Global identification and functional prediction of cold-related lncRNAs in eggplant

Yan YANG, Jingxian ZHANG, Jun LIU, Xiaohui ZHOU, Shuqing LIANG, Songyu LIU, Yong ZHUANG*

*Institute of Vegetable Crops, Jiangsu Academy of Agricultural Sciences, Nanjing, China; yzyangyan890618@163.com; zjx19905161402@163.com; kehl_lau@foxmail.com; xhzhou1984@sina.com; 1023435796@qq.com; liusongyu1@126.com; jaaszy@163.com (*corresponding author)*

Abstract

Long noncoding RNAs (lncRNAs) play critical roles in plant development and stress responses. So far, identification of lncRNA in eggplant response to stresses has been limited and the role in mediating response to cold stress is yet to be characterized in eggplant. In this study, there is reported the first dataset of lncRNAs responsive to cold stress in the cold tolerant and sensitive eggplants using RNA sequencing (RNA-seq). 227 and 225 differentially expressed (DE) lncRNAs were obtained in two genotypes with differential cold-tolerance. Functional characterization through gene ontology (GO) analysis indicated that target genes were particularly related to acyl-CoA dehydrogenase activity and pseudouridine synthase activity, which could result in the tolerant phenotypes. Kyoto Encyclopaedia of Genes and Genomes (KEGG) showed that target genes in both sensitive and tolerant eggplants were mainly involved in cold responsive pathways such as oxidative phosphorylation, peroxisome, protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis and so on. However, the enriched pathways obtained by enrichment analysis in cold-tolerant eggplant were different from those in cold-sensitive eggplant, which further indicated the reason for different tolerances. Our findings highlight the potential contributions of lncRNAs in regulating eggplant response to cold stress and difference in cold tolerance.

Keywords: cold stress; eggplant; functional analysis; lncRNA; target gene

Introduction

In eukaryotes, many transcripts are non-coding RNAs (ncRNA) which lack protein-encoding potential (Chekanova *et al.*, 2007). lncRNA is a type of ncRNA with length of at least 200 nucleotides (nt) (Zhang *et al.*, 2021). LncRNAs are poorly conserved in different species. Like messenger RNAs (mRNA), most lncRNAs are transcribed by RNA polymerase II, while some lncRNAs are transcribed by RNA polymerase III, IV and V in plants. In terms of expression pattern, lncRNA have tissue-specificity. Moreover, according to the location relationship between lncRNAs and adjacent protein-coding genes in the genome, lncRNAs can be divided into five categories as follows: (1) sense lncRNA overlapping one or more exons of a protein-coding gene in the same strand; (2) antisense lncRNA, which overlaps one or more exons of a protein-coding gene in the

complementary strand; (3) intronic lncRNA, which originates from the introns of protein-coding genes; (4) intergenic lncRNA (lincRNA), which is located within the intergenic region of two protein-coding genes; (5) bidirectional lncRNA, which is expressed in opposite direction to adjacent coding genes on the complementary strand (Ponting *et al.*, 2009; Wu *et al.*, 2020).

LncRNA is once considered as transcriptional noise in genome. Emerging evidences showed that lncRNA functions well in plant development and stress responses at transcriptional, post-transcriptional and epigenetic level (Lucero *et al.*, 2021). The functional mechanisms of lncRNA are diverse (Heo and Sung, 2011; Wang and Chang, 2011; Zhu and Wang, 2012). *COOLAIR (COLD INDUCED LONG ANTISENSE INTRAGENIC RNA)* and *COLDAIR (COLD ASSISTED INTRONIC NONCODING RNA)*, the two classical plant lncRNAs, act as signal molecules for floral transition and regulate the expression of *FLC* and flowering time at epigenetic and posttranscriptional levels (Swiezewski *et al.*, 2009; Heo and Sung, 2011). However, a natural antisense transcript (NAT) *MAS* positively regulates flowering time by interacting with and recruiting WDR5a to activate *MAF4* (*MADS AFFECTING FLOWERING4*) (Zhao *et al.*, 2018). LncRNA is also reported as the precursor of miRNA to influence miRNA biosynthesis or as endogenous target mimic (eTM) for microRNA to compromise its repression for target genes. For example, lncRNA *PMS1T* can be targeted by *miR2118* to produce 21-nt small-interfering RNAs to regulate male sterility in rice. *LncRNA354* functions as eTM of *miR160b* to inhibit *miR160b*-induced degradation of ARF17/18 in upland cotton (*Gossypium hirsutum*) (Zhang *et al.*, 2021). In addition, lncRNA plays a vital role in regulating the plant tolerance to cold stress in various manners. LncRNA *SVALKA* is induced by cold treatment and transcribed on the antisense strand between *CBF3* and *CBF1* in *Arabidopsis thaliana*. Read-through transcription of *SVALKA* results in *asCBF1*, which triggered RNAPII collision over the *CBF1* gene body leading to the termination of *CBF1* transcription (Kindgren *et al.*, 2018). Li *et al.* (2022) characterized an intergenic lncRNA 1 (CRIR1) as a novel positive regulator of cassava (*Manihot esculenta*) response to cold stress through modulating the expression of stress-responsive genes and interacting with MeCSP5 to improve the translation efficiency of mRNA.

Eggplant (*Solanum melongena* L.) is a thermophilic species, and is more sensitive to cold stress than other Solanaceae plants. Given the adverse impacts induced by cold stress, cold tolerance is a primary goal of genetic improvement in eggplant. Therefore, it is essential to explore the key regulators in mediating cold-response for genetic improvement. The application of RNA-seq has facilitated the identification of mRNAs and lncRNAs mediating stress response in plants (Li *et al.*, 2018; Shen *et al.*, 2018; Wang *et al.*, 2019; Zhang *et al.*, 2019; Sun *et al.*, 2020). Previously, we found that two eggplant genotypes performed oppositely in response to cold stress (Yang *et al.*, 2020). The DE mRNAs have been explored and analysed using RNA-seq in the two phenotypic differential eggplants. To further explore cold-related lncRNAs, we performed RNA-seq on cold-responsive transcripts to isolate lncRNA that might be involved in cold-tolerance. We identified 452 cold-related lnRNAs and predicted the target genes of those lncRNAs in cis- and trans-regulatory relationship. The functions and pathways of target genes were investigated to realize the potential roles of lncRNAs during eggplant response to cold stress. This study will provide insights into new regulators and regulatory mechanisms for eggplant cold-tolerance.

Materials and Methods

Plant growth, stress treatment and sample collection
In this study, cold-tolerant genotype 'CGN22911' and cold-sensitive genotype 'Chengdumoqie' were used for RNA-seq. 'CGN22911' and 'Chengdumoqie' were obtained from the Centre for Genetic Resources, the Netherlands (CGN) and Chinese Vegetable Germplasm Resource Centre, respectively. These two types of

eggplants were germinated on a soil mixture (soil/ perlite, in a ratio of 3:1) and grown under controlled atmospheric greenhouse conditions (28/24 °C, 16 h/8 h light–dark cycle). These seedlings were transferred to 4 °C when the fourth leaf fully expanded. The samples were collected as a previous study (Yang *et al*., 2020). Three independent biological replicates of both treatment and control plants were used. All collected tissue samples were frozen in liquid nitrogen and kept at −80 °C for future analysis.

RNA extraction, library construction and sequencing

Total RNA of each sample was isolated using the RNAsimple Total RNA Kit (Tiangen, China) according to the manufacturer's protocol. RNA degradation was monitored on 1% agarose gel, followed by checking RNA purity by NanoPhotometer® spectrophotometry (IMPLEN,CA,USA), RNA concentration using Qubit® RNA Assay Kit in Qubit® 2.0 and RNA integrity through RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Ribosomal RNA was firstly removed by Epicentre Ribo-zero™ rRNA Removal Kit (Epicentre, USA), and rRNA free residue was cleaned up by ethanol. Subsequently, sequencing libraries were generated using the rRNA-depleted RNA by NEBNext® Ultra™ Directional RNA Library Prep Kit following manufacturer's recommendations. The libraries were sequenced on an Illumina novaseq 6000 platform.

Reads mapping, transcript assembly and lncRNAs identification

Clean reads were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data and then mapped to the *Solanum melongena* L. genome (Eggplant genome consortium V3) using TopHat software v2.1.1 (Kim *et al*., 2015). The mapped reads were assembled by both Scripture (beta2) (Guttman *et al*., 2010) and Cufflinks (v2.2.1) (Trapnell *et al*., 2010). Then the protein-coding capacity of novel transcripts with length more than 200 nt was identified using Coding-Non-Coding-Index (CNCI) (Sun *et al*., 2013) and Coding Potential Calculator (CPC) (Kong *et al*., 2007), respectively. Pfam database was used to ensure that the predicted transcripts have no protein-coding domains (Finn *et al*., 2010). The transcripts remained after filtering were regarded as the lncRNAs.

Prediction of target genes, differentially expression and functional analysis

For cis-regulation, the coding genes 100 kb upstream or downstream of lncRNAs were considered as cis-target genes (Qing *et al*., 2022). For trans-regulation, the coding genes which had co-expression with lncRNAs were determined as trans-target genes (Zhao *et al*., 2018). FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) was employed to evaluate the lncRNA expression. Subsequently, DElncRNA analysis was performed using DESeq2 Rpackage. The lncRNAs and target genes with a fold change and a threshold of false discovery rates (FDR < 0.01) were assigned as DElncRNAs and DE target genes. GO (Gene Ontology) enrichment analysis of DE target genes was conducted by the GOseq R package (Young *et al*., 2010). We used KOBAS2.0 software to test the statistical enrichment of DE target genes in KEGG pathways (Mao *et al*., 2005).

Validation of selected lncRNAs by quantitative RT-PCR (qPCR)

qPCR was performed to analyse the expression of lncRNAs following the methods by a previous study (Yang *et al*., 2020). Specific primers used in the qPCR experiment are listed in Table S1.

Results

Identification of lncRNA in eggplant

In this study, to identify the lncRNAs involved in mediating eggplant response to cold stress, the leaves from 'CGN22911' (a tolerant genotype) and 'Chengdumoqie' (a sensitive genotype) were collected after 12 h of cold stress. Subsequently, RNA-seq was performed using these samples. A total of 936,823 transcripts were assembled from all the samples. The transcripts were classified with class code 'u', 'j', 'x' and 'o' category and so on (Figure 1A). All the assembled transcripts were subjected to online tools to predict their potential coding capacity, such as CPC, CNCI and Pfam blast. After filtering, 4,374 transcripts were considered as lncRNAs in eggplant. According to the classification criteria, there were about 1,118 lncRNAs including about 180 lncRNAs with low abundance, 923 lncRNAs with medium abundance and about 15 lncRNAs with high abundance for each sample (Figure 1B) (Cao *et al.*, 2021). The lncRNAs regulate the gene expression through either cis- or trans-regulation. To determine the possible relationship of neighboring genes to lncRNAs, we mapped the identified lncRNAs in the genome of eggplant. These expressed lncRNAs were almost evenly distributed across all chromosomes of the eggplant genome (Figure 1C). Based on their chromosomal location, these eggplant lncRNAs were categorized as anti-sense lncRNA, intronic lncRNA and intergenic lncRNA in the percentage of 32.42%, 1.16% and 66.42% respectively (Figure 1D).
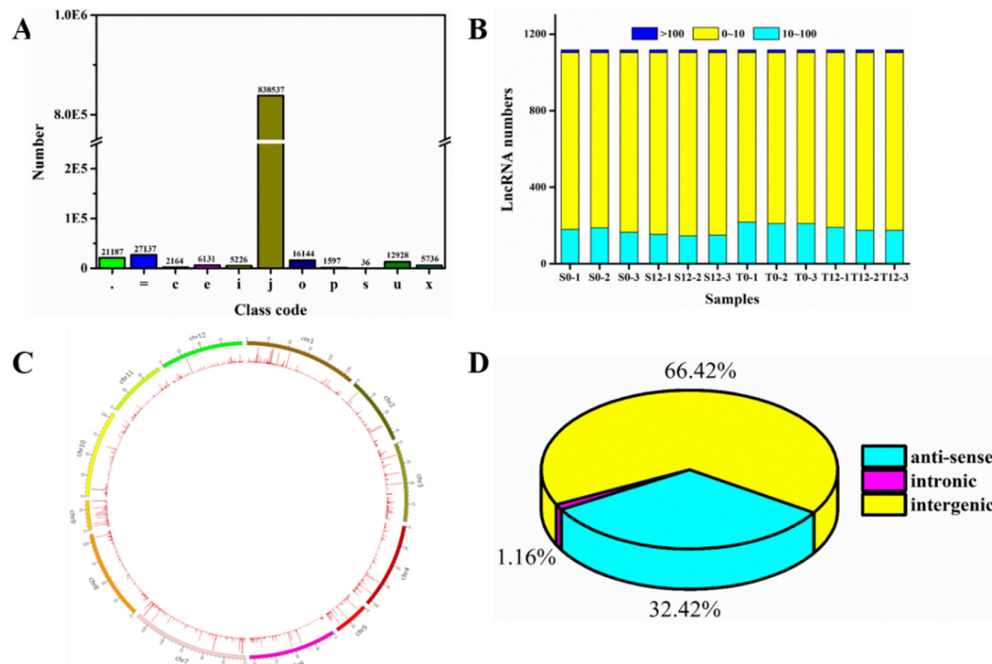

Figure 1. The characteristics of lncRNAs in eggplant
A, The statistics of class codes for eggplant lncRNAs. '.' indicated multiple classification. '=' indicated complete match of intron chain. 'c' indicated contained. 'e' indicated single exon transfrag overlapping a reference exon and at least 10 bp of a reference intron indicating a possible pre-mRNA fragment. 'i' indicated a transfrag falling entirely within a reference intron. 'j' indicated potentially novel isoform (fragment): at least one splice junction is shared with a reference transcript. 'o' indicated generic exonic overlap with a reference transcript. 'p' indicated possible polymerase run-on fragment (within 2Kbases of a reference transcript). 's' indicated an intron of the transfrag overlaps a reference intron on the opposite strand (likely due to read mapping errors). 'u' indicated unknown and intergenic transcript. 'x' indicated exonic overlap with reference on the opposite strand. B, The numbers of expressed lncRNAs in each sample. Transcripts per kilobase of exon model per million mapped reads (TPM value) between 1 ~ 10, 10 ~ 100 and >100 indicated low abundance, medium abundance and high abundance, respectively. LncRNAs with different expression abundance were shown in different colors. C, Chromosome distribution of expressed lncRNAs. D, Types of eggplant lncRNAs according to the location relationship with adjacent protein-coding genes.

Variation in lncRNA expression upon cold stress

To obtain the lncRNAs involved in eggplant response to cold stress, DElncRNAs were the research focus. FPKM was calculated to understand the expression of lncRNA and DEseq2 was used to analyse the DElncRNAs between sample groups. A total of 452 DElncRNAs were screened out with the threshold of |Fold Change|≥2 and FDR < 0.01 (Table S2). Among these 452 lncRNAs, 227 (70 up-regulated, 157 down-regulated) and 225 (55 up-regulated, 170 down-regulated) DElncRNAs were found in *S0 vs S12* and *T0 vs T12*, respectively (Figure 2A). Through venn analysis, 144 common DElncRNAs were confirmed between the two groups (Figure 2B). Simultaneously, we found 83 and 81 unique DElncRNAs in *S0 vs S12* and *T0 vs T12*, respectively (Figure 2B). In sensitive eggplant, *XLOC_008979* was the lncRNA with the greatest down-regulation after the cold treatment, while *XLOC_004912* was the greatest up-regulation followed by *XLOC_002192*. *XLOC_029330* and *XLOC_019776* were the lncRNAs with the greatest down- and up-regulation in *T0 vs T12* after low temperature, respectively (Table S2).
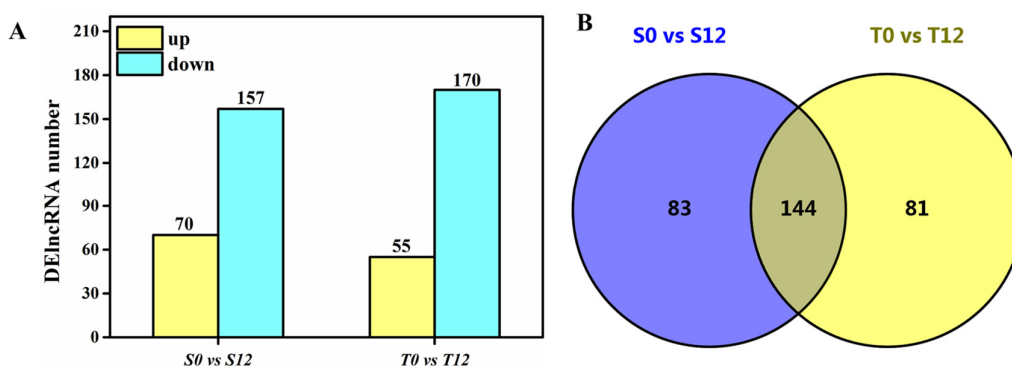

Figure 2. The number of DElncRNAs in cold-sensitive and tolerant eggplants
A, The number of up-regulated and down-regulated lncRNAs. B, venn diagram of DElncRNAs in cold-sensitive and tolerant eggplants. S0 and T0 represented the sensitive and tolerant genotypes before cold treatment, respectively. S12 and T12 represented the sensitive and tolerant genotypes treated with 12 h of cold stress, respectively.

Prediction of target genes of cold-related lncRNA in cis-regulatory relationship

LncRNAs regulate gene expression in cis or trans-manner (Yu *et al.*, 2008). Therefore, to explore the possible function of lncRNAs in eggplant response to cold stress, the potential target genes of DElncRNAs in cis-regulatory were firstly predicted. The protein-coding genes located within 100kb upstream and downstream of the identified lncRNAs were screened out as the cis-targeted genes. 1163 and 1147 mRNAs were obtained according to the location for DElncRNAs in *S0 vs S12* and *T0 vs T12*, respectively (Table 1, Table S3). All the targeted genes were annotated to realize their function. 1147 (156 up-regulated and 73 down-regulated) and 1128 (126 up-regulated and 84 down-regulated) genes gained annotation (Table 1, Figure 3A). Lots of DElnRNAs targeted cold stress-associated genes such as auxin-responsive protein genes (*SMEL_011g362910.1*, *SMEL_005g234100.1* and *SMEL_011g363410.1*), sugar transporter gene *ERD SMEL_009g329020.1*, autophagy-related protein gene ATG8f (SMEL_008g316860.1), mitogen-activated protein kinase (MAPK) gene *SMEL_007g294740.1* and calmodulin gene (*SMEL_001g141890.1*) (Table S4).

Table 1. The number of different types of target genes in cis- or trans-regulatory relationship

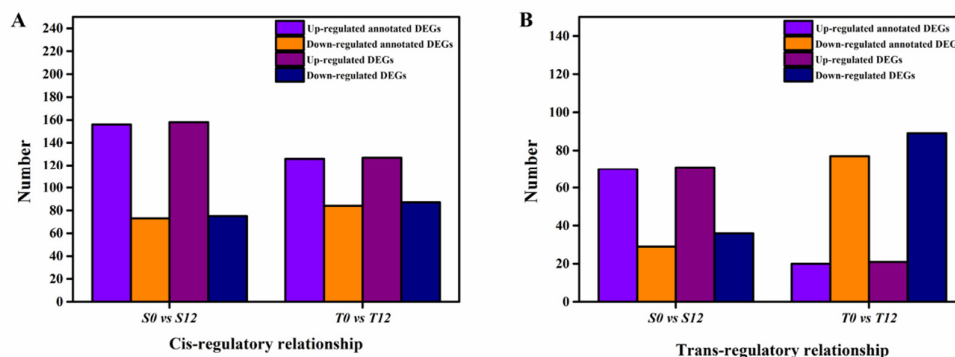| Types	*S0 vs S12*		*T0 vs T12*	
	cis	trans	cis	trans
Target gene	1163	231	1147	205
Annotated target gene	1147	206	1128	170
Differentially expressed target gene	233	107	214	110

Figure 3. The number of target genes of cold-responsive lncRNAs in eggplant
A, The number of target genes in cis-regulatory relationship. B, The number of target genes in cis-regulatory relationship.

To explore the function of lncRNAs, differentially expressed targeted mRNAs were firstly filtered out based on with the threshold. 233 (158 up-regulated and 75 down-regulated) and 214 (127 up-regulated and 87 down-regulated) cis-target genes were differentially expressed in *S0 vs S12* and *T0 vs T12*, respectively (Table 1, Table S2, Figure 3A). The expression level of some targeted genes showed positively relationship with the lncRNAs, while the expression level of some other cis-acting genes was negatively related to lncRNAs in both *S0 vs S12* and *T0 vs T12* groups (Figure 4A, 4B, Figure S1A, S1B). For example, in *S0 vs S12*, *XLOC_000587* and its targeted genes (*SMEL_001g125800.1* and *SMEL_001g125840.1*) were induced by cold stress, whilst *XLOC_035795* was up-regulated and its targeted genes (*SMEL_011g363430.1* and *SMEL_011g3749.1*) were down-regulated under cold stress compared with the untreated plants. In *T0 vs T12*, *XLOC_039560* exhibited down-regulated expression, while its target genes *SMEL_012g396740.1* and *SMEL_012g396840.1* were up- and down- regulated respectively. To understand the expression correlation between the lncRNAs and the cis-regulatory genes, the correlation coefficients of Pearson were calculated using the SPSS software. As a result, the correlation coefficients of Pearson in the two groups were 0.087 and 0.084 respectively, which showed that the expression level of lncRNAs has irrelevance with their cis-target genes (Figure 4A, 4B, Table 2).

Table 2. The correlation coefficient between lncRNAs and their target genes in cis- or trans-regulatory relationship

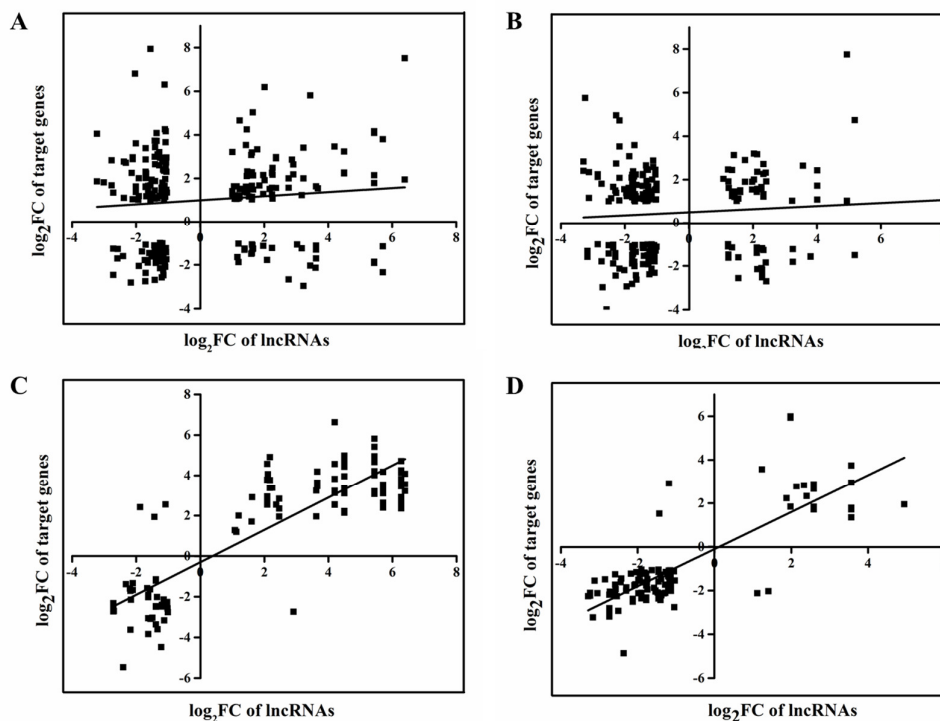|	lncRNA-S	lncRNA-T	cis-S	cis-T	trans-S	trans-T
lncRNA-S	1	0.071	0.087	0.012	0.828**	-0.110
lncRNA-T		1	-0.007	0.084	0.037	0.798**
cis-S			1	0.039	-0.010	-0.100
cis-T				1	0.080	0.114
trans-S					1	0.080
trans-T						1

**significant at 0.01 probability level

Figure 4. The correlation between the expression of lncRNAs and their target genes
A, Cis-regulatory target genes and lncRNAs in cold-sensitive eggplant. B, Cis-regulatory target genes and lncRNAs in cold-tolerant eggplant. C, Trans-regulatory target genes and lncRNAs in cold-sensitive eggplant. D, Trans-regulatory target genes and lncRNAs in cold-tolerant eggplant.

Prediction of target genes of cold-related lncRNAs in trans-regulatory relationship

To investigate the possible functions of eggplant lncRNAs, we also predicted the potential targets of lncRNAs in trans-regulatory. As a result, a total of 231 and 205 genes in trans-regulatory were predicted in *S0 vs S12* and *T0 vs T12*, respectively (Table 1, Table S5). To understand the function of targeted genes, we performed an annotation analysis for trans-regulatory genes. 206 (70 up-regulated and 29 down-regulated) and 179 (20 up-regulated and 77 down-regulated) genes gained annotation (Table 1, Figure 3B). Unlike the cis-regulatory function, 32 (15 in *S0 vs S12* and 17 in *T0 vs T12*) new genes arose in trans-regulatory function (Table S6). Target genes encoded many stress-related proteins. For instance, NAC transcription factor (*SMEL_002g168980.1*) ethylene-responsive transcription factor (*SMEL_005g235450.1*), cytochrome P450 (*SMEL_003g189300.1*) and auxin-responsive protein (*SMEL_001g116470.1*) (Table S6).

The filtering results of genes showed 107 (71 up-regulated and 36 down-regulated) and 110 (21 up-regulated and 89 down-regulated) differentially expressed trans-regulated genes in *S0 vs S12* and *T0 vs T12*, respectively (Table 1, Figure 3B). A majority of the targeted genes showed a positive relationship with lncRNAs in the expression level, while only few of the targeted genes are negatively related to lncRNAs such as the trans-regulatory gene *SMEL_007g279670.1*, *SMEL_004g220310.1*, *SMEL_003g194470.1*, *SMEL_010g339130.1*, *SMEL_001g139840.1*, *SMEL_008g303270.1*, *SMEL_004g218250.1*, *SMEL_004g215800.1*, *SMEL_004g215770.1*, *SMEL_008g312370.1* and *SMEL_010g346990.1* (Figure 4C, 4D, Figure S1C, S1D). Furthermore, we calculated the correlation coefficient between the fold changes of lncRNAs and their target genes in trans-regulatory relationships after cold stress treatment. As shown in Table 2, the correlation coefficient of *S0 vs S12* and *T0 vs T12* was as high as 0.828 and 0.798 respectively, indicating

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that genes with trans-regulatory relationships were positively related to lncRNAs in eggplant under cold stress. Moreover, these positive correlation relationships were significant at level of 0.01.

Functional analysis of cis-acting genes regulated by DElncRNAs

Go enrichment was carried out to analyse the targeted genes to understand the function of lnRNAs. As reports in other studies, the coding genes targeted by lncRNAs in both *S0 vs S12* and *T0 vs T12* were classified into three categories of ontologies including "molecular function" (MF), "biological process" (BP) and "cellular component" (CC). The top 20 significant GO terms were further considered. As shown in Figure 5A, we found that the categories in cis-target genes of *S0 vs S12* were mainly related to malate synthase activity, L-lactate dehydrogenase activity and polyubiquitin modification-dependent protein binding ability for MF. De-etiolation, recognition of pollen and glyoxylate cycle were the most highly enriched BP terms of the cis-acting genes (Figure 5A). In the T0 and T12 comparison, the highly enriched terms of MF were pseudouridine synthase activity, polyubiquitin modification-dependent protein binding and acyl-CoA dehydrogenase activity (Figure 5A). Consistent with cis-target genes in *S0 vs S12*, the cis-target genes of *T0 vs T12* mainly enriched in de-etiolation, recognition of pollen and pseudouridine synthesis for BP (Figure 5A).
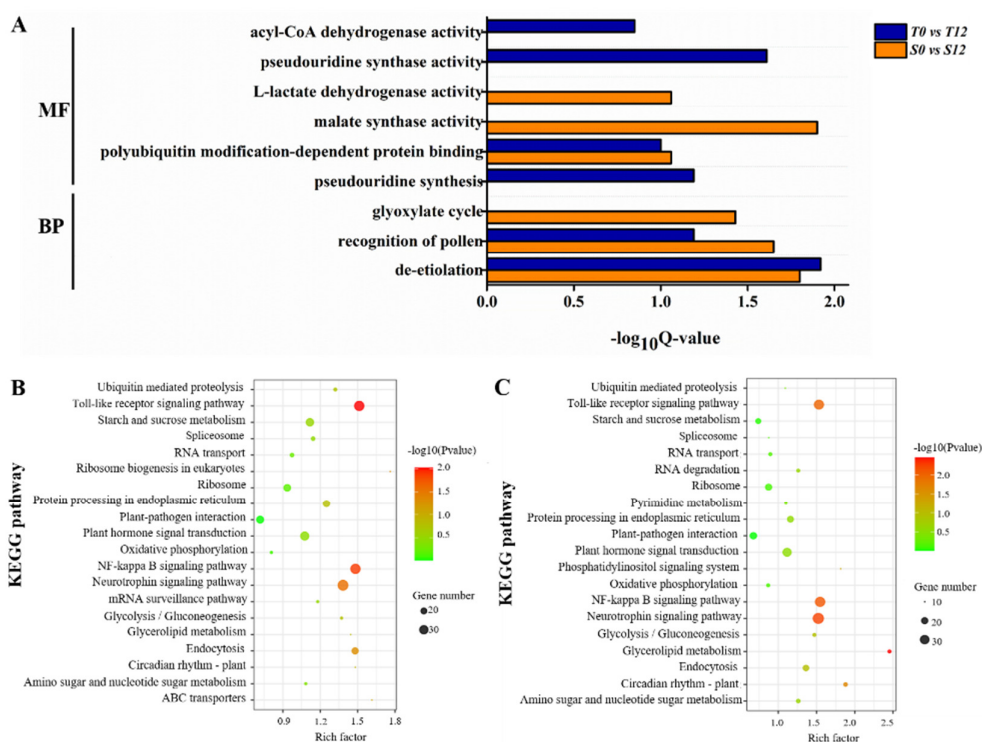

Figure 5. Functional analysis of cis-acting genes of cold responsive lncRNAs in *S0 vs S12* and *T0 vs T12*
A, The representative GO terms of target genes in cis-regulatory relationship. B, KEGG pathway enrichment analysis of cis-regulatory target genes in cold-sensitive eggplant. C, KEGG pathway enrichment analysis of cis-regulatory target genes in cold-tolerant eggplant.

To identify the major biochemical and signal transduction pathways in which the lncRNAs were involved, the target genes in cis-regulatory relationship were blasted to the KEGG database and pathway enrichment analysis was conducted (Kanehisa *et al*., 2007). Out of the top 20 KEGG pathways, the proportion of the matched pathways between the two comparisons had risen to 85% (Figure 5B, 5C). The common pathways were mainly stress-related, including plant hormone signal transduction, protein processing in endoplasmic reticulum, ubiquitin mediated proteolysis, starch and sucrose metabolism, amino sugar and

nucleotide sugar metabolism and so on (Figure 5B, 5C). To understand the most possible pathways in which cis-target genes were involved, the enrichment analysis was conducted. We found that the enriched pathways of cis-regulatory genes in *S0 vs S12* were endocytosis and ribosome biogenesis in eukaryotes, while the enriched pathways in *T0 vs T12* were glycerolipid metabolism, circadian rhythm and phosphatidylinositol signaling system (Figure 5B, 5C).

Functional analysis of trans-acting genes regulated by DElncRNAs

For trans-acting genes in *S0 vs S12*, the three overrepresented terms of MF were SNARE binding, ADP binding and transferase activity and transferring hexosyl groups (Figure 6A). SNARE complex and integral component of membrane were the two enriched CC terms (Figure 6A). Defense response, vesicle fusion and vesicle docking were the most likely biological process in which the trans-acting genes of *S0 vs S12* participated (Figure 6A). Moreover, in addition to SNARE binding and ADP binding, SNARE receptor activity was the relatively enriched GO term of MF for the trans-acting genes in *T0 vs T12* (Figure 6A). Similar to the comparison between S0 and S12, defense response, vesicle fusion and vesicle docking were also the most likely biological process in *T0 vs T12* (Figure 6A). Moreover, the trans-acting genes might also encode SNARE complex (Figure 6A).
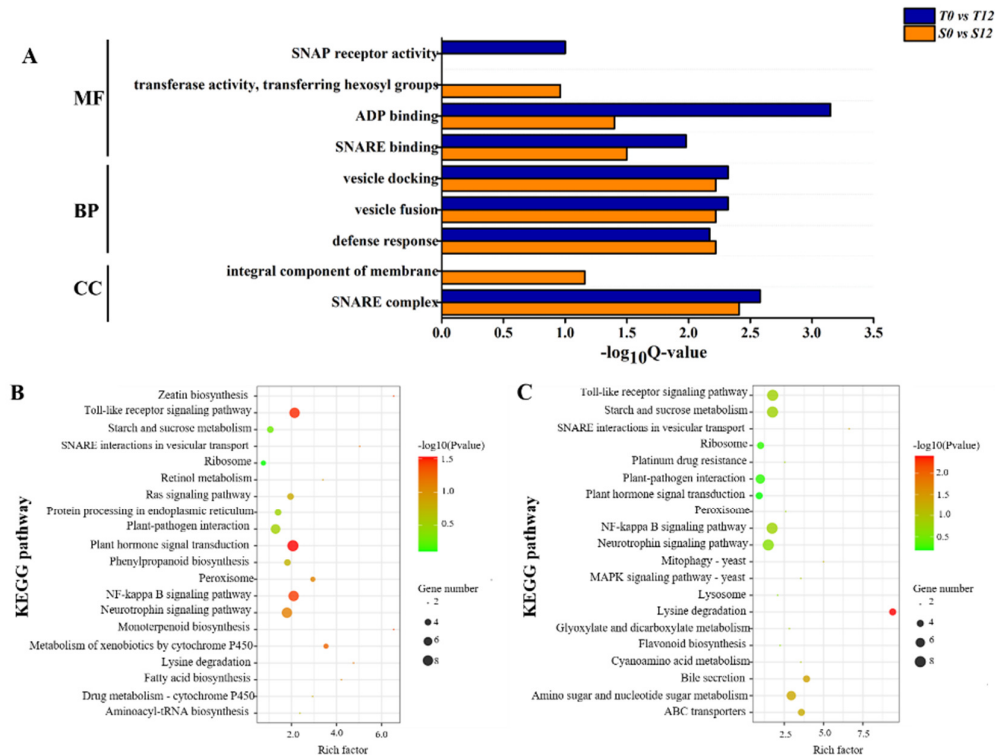

Figure 6. Functional analysis of trans-acting genes of cold responsive lncRNAs in *S0 vs S12* and *T0 vs T12* A, The representative GO terms of target genes in trans-regulatory relationship. B, KEGG pathway enrichment analysis of trans-regulatory target genes in cold-sensitive eggplant. C, KEGG pathway enrichment analysis of trans-regulatory target genes in cold-tolerant eggplant.

KEGG pathway analysis was also performed according to the target genes in trans-regulatory relationship to further explore the biological functions of lncRNAs. As shown Figure 6B and Figure 6C, several pathways were overlapped including plant hormone signal transduction, SNARE interactions in vesicular transport, starch and sucrose metabolism, peroxisome and so on. After enrichment analysis, we found that

common pathways were not simultaneously enriched in both genotypes (Figure 6B, Figure 6C). Plant hormone signal transduction, zeatin biosynthesis and monoterpenoid biosynthesis were found to be the unique enriched pathways in *S0 vs S12* compared with *T0 vs T12*, while the genes in *T0 vs T12* comparison were particularly associated with lysine degradation, SNARE interactions and amino sugar and nucleotide sugar metabolism (Figure 6B, 6C).

Validation of lncRNA expression using qPCR

We performed qPCR analysis to validate the RNA-seq results for nine randomly selected lncRNAs (Figure 7). As a result, the expression patterns of the stress-responsive lncRNAs as investigated by RNA-seq and qPCR were relatively consistent with similar trends. *XLOC_008350* and *XLOC_029091* were significantly induced by cold stress in both tolerant and sensitive genotypes, while the expression of *XLOC_010165* and *XLOC_033802* were suppressed by cold stress in both genotypes. Furthermore, *XLOC_004330*, *XLOC_007272* and *XLOC_032705* were only differentially expressed in tolerant eggplant. *XLOC_014200* was the unique DElncRNA of *S0 vs S12* in qPCR in accordance with sequencing result. The correlation coefficient of lncRNAs expression detected by RNA-seq and qPCR was 0.709 (Figure 8). These demonstrated that the lncRNAs identified by RNA-seq were reliable.
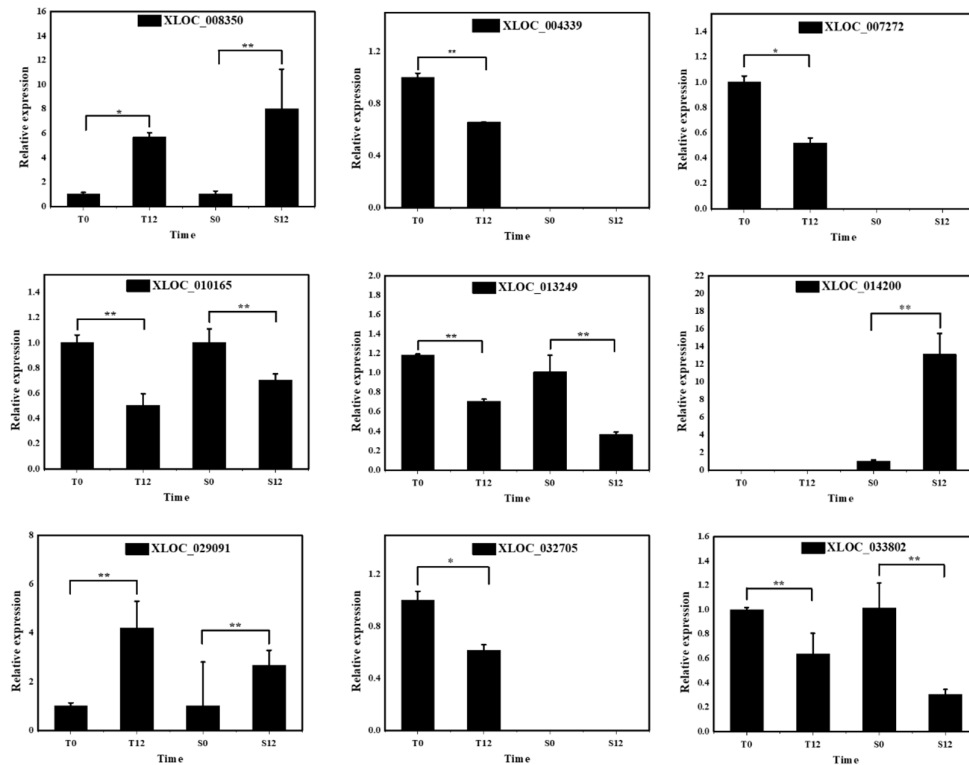

Figure 7. Validation of the expression patterns of lncRNAs obtained by RNA-seq using qPCR
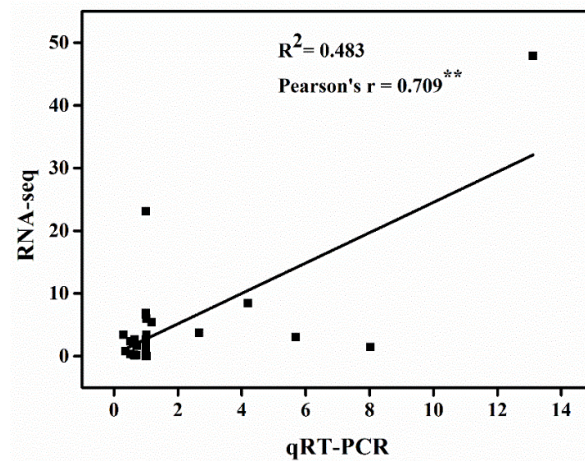
Figure 8. Correlation analysis of RNA-seq and qPCR results

Discussion

With the development of high throughput sequencing, noncoding RNAs were dug out in lots of plants, especially in model plants (Li *et al.*, 2022; Qing *et al.*, 2022). Increasing evidences have indicated the functions of noncoding RNAs including miRNA (Yan *et al.*, 2016; Tang and Thompson, 2019), circular RNA (circRNA) (Gao *et al.*, 2019) and lncRNA (Li *et al.*, 2017; Deng and Wu, 2019) in response to cold stress in plants. In eggplant, Yang *et al.* (2017) identified the cold-responsive miRNAs and their target genes in the wild eggplant species. The involvement of lncRNAs in eggplant cold tolerance remained limited. In this study, we carried out an identification of lncRNAs in eggplant. As a result, 4374 long noncoding transcripts have been obtained, among which 452 lncRNAs (227 and 225 in sensitive and tolerant eggplants respectively) were differentially expressed to response to cold stress (Figure 2). There were no much differences in the number of cold-related lncRNAs between these two genotypes with differential cold tolerance. A large number of cold-responsive lncRNAs have been identified in many plants, such as *Medicago truncatula* (1271 DElncRNAs) (Zhao *et al.*, 2020), cassava (316 DElncRNAs) (Li *et al.*, 2022), wild banana (*Musa itinerans*) (14034 DElncRNAs) (Liu *et al.*, 2018). The number of cold-responsive lncRNAs identified in our study is inconsistent with that in *Medicago truncatula*, cassava and wild banana. This difference may be caused by genome sizes, sequencing methods and sample numbers.

lncRNA is a class of regulatory RNA in a broad range of biological process in plants (Zhu and Wang, 2012). Hence, it is indispensable to identify their potential target genes for understanding the regulatory roles of lncRNAs. We predicted the possible target genes of cold-responsive lncRNAs, finding more target genes in cis-regulatory relationship than in trans-regulatory relationship in both eggplant varieties (Table 1). This indicated that eggplant lncRNAs mainly responded to cold stress by targeting genes in cis-regulation. The target genes in sensitive eggplant were a little more than those in tolerant eggplant (Figure 3). Some target genes of cold-related lncRNAs may be involved in abiotic stress response such as ethylene-responsive transcription factor (ERF), cytochrome P450, MAPK and so on. These target genes were induced by cold stress (Table S4, Table S6). In Zhuo's (2018) reports, a cold responsive ERF enhanced cold tolerance by increasing polyamine turnover, antioxidant protection and proline accumulation in *Medicago falcate*. Previous studies have revealed the response of cytochrome P450 to various abiotic stresses, including salinity, drought, and cold (Bilodeau *et al.*, 1999; Tao *et al.*, 2017). In addition, MAPK have been widely studied and functionally characterized in plants response to cold stresses (Zhao *et al.*, 2013; Yu *et al.*, 2016; Tak *et al.*, 2020). Therefore, we speculated that eggplant lncRNAs mediated cold tolerance by targeting these cold-responsive genes. We also conducted

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the expression correlation between lncRNAs and their target genes (Figure 4) (Table 2). As a result, the expression correlation coefficients between lncRNAs and their cis-regulatory genes were only 0.087 and 0.088 in two eggplant genotypes respectively, suggesting no expression relevance in lncRNA and their cis-regulatory genes. This result did not agree with the finding in grape lncRNAs (Wang *et al*., 2019). In both sensitive and tolerant eggplants, correlation relationships were significantly positive at the level of 0.01. The expression correlation between cold responsive lncRNAs and their target genes in trans-regulatory relationships were much higher than in cis-regulatory relationships (Figure 4). The reason for this phenomenon was that these genes were thought to be the trans-regulatory targets of lncRNAs if these genes were co-expressed with lncRNAs, while cis-regulatory genes were predicted according to their locations (Wang *et al*., 2021; Ye *et al*., 2022).

Functional characterization analysis showed that target genes in cis-regulation of *T0 vs T12* mainly enriched in acyl-CoA dehydrogenase activity, pseudouridine synthase (activity), polyubiquitin modification-dependent protein binding ability, recognition of pollen and de-etiolation, among which acyl-CoA dehydrogenase activity and pseudouridine synthase (activity) were unique GO terms of *T0 vs T12* compared with *S0 vs S12* (Figure 5A). A recent report indicated that pseudouridine synthase was required for rice development at low temperature because of albino phenotype and death led by the deficiency of TCD3 encoding pseudouridine synthase (Lin *et al*., 2020). The target genes of *S0 vs S12* were particularly related to L-lactate dehydrogenase activity, malate synthase activity and glyoxylate cycle. These different GO terms may be responsible for the difference in the cold tolerance between two eggplant genotypes. The enriched GO terms of trans-regulatory genes in both *T0 vs T12* and *S0 vs S12* showed that target genes in trans-acting manner were connected with cargos trafficking, including encoding SNARE complex, stabilizing SNARE activity and SNARE binding, and participating vesicle docking and fusion (Figure 6A). Several genetic and biochemical studies have reported that SNAREs are the key regulators control trafficking of cargo proteins to their final destinations and plays key role in plants, due to their performances in the final membrane fusion step of membrane traffic in plant through forming a SNARE complex (Sun *et al*., 2013; Zhu *et al*., 2019; Kwon *et al*., 2020). Based on our analysis and previous reports, we hypothesized that eggplant lncRNAs could regulated the cargo transport to mediate cold response.

Plants have evolved responsive pathways to cold stress. Target genes of lncRNAs were categorized into pathway terms based on their annotations, which will help us to understand the potential functions of eggplant lncRNAs in response to cold treatment. Regardless of regulatory manner, target genes of lncRNAs in both eggplant varieties were involved in plant hormone signal transduction, which was consistent with our finding in transcriptome analysis (Figure 5B, 5C, 6B, 6C) (Yang *et al*., 2020). It is widely reported that several pathways such as ubiquitin mediated proteolysis, starch and sucrose metabolism, oxidative phosphorylation and protein processing in endoplasmic reticulum were activated and respond to cold stress (Bar-Peled *et al*., 2007; Zhang *et al*., 2017; Rurek *et al*., 2018; Boulc *et al*., 2020). In our study, a majority of genes in cis-regulatory manner in both eggplant varieties took participate in those pathways (Figure 5B,5C), which indicated that lncRNAs could mediate eggplant cold response by cis-regulating these pathways. A common consequence of cold stress is accumulation of macromolecular components including unfolded or misfolded proteins (Park and Seo, 2015; Bao *et al*., 2017). Prevention aggregation of damaged proteins to maintain cell homeostasis is important for cell survival under stress. Pathway analysis in this study revealed that under cold stress eggplant cis-regulatory genes were involved in ubiquitin mediated proteolysis, protein processing in endoplasmic reticulum and endocytosis, whilst trans-regulatory genes participated in SNARE interactions in vesicular transport apart from protein processing in endoplasmic reticulum (Figure 5B, 5C, 6B, 6C). Furthermore, pathways in cis- or trans-acting manner were differential in the two eggplant genotypes, although starch and sucrose metabolism, vesicular transport, ribosome and lysine degradation were common. For example, the enriched pathways were different (Figure 5B, 5C, 6B, 6C). Notably, plant hormone signal transduction was enriched in sensitive, not tolerant eggplant according enrichment analysis, which demonstrated that cis-acting genes of lncRNAs in sensitive

eggplant were mainly associated with plant hormone signal transduction. All of those differentials may account for different phenotypes to cold stress in two eggplant varieties.

Conclusions

In this study, the lncRNA profiling of two eggplant genotypes with differential tolerance to cold stress were performed. The numbers of DElncRNAs in two eggplant varieties were approximate despite of the extreme phenotypes to cold stress. Functional characterization revealed that a majority of target genes functioned similarly in two eggplant genotypes due to the same GO annotations and involved KEGG pathways. There were only a minority of differentials in molecular function including acyl-CoA dehydrogenase activity and pseudouridine synthase activity and in enriched pathways such as plant hormone signal transduction reported as our previous study. Our findings will facilitate further experimental studies and functional classifications of these genes, and provide insights into new regulators and regulatory mechanisms for eggplant cold-tolerance.

Authors' Contributions

Conceptualization: YZ; Data curation and Formal analysis: YY and JXZ; Funding acquisition: YY and YZ; Methodology: JL and SQL; Writing - original draft: YY; Writing - review and editing: XHZ and SYL. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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