism', etc.

3.5. Association of heat- and drought- responsive lncRNAs with transcription factors in B. juncea

We identified transcription factor (TF) families co-expressing with heat- and drought-responsive *B. juncea* lncRNAs based on the sequence information available for *B. rapa* in the Plant TF database (PlantTFDB) v4.0. It was observed that 21 lncRNAs co-expressed with 10 TF families indicating their potential involvement in response to heat and drought (Fig. 5). The highest percentage of co-expressing lncRNA-TF pairs was observed for MIKC-MADS, which is a regulator of stress-related responses including developmental plasticity (Castelán-Muñoz et al., 2019) and NAC families (a plant-specific TF family) followed by MYB family, which are positive regulators of plant stress tolerance (Hoang et al., 2017).

3.6. Interactions of B. juncea heat- and drought-responsive lncRNAs with miRNAs

In order to gain a comprehensive view of the possible *B. juncea* lncRNA-miRNA interaction, we downloaded the available *B. rapa*-specific 157 mature miRNAs from mirBase (miRNA database) and *B. juncea*-specific conserved (51) and true novel (37) miRNAs as reported by Bhardwaj et al., 2014. Next, using plant small RNA target analysis server, psRNATarget, we identified those heat- and drought-responsive *B. juncea* lncRNAs that could act as putative targets of miRNAs. Based on our analyses, 795, 379, and 287lncRNAs were predicted to be targeted by 152 *B. rapa*-specific, 50 *B. juncea* conserved and 36*B. juncea* novel miRNAs, respectively (Supplementary File 4).Further, 7, 6, and 18lncRNAswere predicted to be endogenous target mimics (eTMs) for 8 *B. rapa*-specific, 6*B. juncea* conserved and 10 *B. juncea* novel miRNAs. Fig. 6A and B show the common and exclusive *B. juncea* lncRNAs as targets and eTMs with respect to *B. juncea* and *B. rapa* miRNAs. Interestingly, we identified lncRNAs that potentially interact with miRNAs, which have been studied in response to abiotic stress conditions, particularly, drought and heat stress; for instance, miR156, miR159, miR172, miR319, and miR399. Fig. 6C and D represent the examples of secondary structure prediction of lncRNAs as putative targets and eTMs of miRNAs, respectively. Lastly, the interactions between miRNAs and lncRNAs were visualized, which revealed the intricacy of their relationships (Fig. 7, Supplementary File 5).

3.7. Expression analysis of select lncRNA candidates using qRT-PCR

Of the highly expressing (FPKM > 100) abiotic stress-responsive lncRNAs, we randomly selected candidates to validate their expression profile using quantitative real time polymerase chain reaction (qRT-PCR). Based on the analysis, similar trends of expression were observed as those observed using the RNA-seq data (Fig. 8). Especially for lncRNAs TCONS_00051908 and TCONS_00088973, higher expressions were observed under heat and drought stress conditions, respectively, compared to the control in both qRT-PCR and RNA-seq data based analysis.

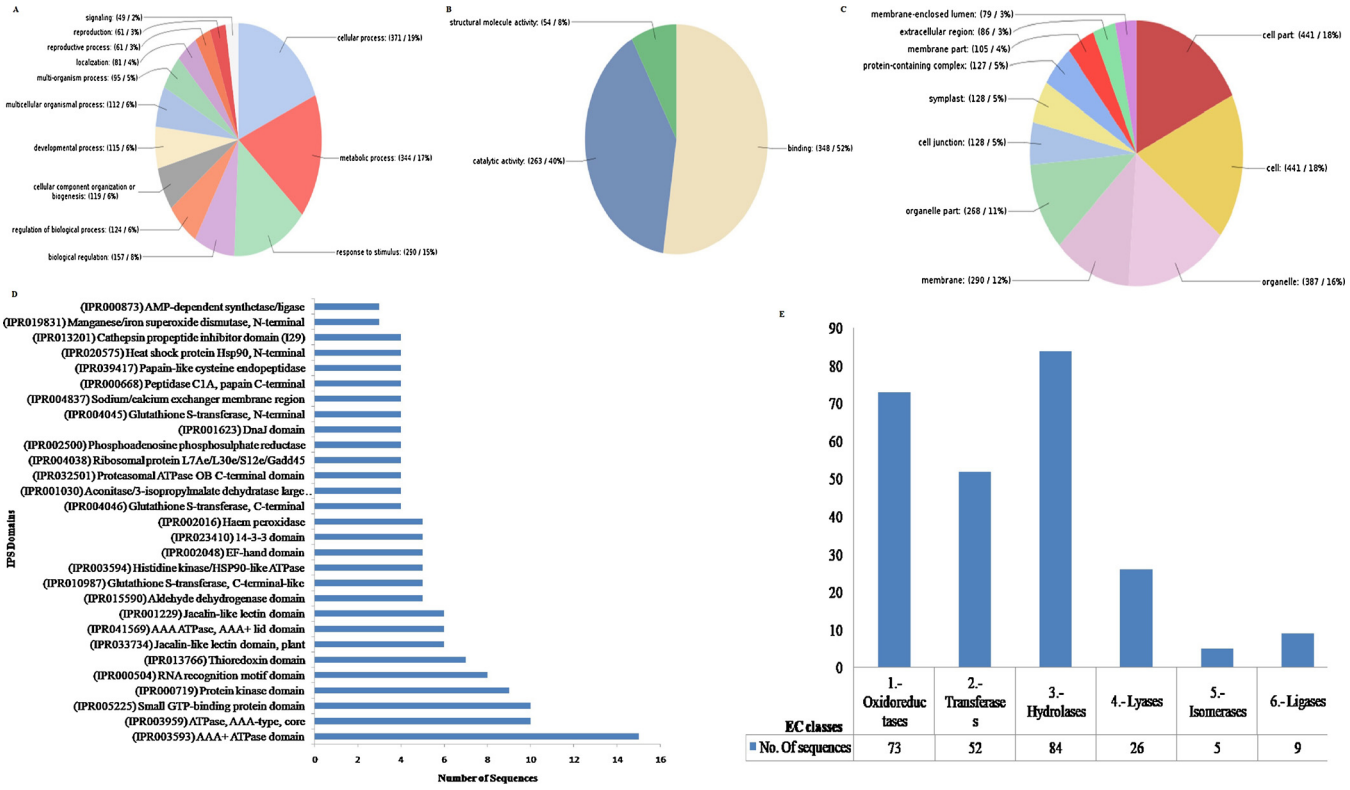

Fig. 4. Top gene ontology (GO) Terms showing enrichment for lncRNAs co-expressing with mRNAs: The enrichment is represented in three categories: (A) BP, biological process; (B) MF, molecular function; and(C) CC, cellular component. (D) Domain distribution of co-expressing mature mRNAs with lncRNAs into different categories as per GO terms. (E)Enzyme code classification of mature mRNAs with lncRNAs.

4. Discussion

Over the last couple of years, several studies have been conducted to identify and characterize lncRNAs in different members of Brassicaceae. In fact, a majority of the earliest studies to understand lncRNAs in plants were centred on *Arabidopsis* (Ben Amor et al., 2009; Franco-Zorrilla et al., 2007; Liu et al., 2012; Wang et al., 2014; Zhu et al., 2014). Amongst subsequent studies based on abiotic stress response in *Brassica* species, lncRNAs have been reported in *B. rapa* ssp. *chinensis* in response to cold and heat stress (Song et al., 2016; Wang et al., 2019) and in *B. napus* in response to cadmium toxicity (Feng et al., 2016). To our knowledge, this is the first study where we have examined the role of lncRNAs in *B. juncea* in response to heat and drought. These abiotic stress conditions impede plant growth and development while imposing

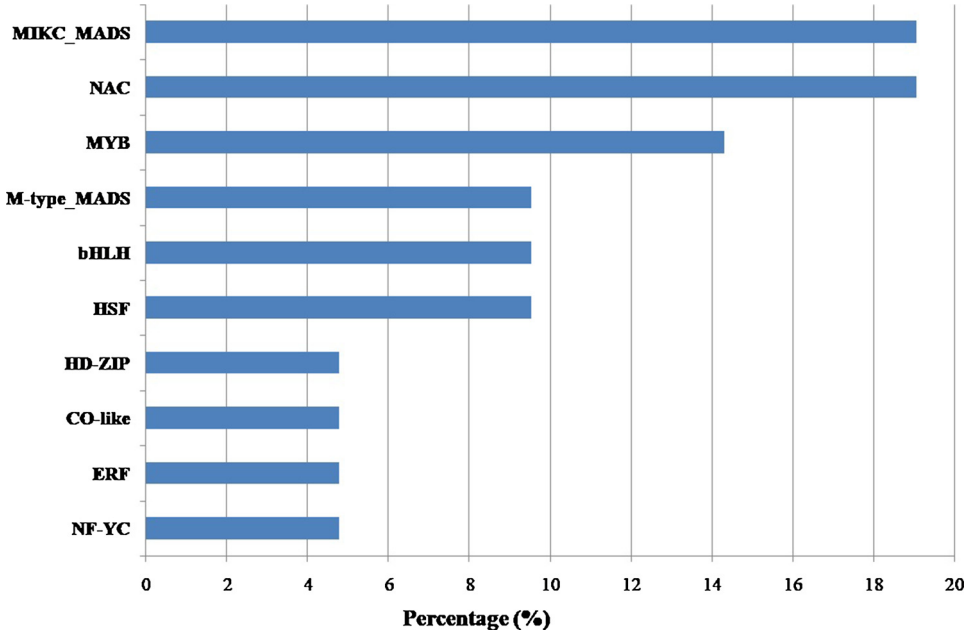

Fig. 5. Transcription factor (TF) families co-expressing with heat- and drought-responsive *B. juncea* lncRNAs. The highest percentage of co-expressing lncRNA-TF pairs was observed for MIKC-MADS and NAC families.

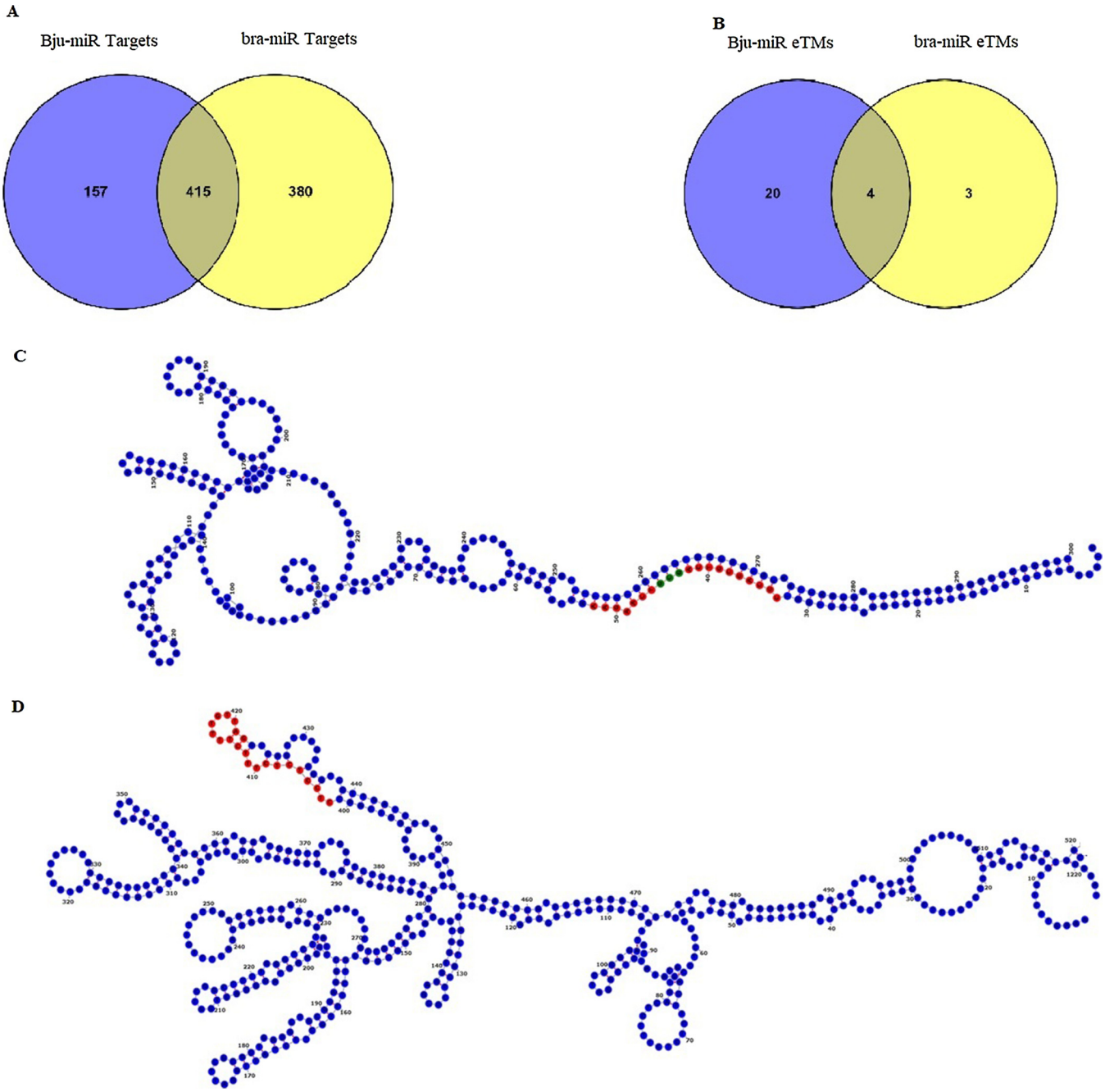

Fig. 6. LncRNAsas putative targets and endogenous target mimics (eTMs) of miRNAs. (A) A Venn diagram showing lncRNAs that can act as targets of Bju-miRNAs and bra-miRNAs. (B) A Venn diagram showing lncRNAs that can act as endogenous target mimics of Bju-miRNAs and bra-miRNAs. (C) Secondary structure of an lncRNA (TCONS_00081575) shown in blue, which acts as a putative eTM for miRNA (Bju-miR172_1) shown in red. The characteristic 3-nt bulge is shown in green. (D) Secondary structure of an lncRNA (TCONS_00088622) shown in blue, which acts as a putative target of miRNA (Bju-miR156_4) shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

deleterious effects on crop production and yield (Fahad et al., 2017). Therefore, it is important to understand how plant response to such conditions is regulated in order to provide novel candidates for breeding to enhance stress tolerance.

In the present study, we identified 7613 lncRNAs using a computational pipeline based on RNA-seq data assembly and by applying length, coding potential, and homology-based filters. Foremost, *in silico* characterization of these lncRNAs was conducted, which revealed that nearly 87% of these could be attributed to the 18 chromosomes of the plant. Of these, 54 % were found to be distributed on the B subset of chromosomes despite being less in number compared to the A subset, whereas this trend was opposite in case of mRNAs. It would be interesting to further delve into this observation in the future studies because *B. nigra* (as the B sub-genome contributor) is known for its weed populations (Westman and Kresovich, 1999) and is yet to be examined for its long non-coding RNA landscape. The length distribution of the identified *B. juncea* lncRNAs was in sync with that reported for *B. rapa* ssp. *chinensis* and *B. napus* lncRNAs, that is, the majority were > 200 and < 1000 nt in length (Feng et al., 2016; Wang et al., 2019). The Cuffcompare program mediated categorization of lncRNAs performed on the basis of genomic locations with respect to the protein coding genes revealed a greater proportion of transcripts in "=" category as compared to negligible representation in "c" category. While the "=" category can be treated as complete transcripts, "c" category represents

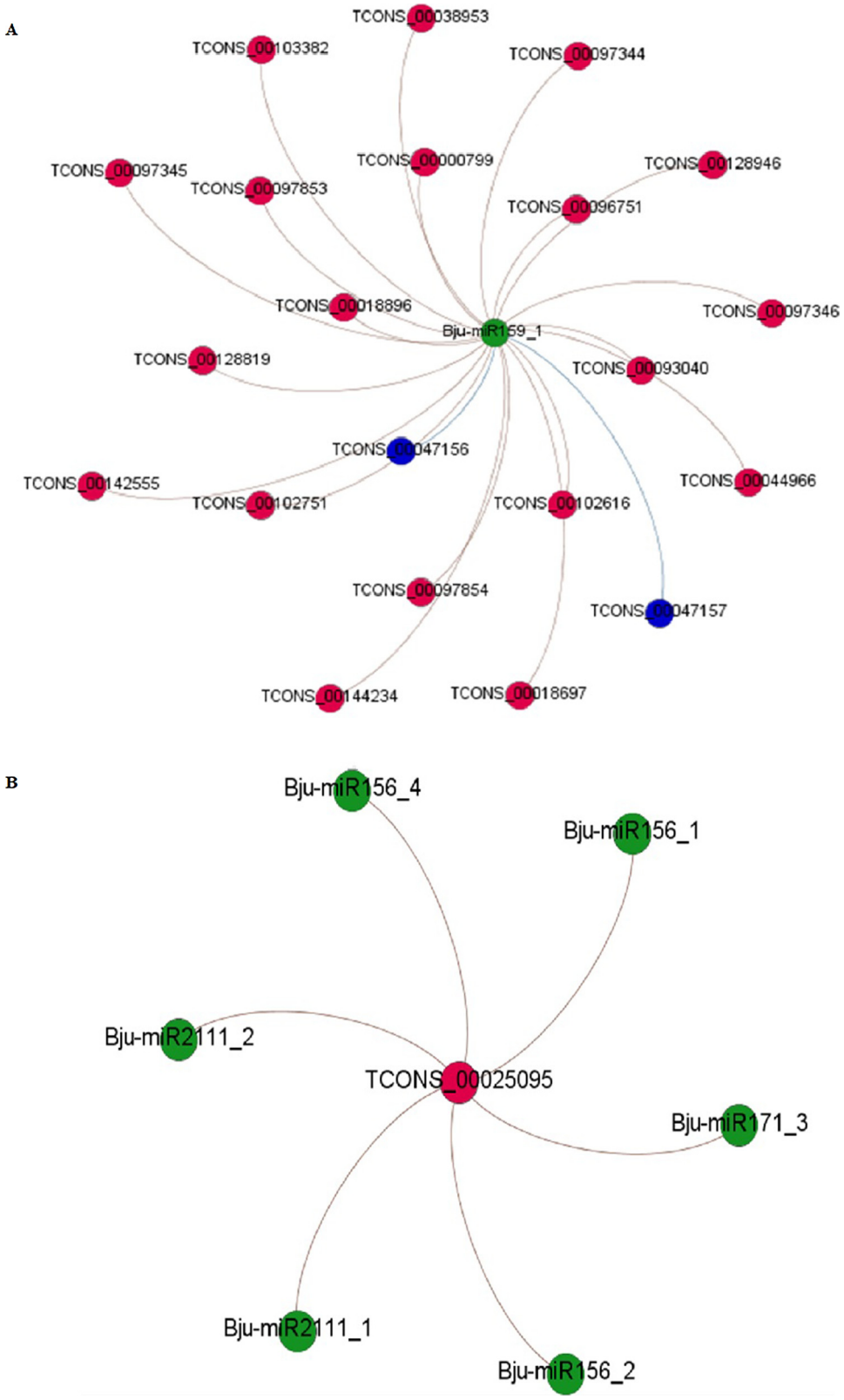

Fig. 7. Interaction network analysis representing (A) an miRNA (green) with multiple lncRNAs (red). A potential endogenous target mimic (eTM) is marked in blue. (B) Interaction of an lncRNA (red) with multiple miRNAs (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

partial assemblies (Sun et al., 2012). The "j" category could be exploited to identify the long non-coding isoforms of the known genes since these transcripts share at least one spliced site with reference transcripts.

Further, we explored the expression profile of mature mRNAs and lncRNAs to mine out the differentially expressed transcripts in response to heat and drought stress. We found1614 abiotic-stress responsive lncRNAs and our subsequent analysis was focused on understanding the

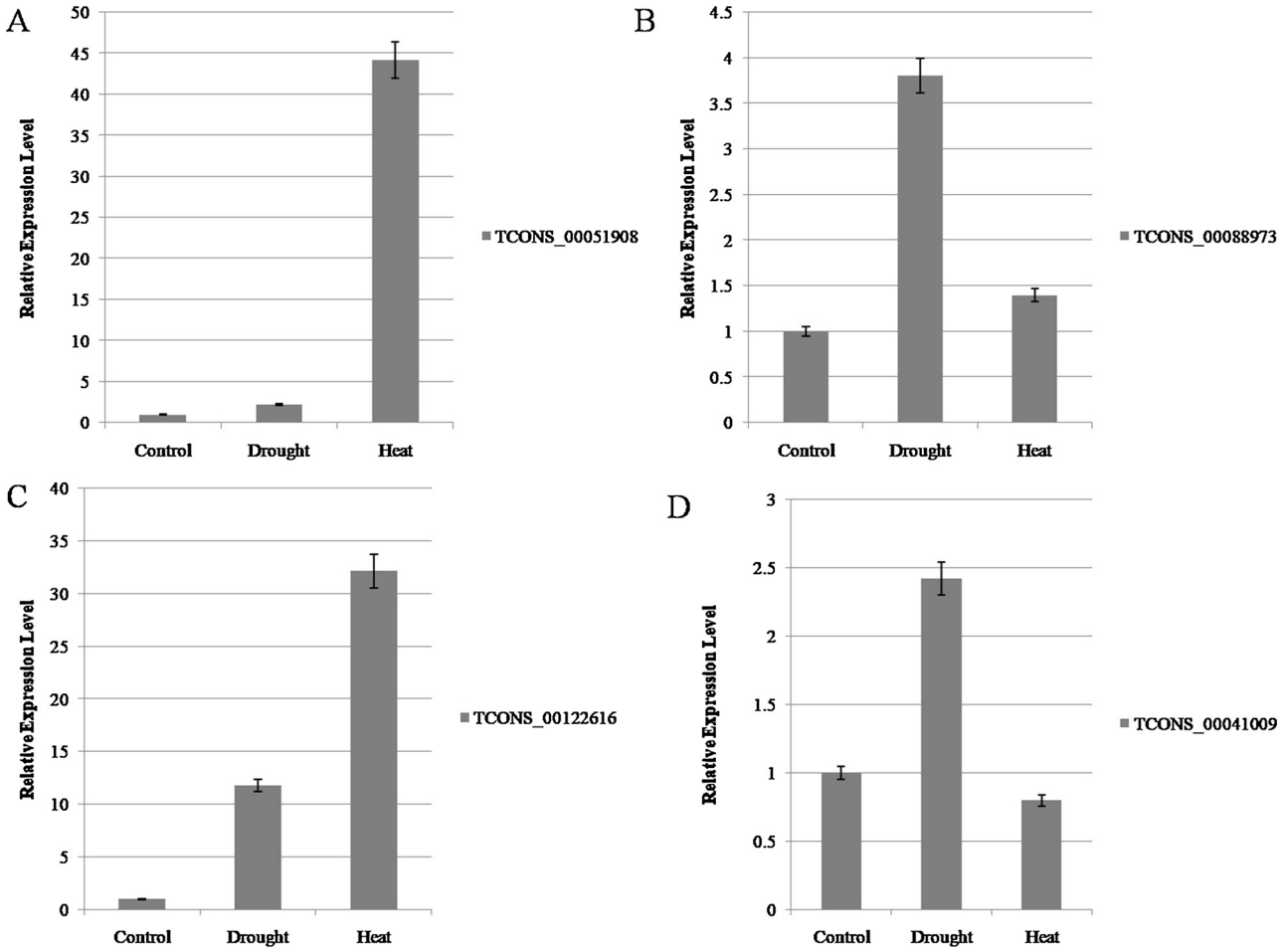

Fig. 8. Relative expression analysis of select lncRNAs using quantitative real time polymerase reaction. *Tonoplastic Intrinsic Proteins-41 (TIPS-41)* was used for normalization of gene expression.

putative functions these transcripts could be associated with in the plant. Firstly, co-expression analysis was conducted for *B. juncea* lncRNAs and mature mRNAs, and the former were predicted to be associated with important biological processes in response to drought and heat stress. The domain analysis results for these co-expressing mature mRNAs further highlighted the potentially important roles of these lncRNAs. For instance, domains of key ROS scavengers in plants such as manganese/iron superoxide dismutase (Verma et al., 2019), heme-containing peroxidases such as catalase and ascorbate peroxidase (Anjum et al., 2016), and glutathione S-transferase (Kumar and Trivedi, 2018) were observed. This suggests the involvement of co-expressing lncRNAs in restoring the cellular redox homeostasis disturbed during stress conditions like drought. Likewise, protein kinases (protein kinase domain)were prominently observed, which have been reported to be responsive to heat, drought, and cold stress during transcriptomic profiling studies in *B. juncea* (Bhardwaj et al., 2015; Sinha et al., 2015), and the co-expressing lncRNAs could potentially be involved in regulation of the stress signaling pathways. In fact, our results also included the highly conserved histidine kinase domain, which is suggestive of the role of respective co-expressing lncRNAs in regulating response to abiotic stress conditions such as enhancing drought tolerance (Tran et al., 2007). Moreover, domain results for heat shock protein (HSP) 90 indicate the involvement of co-expressing lncRNAs in protection of proteins during heat stress.

In addition to the aforementioned enzymatic ROS scavengers, non-enzymatic systems are available in plants to balance the oxidized and reduced states of the cells, thereby avoiding adverse conditions/plant cell death. These comprise the antioxidants such as glutathione, proline, cysteine, ascorbic acid, flavonoids, phenolic acids, etc. (Foyer and Noctor, 2011; Sharma et al., 2019a). Based on pathways enrichment analysis, we found lncRNAs co-expressing with different enzymes involved in key pathways associated with the metabolism of these antioxidants. For instance, lncRNAs were found to be associated with metabolic pathways of glutathione and ascorbate, particularly co-expressing with gluthathione S-transferase (EC 2.5.1.18), phospholipid-hydroperoxide glutathione peroxidase (EC 1.11.1.12) and L-ascorbate peroxidase (EC 1.11.1.11) (Supplementary Fig. 3). Furthermore, lncRNAs were also associated with cysteine metabolism pathway. Cysteine acts as a sulphur donor for glutathione, which eventually plays a key role in quenching ROS through glutathione-ascorbate cycle and as an electron donor to glutathione peroxidase (Zagorchev et al., 2013). Another stress modulator, proline, acts as an osmolyte, signaling molecule, and is known to accumulate as an antioxidant in response to water deficit (Hayat et al., 2012) and we found lncRNAs co-expressing with enzymes involved in its metabolism. Similarly, lncRNAs were found to be associated with biosynthetic pathways of phenolic acids and flavonoids, which have been shown to accumulate in response to drought stress in plants. In *B. napus*, an increase in phenylalanine ammonia-lyase activity along with its increased expression under drought stress has already been reported (Rezayian et al., 2018). Clearly, based on this analysis, the identified *B. juncea* lncRNAs potentially aid in regulation of pathways activated to tolerate the abiotic stress conditions in the plant.

In earlier transcriptomic studies in response to abiotic stress in *B. juncea*, in addition to protein kinases, transcription factors (TFs) have been found to be majorly differentially regulated (Bhardwaj et al.,

2015; Sinha et al., 2015). Since lncRNAs are primarily involved in regulation of gene expression, we explored their possible association with TFs to understand whether the two regulatory moieties act in sync in response to abiotic stress. We identified *B. juncea* lncRNAs co-expressing with *B. rapa* TFs (since comprehensive information about *B. juncea* TFs was not available in the Plant TF database (PlantTFDB) v4.0). The identified co-expressing lncRNA-TF pairs can be further studied for detailed RNA-protein interactions to better understand the underlying mechanisms of stress tolerance in the plant.

Finally, the identified lncRNAs were examined for their possible interaction with miRNAs, which revealed that the former can act as both the targets and endogenous target mimics (eTMs) for the latter. The mechanism of target mimicry has been studied in plants such as *Arabidopsis* (Franco-Zorrilla et al., 2007), where lncRNAs sequester miRNAs to rescue the original target mRNAs. In fact, in our previous study on lncRNAs in *Vitis vinifera,* eTMs which could regulate TFs via sequestration/sponging of miRNAs were identified. For instance, lncRNA 'TR123921' was predicted to sequester 'vvi-miR156h', thereby sparing its originally targeted TF 'Squamosa Promoter Binding Protein' (SBP) mRNA important for inflorescence development (Bhatia et al., 2019). In the present study, based on *in silico* analysis, we observed lncRNA 'TCONS_00081575' as an eTM of 'Bju-miR172_1'. Under normal conditions, miR172 have been known to target *APETELA*2–*LIKE* (AP2-like) TFs such as *TARGET OF EAT*1 (*TOE1*), *TOE2*, etc., hence, promoting adult epidermal identity or shoot maturation (Wu et al., 2009). However, under heat stress, miR172 has been found to be down-regulated in plants and its targets *TOE1, TOE2,* etc. have been found to be up-regulated, thereby delaying flowering (Li et al., 2014; May et al., 2013). The putative role of lncRNA as an eTM in this case seems to be a possible explanation to the above-mentioned observations. Another interesting interaction observed in the present study includes an lncRNA 'TCONS_00047156', which is a putative eTM for 'Bju-miR159_1' that originally targets *MYB* TF mRNAs. In rice, Wang et al. have shown that overexpression of miR159 renders the plant more sensitive to heat stress and have suggested down-regulation of the same to avoid miRNA-mediated cleavage of *MYB* TF mRNA (Wang et al., 2012). We propose that by over-expressing the eTM and the subsequent sequestering of the miRNA by virtue of target mimicry, heat stress tolerance of the plant could be enhanced. Furthermore, over expression of MYB TF has shown to enhance drought tolerance in plants such as *Boea crassifolia* (Chen et al., 2005). Also, TF-miRNA-gene network analysis for *Arabidopsis* transcriptomic data under abiotic stress conditions has shown miR159 as one of the 'strongly connected components' in response to different abiotic stress conditions including drought (Sharma et al., 2019b). In view of this, target mimicry could also help enhance drought tolerance. In future, it would be interesting to revisit the regulatory networks associated with miRNAs, TFs, and lncRNAs to provide candidates for breeding to enhance stress tolerance in important crop plants.

Along with the comprehensive *in silico* analysis, the qRT-PCR based expression analysis also suggested the differential expression profile of the identified heat and drought stress-responsive *B. juncea* lncRNAs. Particularly, TCONS_00051908 and TCONS_00088973, for which higher expression was observed under heat and drought stress conditions, respectively, could be further analyzed. Interestingly, both these lncRNAs were classified into "j" category (Cuffcompare-based classification) indicating these could be alternative long non-coding isoforms of known genes (transcripts in this category share at least one spliced site with the reference transcripts) (Sun et al., 2012). Based on this study, subsequent experiments could be planned with focus on unraveling the molecular mechanisms of the selected lncRNA candidates and understanding the regulation of corresponding gene expression in response to these abiotic stress conditions. Moreover, the regulation of specific pathways/processes mediated by interaction of lncRNAs with other regulators such as TFs and miRNAs could be studied in *B. juncea* by *in vivo/in planta* studies.

5. Conclusion

We identified 1614 heat and drought responsive lncRNAs in *B. juncea* using RNA-seq data based computational pipeline and digital expression analysis. The predicted lncRNAs were thoroughly characterized *in silico*, and their functional roles were explored based on co-expressing mature mRNAs. The subsequent functional annotation analysis revealed their association with ROS scavenging enzymatic (such as superoxide dismutases, peroxidises, glutathione S-transferases, *etc.*) and non-enzymatic defence mechanisms mediated by biosynthesis of cysteine, proline, ascorbate, phenolics, flavonoids, etc. Moreover, lncRNA- miRNA interaction network analysis was performed and the possibility of target mimicry in regulation of the underlying mechanisms of heat and drought tolerance was explored. Since, transcription factors are key modulators of gene expression, their association with lncRNAs was examined to understand the putative TF-lncRNA and/or TF-miRNA-lncRNA interactions. Overall, regulation of *B. juncea* response to heat and drought can be further explored in view of the identified lncRNAs and their predicted associations to enhance stress tolerance in the plant.

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Author contributions

GB, AJ collected the data, performed all the analysis. GB wrote the manuscript. DV performed the plant treatments and real-time PCR analysis. SS helped in analysis of data, co-expression analysis etc. KS conceived the idea, designed the experiments and finalized the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.envexpbot.2019.103922.

References

Altschul, S., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402. https://doi.org/10.1093/nar/25.17.3389.

Anjum, N.A., Sharma, P., Gill, S.S., Hasanuzzaman, M., Khan, E.A., Kachhap, K., Mohamed, A.A., Thangavel, P., Devi, G.D., Vasudhevan, P., Sofo, A., Khan, N.A., Misra, A.N., Lukatkin, A.S., Singh, H.P., Pereira, E., Tuteja, N., 2016. Catalase and ascorbate peroxidase—representative H2O2-detoxifying heme enzymes in plants. Environ. Sci. Pollut. Res. 23, 19002–19029. https://doi.org/10.1007/s11356-016-7309-6.

Bastian, M., Bastian, M., Heymann, S., Jacomy, M., 2009. Gephi: an Open source software for exploring and manipulating networks. Third Int. AAAI Conf. WEBLOGS Soc. Media.

Ben Amor, B., Wirth, S., Merchan, F., Laporte, P., D'Aubenton-Carafa, Y., Hirsch, J., Maizel, A., Mallory, A., Lucas, A., Deragon, J.M., Vaucheret, H., Thermes, C., Crespi, M., 2009. Novel long non-protein coding RNAs involved in Arabidopsis

differentiation and stress responses. Genome Res. 19, 57–69. https://doi.org/10.1101/gr.080275.108.
Bhardwaj, A.R., Joshi, G., Kukreja, B., Malik, V., Arora, P., Pandey, R., Shukla, R.N., Bankar, K.G., Katiyar-Agarwal, S., Goel, S., Jagannath, A., Kumar, A., Agarwal, M., 2015. Global insights into high temperature and drought stress regulated genes by RNA-Seq in economically important oilseed crop Brassica juncea. BMC Plant Biol. 15, 1–15. https://doi.org/10.1186/s12870-014-0405-1.
Bhardwaj, A.R., Joshi, G., Pandey, R., Kukreja, B., Goel, S., Jagannath, A., Kumar, A., Katiyar-Agarwal, S., Agarwal, M., 2014. A genome-wide perspective of miRNAome in response to high temperature, salinity and drought stresses in Brassica juncea (Czern) L. PLoS One 9, e92456. https://doi.org/10.1371/journal.pone.0092456.
Bhatia, G., Goyal, N., Sharma, S., Upadhyay, S., Singh, K., 2017. Present scenario of long non-coding RNAs in plants. Non-Coding RNA 3, 16. https://doi.org/10.3390/ncrna3020016.
Bhatia, G., Sharma, S., Upadhyay, S.K., Singh, K., 2019. Long non-coding RNAs coordinate developmental transitions and other key biological processes in grapevine. Sci. Rep. 9, 3552. https://doi.org/10.1038/s41598-019-38989-7.
Bita, C.E., Gerats, T., 2013. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. Front. Plant Sci. 4, 273. https://doi.org/10.3389/fpls.2013.00273.
Bonnet, E., He, Y., Billiau, K., Van de Peer, Y., 2010. TAPIR, a web server for the prediction of plant microRNA targets, including target mimics. Bioinformatics 26, 1566–1568. https://doi.org/10.1093/bioinformatics/btq233.
Castelán-Muñoz, N., Herrera, J., Cajero-Sánchez, W., Arrizubieta, M., Trejo, C., García-Ponce, B., Sánchez, M., de la, P., Álvarez-Buylla, E.R., Garay-Arroyo, A., 2019. MADS-box genes are key components of genetic regulatory networks involved in abiotic stress and plastic developmental responses in plants. Front. Plant Sci. 10, 853. https://doi.org/10.3389/FPLS.2019.00853.
Chen, B.-J., Wang, Y., Hu, Y.-L., Wu, Q., Lin, Z.-P., 2005. Cloning and characterization of a drought-inducible MYB gene from Boea crassifolia. Plant Sci. 168, 493–500. https://doi.org/10.1016/J.PLANTSCI.2004.09.013.
Cheng, F., Liu, S., Wu, J., Fang, L., Sun, S., Liu, B., Li, P., Hua, W., Wang, X., 2011. BRAD, the genetics and genomics database for Brassica plants. BMC Plant Biol. 11, 136. https://doi.org/10.1186/1471-2229-11-136.
Conesa, A., Götz, S., 2008. Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int. J. Plant Genomics 2008, 619832. https://doi.org/10.1155/2008/619832.
Dai, X., Zhuang, Z., Zhao, P.X., 2018. psRNATarget: a plant small RNA target analysis server (2017 release). Nucleic Acids Res. 46, W49–W54. https://doi.org/10.1093/nar/gky316.
Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635.
Fahad, S., Bajwa, A.A., Nazir, U., Anjum, S.A., Farooq, A., Zohaib, A., Sadia, S., Nasim, W., Adkins, S., Saud, S., Ihsan, M.Z., Alharby, H., Wu, C., Wang, D., Huang, J., 2017. Crop production under drought and heat stress: plant responses and management options. Front. Plant Sci. 8, 1–16. https://doi.org/10.3389/fpls.2017.01147.
Feng, S.J., Zhang, X.D., Liu, X.S., Tan, S.K., Chu, S.S., Meng, J.G., Zhao, K.X., Zheng, J.F., Yang, Z.M., 2016. Characterization of long non-coding RNAs involved in cadmium toxic response in Brassica napus. RSC Adv. 6, 82157–82173. https://doi.org/10.1039/C6RA05459E.
Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol. 155, 2–18. https://doi.org/10.1104/PP.110.167569.
Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., García, J.A., Paz-Ares, J., 2007. Target mimicry provides a new mechanism for regulation of microRNA activity. Nat. Genet. 39, 1033–1037. https://doi.org/10.1038/ng2079.
Ghawana, S., Paul, A., Kumar, H., Kumar, A., Singh, H., Bhardwaj, P.K., Rani, A., Singh, R.S., Raizada, J., Singh, K., Kumar, S., 2011. An RNA isolation system for plant tissues rich in secondary metabolites. BMC Res. Notes 4, 85. https://doi.org/10.1186/1756-0500-4-85.
Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., Ahmad, A., 2012. Role of proline under changing environments. Plant Signal. Behav. 7, 1456–1466. https://doi.org/10.4161/psb.21949.
He, X., Guo, S., Wang, Y., Wang, L., Shu, S., Sun, J., 2019. Systematic identification and analysis of heat-stress-responsive lncRNAs, circRNAs and miRNAs with associated co-expression and ceRNA networks in cucumber (Cucumis sativus L.). Physiol. Plant. https://doi.org/10.1111/ppl.12997.
Hoang, X.L.T., Nhi, D.N.H., Thu, N.B.A., Thao, N.P., Tran, L.-S.P., 2017. Transcription factors and their roles in signal transduction in plants under abiotic stresses. Curr. Genomics 18. https://doi.org/10.2174/1389202918666170227150057.
Hofacker, I.L., 2003. Vienna RNA secondary structure server. Nucleic Acids Res. 31, 3429–3431. https://doi.org/10.1093/nar/gkg599.
Hussain, H.A., Men, S., Hussain, S., Chen, Y., Ali, S., Zhang, S., Zhang, K., Li, Y., Xu, Q., Liao, C., Wang, L., 2019. Interactive effects of drought and heat stresses on morpho-physiological attributes, yield, nutrient uptake and oxidative status in maize hybrids. Sci. Rep. 9, 3890. https://doi.org/10.1038/s41598-019-40362-7.
Jin, J., Tian, F., Yang, D.-C., Meng, Y.-Q., Kong, L., Luo, J., Gao, G., 2017. PlantTFDB 4.0: toward a central hub for transcription factors and regulatory interactions in plants. Nucleic Acids Res. 45, D1040–D1045. https://doi.org/10.1093/nar/gkw982.
Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., Morishima, K., 2017. KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45, D353–D361. https://doi.org/10.1093/nar/gkw1092.
Kong, L., Zhang, Y., Ye, Z.-Q., Liu, X.-Q., Zhao, S.-Q., Wei, L., Gao, G., 2007. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic Acids Res. 35, W345–W349. https://doi.org/10.1093/nar/gkm391.
Kozomara, A., Birgaoanu, M., Griffiths-Jones, S., 2019. miRBase: from microRNA sequences to function. Nucleic Acids Res. 47, D155–D162. https://doi.org/10.1093/nar/gky1141.
Kumar, S., Trivedi, P.K., 2018. Glutathione S-transferases: role in combating abiotic stresses including arsenic detoxification in plants. Front. Plant Sci. 9, 751. https://doi.org/10.3389/fpls.2018.00751.
Li, S., Liu, J., Liu, Z., Li, X., Wu, F., He, Y., 2014. HEAT-INDUCED TAS1 TARGET1 mediates thermotolerance via heat stress transcription factor A1a-directed pathways in Arabidopsis. Plant Cell 26, 1764–1780. https://doi.org/10.1105/tpc.114.124883.
Liu, F., Wang, W., Sun, X., Liang, Z., Wang, F., 2014. RNA-Seq revealed complex response to heat stress on transcriptomic level in Saccharina japonica (Laminariales, Phaeophyta). J. Appl. Phycol. 26, 1585–1596. https://doi.org/10.1007/s10811-013-0188-z.
Liu, J., Jung, C., Xu, J., Wang, H., Deng, S., Bernad, L., Arenas-Huertero, C., Chua, N.-H., 2012. Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. Plant Cell 24, 4333–4345. https://doi.org/10.1105/tpc.112.102855.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408. https://doi.org/10.1006/meth.2001.1262.
May, P., Liao, W., Wu, Y., Shuai, B., Richard McCombie, W., Zhang, M.Q., Liu, Q.A., 2013. The effects of carbon dioxide and temperature on microRNA expression in Arabidopsis development. Nat. Commun. 4, 2145. https://doi.org/10.1038/ncomms3145.
Nazarov, P.V., Reinsbach, S.E., Muller, A., Nicot, N., Philippidou, D., Vallar, L., Kreis, S., 2013. Interplay of microRNAs, transcription factors and target genes: linking dynamic expression changes to function. Nucleic Acids Res. 41, 2817–2831. https://doi.org/10.1093/nar/gks1471.
Nejat, N., Mantri, N., 2018. Emerging roles of long non-coding RNAs in plant response to biotic and abiotic stresses. Crit. Rev. Biotechnol. 38, 93–105. https://doi.org/10.1080/07388551.2017.1312270.
Pandey, P., Ramegowda, V., Senthil-Kumar, M., 2015. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. Front. Plant Sci. 6. https://doi.org/10.3389/fpls.2015.00723.
Patel, R.K., Jain, M., 2012. NGS QC toolkit: a toolkit for quality control of next generation sequencing data. PLoS One 7, e30619. https://doi.org/10.1371/journal.pone.0030619.
Qi, X., Xie, S., Liu, Y., Yi, F., Yu, J., 2013. Genome-wide annotation of genes and non-coding RNAs of foxtail millet in response to simulated drought stress by deep sequencing. Plant Mol. Biol. 83, 459–473. https://doi.org/10.1007/s11103-013-0104-6.
Qin, T., Zhao, H., Cui, P., Albesher, N., Xionga, L., 2017. A nucleus-localized long non-coding rna enhances drought and salt stress tolerance. Plant Physiol. 175, 1321–1336. https://doi.org/10.1104/pp.17.00574.
Qiu, C.W., Zhao, J., Chen, Q., Wu, F., 2019. Genome-wide characterization of drought stress responsive long non-coding RNAs in Tibetan wild barley. Environ. Exp. Bot. 164, 124–134. https://doi.org/10.1016/j.envexpbot.2019.05.002.
Rezayian, M., Niknam, V., Ebrahimzadeh, H., 2018. Differential responses of phenolic compounds of Brassica napus under drought stress. Plant Physiol. 8, 2417–2425. https://doi.org/10.22034/IJPP.2018.540887.
Sahay, S., Khan, E., Gupta, M., 2019. Nitric oxide and abscisic acid protects against PEG-induced drought stress differentially in Brassica genotypes by combining the role of stress modulators, markers and antioxidants. Nitric Oxide - Biol. Chem. 89, 81–92. https://doi.org/10.1016/j.niox.2019.05.005.
Sharma, A., Shahzad, B., Rehman, A., Bhardwaj, R., Landi, M., Zheng, B., 2019a. Response of phenylpropanoid pathway and the role of polyphenols in plants under abiotic stress. Molecules 24, 2452. https://doi.org/10.3390/molecules24132452.
Sharma, R., Upadhyay, S., Bhat, B., Singh, G., Bhattacharya, S., Singh, A., 2019b. Abiotic stress induced miRNA-TF-gene regulatory network: a structural perspective. Genomics. https://doi.org/10.1016/J.YGENO.2019.03.004.
Shuai, P., Liang, D., Tang, S., Zhang, Z., Ye, C.Y., Su, Y., Xia, X., Yin, W., 2014. Genome-wide identification and functional prediction of novel and drought-responsive lincRNAs in Populus trichocarpa. J. Exp. Bot. 65, 4975–4983. https://doi.org/10.1093/jxb/eru256.
Sinha, S., Raxwal, V.K., Joshi, B., Jagannath, A., Katiyar-Agarwal, S., Goel, S., Kumar, A., Agarwal, M., 2015. De novo transcriptome profiling of cold-stressed siliques during pod filling stages in Indian mustard (Brassica juncea L.). Front. Plant Sci. 6, 932. https://doi.org/10.3389/fpls.2015.00932.
Song, X., Liu, G., Huang, Z., Duan, W., Tan, H., Li, Y., Hou, X., 2016. Temperature expression patterns of genes and their coexpression with LncRNAs revealed by RNA-Seq in non-heading Chinese cabbage. BMC Genomics 17, 1–15. https://doi.org/10.1186/s12864-016-2625-2.
Sun, L., Zhang, Z., Bailey, T.L., Perkins, A.C., Tallack, M.R., Xu, Z., 2012. Prediction of novel long non-coding RNAs based on RNA-Seq data of mouse Klf1 knockout study. BMC Bioinf. 13 (331). https://doi.org/10.1186/1471-2105-13-331.
Sun, X., Zheng, H., Sui, N., 2018. Regulation mechanism of long non-coding RNA in plant response to stress. Biochem. Biophys. Res. Commun. 503, 402–407. https://doi.org/10.1016/j.bbrc.2018.07.072.
Szcześniak, M.W., Rosikiewicz, W., Makałowska, I., 2016. CANTATAdb: a collection of plant long non-coding RNAs. Plant Cell Physiol. 57, e8. https://doi.org/10.1093/pcp/pcv201.
Tran, L.-S.P., Urao, T., Qin, F., Maruyama, K., Kakimoto, T., Shinozaki, K., Yamaguchi-Shinozaki, K., 2007. Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. 104, 20623–20628. https://doi.org/10.1073/pnas.0706547105.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., Pachter, L., 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511–515. https://doi.org/10.1038/nbt.1621.

Verma, D., Lakhanpal, N., Singh, K., 2019. Genome-wide identification and characterization of abiotic-stress responsive SOD (superoxide dismutase) gene family in Brassica juncea and B. rapa. BMC Genomics 20, 1–18. https://doi.org/10.1186/s12864-019-5593-5.

Wang, A., Hu, J., Gao, C., Chen, G., Wang, B., Lin, C., Song, L., Ding, Y., Zhou, G., 2019. Genome-wide analysis of long non-coding RNAs unveils the regulatory roles in the heat tolerance of Chinese cabbage (Brassica rapa ssp. chinensis). Sci. Rep. 9, 1–14. https://doi.org/10.1038/s41598-019-41428-2.

Wang, H., Chung, P.J., Liu, J., Jang, I.-C., Kean, M.J., Xu, J., Chua, N.-H., 2014. Genome-wide identification of long noncoding natural antisense transcripts and their responses to light in Arabidopsis. Genome Res. 24, 444–453. https://doi.org/10.1101/gr.165555.113.

Wang, Y., Sun, F., Cao, H., Peng, H., Ni, Z., Sun, Q., Yao, Y., 2012. TamiR159 directed wheat TaGAMYB cleavage and its involvement in anther development and heat response. PLoS One 7, e48445. https://doi.org/10.1371/journal.pone.0048445.

Westman, A.L., Kresovich, S., 1999. Simple sequence repeat (SSR)-based marker variation in Brassica nigra genebank accessions and weed populations. Euphytica 109, 85–92. https://doi.org/10.1023/A:1003637814963.

Wu, G., Park, M.Y., Conway, S.R., Wang, J.-W., Weigel, D., Poethig, R.S., 2009. The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. Cell 138, 750–759. https://doi.org/10.1016/j.cell.2009.06.031.

Xiao, L., Shang, X.-H., Cao, S., Xie, X.-Y., Zeng, W.-D., Lu, L.-Y., Chen, S.-B., Yan, H.-B., 2019. Comparative physiology and transcriptome analysis allows for identification of lncRNAs imparting tolerance to drought stress in autotetraploid cassava. BMC Genomics 20, 514. https://doi.org/10.1186/s12864-019-5895-7.

Yang, J., Liu, D., Wang, X., Ji, C., Cheng, F., Liu, B., Hu, Z., Chen, S., Pental, D., Ju, Y., Yao, P., Li, X., Xie, K., Zhang, J., Wang, J., Liu, F., Ma, W., Shopan, J., Zheng, H., Mackenzie, S.A., Zhang, M., 2016. The genome sequence of allopolyploid Brassica juncea and analysis of differential homoeolog gene expression influencing selection. Nat. Genet. 48, 1225–1232. https://doi.org/10.1038/ng.3657.

Yang, Z., Li, W., Su, X., Ge, P., Zhou, Y., Hao, Y., Shu, H., Gao, C., Cheng, S., Zhu, G., Wang, Z., 2019. Early response of radish to heat stress by strand-specific transcriptome and miRNA analysis. Int. J. Mol. Sci. 20, 3321. https://doi.org/10.3390/ijms20133321.

Zagorchev, L., Seal, C., Kranner, I., Odjakova, M., 2013. A central role for thiols in plant tolerance to abiotic stress. Int. J. Mol. Sci. 14, 7405–7432. https://doi.org/10.3390/ijms14047405.

Zhang, W., Han, Z., Guo, Q., Liu, Y., Zheng, Y., Wu, F., Jin, W., 2014. Identification of maize long non-coding RNAs responsive to drought stress. PLoS One 9. https://doi.org/10.1371/journal.pone.0098958.

Zhu, Q.-H., Stephen, S., Taylor, J., Helliwell, C.A., Wang, M.-B., 2014. Long noncoding RNAs responsive to *Fusarium oxysporum* infection in *Arabidopsis thaliana*. New Phytol. 201, 574–584. https://doi.org/10.1111/nph.12537.