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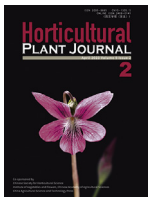

Involvement of long non-coding RNAs in pear fruit senescence under high- and low-temperature conditions

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A B S T R A C T

Pear fruit senescence under high- and low-temperature conditions has been reported to be mediated by microRNAs. Long non-coding RNAs (lncRNAs), which can function as competing endogenous RNAs that interact with microRNAs, may also be involved in temperature-affected fruit senescence. Based on the transcriptome and microRNA sequencings, in this study, 3 330 lncRNAs were isolated from *Pyrus pyrifolia* fruit. Of these lncRNAs, 2 060 and 537 were responsive to high- and low-temperature conditions, respectively. Of these differentially expressed lncRNAs, 82 and 24 correlated to the mRNAs involved in fruit senescence under high- and low-temperature conditions, respectively. Moreover, three lncRNAs were predicted to be competing endogenous RNAs (ceRNAs) that interact with the microRNAs involved in fruit senescence, while one and two ceRNAs were involved in fruit senescence under high- and low-temperature conditions, respectively. A dual-luciferase assay showed that the interaction of an lncRNA with a microRNA disrupts the action of the microRNA on the expression of its target mRNA(s). Furthermore, four alternative splicing-derived lncRNAs interacted with *miR172i* homologies (*Novel_88* and *Novel_69*) to relieve the repressed expression of their target and produce an *miR172i* precursor. Correlation analysis of microRNA expression suggested that *Novel_69* is likely involved in the cleavage of the pre-*miR172i* hairpin to generate mature *miR172i*. Taken together, lncRNAs are involved in pear fruit senescence under high- or low-temperature conditions through ceRNAs and the production of microRNA.

Keywords: *Pyrus pyrifolia*; Long non-coding RNA (lncRNA); Fruit senescence; High-temperature; Low-temperature; lncRNA-microRNA-mRNA interaction

1. Introduction

Senescence is unavoidable in fruit life cycles and directly affects fruit quality, resistance to pathogens, and shelf life, contributing to the loss of the economic value of post-harvest fruit. During fruit senescence, a series of physiological and biochemical reactions in stored fruit arouse dramatic changes in texture, flavor, skin color, biochemical composition, and pathogen susceptibility (Tian et al., 2013), which lead to declining quality and nutrition (Yoshida, 2003; Tian et al., 2007; Xu et al., 2020). However, these reactions could be blocked by exogenous treatments, including low-temperature, 1-methylcyclopropene, calcium, controlled atmosphere, salicylic acid, ATPase, and oxalic acid (Aghdam et al., 2012; Huang et al., 2013; Wang et al., 2013).

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Presently, low-temperature has been most widely used to delay fruit senescence, which retards firmness loss and slows metabolism during storage (Ali et al., 2004; Yun et al., 2012). In contrast, high-temperature accelerates fruit senescence through a molecular mechanism different from that of low-temperature-inhibited fruit senescence (Gu et al., 2020). In pear fruit, the compounds involved in high-temperature- and low-temperature-induced fruit senescence are divided into anti-senescent (I→III) and pro-senescent (IV→VI) types. LT promotes the accumulation of types I and II anti-senescent compounds and impedes the biosynthesis of types IV and V pro-senescent compounds by affecting the expression level of 530 differentially expressed mRNAs (DEGs; Gu et al., 2020). Whereas high-temperature enhances the biosynthesis of types V and VI pro-senescent compounds and inhibits the accumulation of types II and III anti-senescent compounds by mediating the expression level of 202 DEGs (Gu et al., 2020). Moreover, 14 and 2 microRNAs (non-coding RNAs) are involved in low-temperature- and high-temperature-induced fruit senescence, respectively, by interacting with their target DEGs (Gu et al., 2020). It is unclear whether low-temperature- and high-temperature-induced fruit senescence are regulated by other non-coding RNAs, such as long non-coding RNA (lncRNA).

LncRNAs are usually more than 200 nucleotides in length (Chen, 2009; Rinn and Chang, 2012; Huang et al., 2021) and can be grouped into three classes based on their positional relationship to nearby protein-coding genes: long intergenic non-coding RNAs, nature anti-sense transcripts, and intronic RNAs (Ma et al., 2013). LncRNAs mediate transcript or translation in many ways. For example, lncRNAs regulate the DNA-binding activity of transcription factors (TFs) by polydimerization, phosphorylation, and chromatin modification (Shamovsky et al., 2006; Csorba et al., 2014; Wang et al., 2014a). Enhancer lncRNAs affect the transcriptional activities of their flanking genes by recruiting enhancer-binding TFs (Natoli and Andrau, 2012; Lam et al., 2014). The formation of a stable RNA–DNA triplex controls TF-binding sites (Sun et al., 2013a; Wahba and Koshland, 2013). Alternative splicing competitor lncRNA alters the splicing patterns of transcripts by robbing nuclear speckle RNA-binding proteins (Bardou et al., 2014). Moreover, double-strand RNAs, which are formed by the complementation of lncRNA and mRNA, could be processed into small RNAs and thus are involved in post-transcriptional gene silencing and transcriptional gene silencing (Bologna and Voinnet, 2014; Kim et al., 2014). Notably, lncRNAs also function as competing endogenous RNAs (CeRNAs) that share microRNA recognition elements to relieve the effect of microRNA on the expression of its target (Franco-Zorrilla et al., 2007; Cesana et al., 2011). In addition, lncRNAs can act as a scaffold for the RNA-directed DNA methylation pathway (RdDM; Kim and Sung, 2012; Wierzbicki, 2012) and interact with RNA-binding proteins to regulate protein–protein interactions and protein modifications (Wang et al., 2014a; Yang et al., 2014a). Presently, lncRNAs have been reported to be associated with plant growth and differentiation (Ben Amor et al., 2009; Fitica and Bozzoni, 2014; Kornblihtt, 2014; Wang et al., 2014b), grain yield (Wang et al., 2018), fruit ripening (Zhu et al., 2015; Li et al., 2018; Zhang et al., 2018), and biotic and abiotic stress (Liu et al., 2012; Mach, 2017; Nejat and Mantri, 2018).

Pear is one of the most popular fruit with a worldwide production of more than 23.7 million tons (FAOSTAT, 2018) and it is the third-most important fruit in China (He et al., 2022). In China, pear fruit is harvested in the summer and shows fast senescence because of the high temperature (> 35 °C) in production areas of Yangtze river basin, China. To prolong shelf life, the harvested fruit is usually stored at a low-temperature (approximately 4 °C) to delay senescence. In a previous study (Gu et al., 2020), we treated pear fruit at high-temperature (40 °C ± 2 °C), room-temperature (25 °C ± 1 °C), and low-temperature (4 °C ± 1 °C), and conducted non-targeted metabolome, transcriptome, and small RNA sequencing to identify the compounds, mRNAs, and microRNAs, respectively, involved in high-temperature- or low-temperature-induced fruit senescence. To test the involvement of lncRNAs in high-temperature- or low-temperature-induced fruit senescence, we assembled lncRNA sequences using the clean reads of transcriptome sequencing and identified the differentially expressed lncRNAs (DELs) responsive to fruit senescence under high- or low-temperature conditions. Moreover, we analyzed the positional relationship of DEGs and their correlated DELs and tested the potential lncRNA–microRNA and lncRNA–microRNA–mRNA interactions. This information provides insights into the molecular mechanism of fruit senescence under high- and low-temperature conditions and may be useful for optimizing post-harvest handling methods for extending storage life and maintaining fruit quality during storage.

2. Materials and methods

2.1. Plant materials and treatment

To explore the molecular mechanism of pear fruit senescence, in a previous study (Gu et al., 2020), ripening fruit (RF) of Housui were harvested and then stored at (40 ± 2) °C (high-temperature), (25 ± 1) °C (room-temperature), and (4 ± 1) °C (low-temperature). Based on the threshold of 30% (the decayed fruit) of the stored fruit, the undecayed fruit was deemed senescent. As a result, the stored fruit was senescent at 15 days after treatment (DAT) under high-temperature conditions, 19 DAT under room-temperature conditions, and 105 DAT under low-temperature conditions. Finally, the fruit samples were collected at 0, 10, 15, 19, 80, and 105 DAT. A total of 11 samples were immediately frozen in liquid nitrogen and stored at −80 °C until use. Each sample contained at least 10 fruits.

2.2. Description of transcriptome data

The transcriptome data from the 11 samples (Gu et al., 2020) were used for lncRNA identification herein. The transcriptome libraries were constructed by removing ribosomal RNAs and enriching the cDNA fragments with a size of 150–200 bp. These libraries were sequenced on an Illumina Hiseq 2000 platform (Illumina, San Diego, CA) in 150 bp paired-end reads. Raw data were available on the Sequence Read Archive (Accession number: SRP233477) at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/sra).

2.3. Transcriptome assembly

To obtain the protein-coding and non-coding transcripts, the low-quality raw reads were filtered and the clean reads were assembled with two methods that used the spliced reads to determine exon connectivity. One was Scripture (beta 2), which uses a statistical segmentation model to distinguish expressed

loci from experimental noise (Guttman et al., 2010). The other was Cufflinks (V 2.1.1), which uses a probabilistic model to simultaneously assemble and quantify the expression level of a minimal set of isoforms (Cabili et al., 2011). This isoform provides a maximum likelihood explanation of the expression data in a given locus (Cabili et al., 2011). Scripture was run with default parameters, while Cufflinks was run with 'min-frags-per-transfrag = 0' and '–library-type', with the other parameters set as default.

2.4. Identification of lncRNAs

After merging the transcripts assembled by Scripture and Cufflinks, four steps were conducted to identify lncRNAs. First, transcripts with a size less than 200 bp were removed. Second, the transcripts that overlapped with the annotated exons in the pear database were removed using Cuffcompare V 1.0.4 (Trapnell et al., 2010). Third, the expression level of each transcript was calculated based on fragments per kilobase of exon model per million mapped fragments (FPKM) using Cufflinks V 2.1.1 (Trapnell et al., 2010). The transcripts that had low levels of expression (FPKM $\leq$0.5) in all tested samples were removed. Fourth, the transcripts that encoded any conserved protein domains were removed using the Coding Potential Calculator (CPC; Kong et al., 2007), Pfam-scan (El-Gebali et al., 2019), Coding-Non-Coding Index (CNCI; Sun et al., 2013a), and Predictor of long non-coding RNAs and messenger RNAs based on an improved k-mer scheme (PLEK; Li et al., 2014). Pfam was performed with default parameters of -E 0.001 –domE 0.001 (Bateman et al., 2002), while CPC was run in NCBI eukaryotes' protein database with an *e*-value of 1e−10 as the threshold. CNCI and PLEK were run with the default parameters. Finally, the overlapped transcripts of CPC and Pfam-scan were deemed lncRNAs.

2.5. Quantitative real-time PCR testing of lncRNAs and mRNAs

The first-strand cDNAs were synthesized using TransScript One-Step gDNA Removal and cDNA Synthesis Supermix (TransGen, Beijing, China). Quantitative real-time PCR (qRT-PCR) was carried out in a LightCycler 480® II/96 Thermal Cycler (Roche Diagnostics, Rotkreuz, Switzerland). The reaction mixture and conditions were identical to a previous report (Hao et al., 2018). A pear *TUBULIN* gene was used as an endogenous control (Hao et al., 2018). Moreover, the reaction mixture without the cDNA template was used as a negative control. All primer sequences are listed in Table S1.

2.6. Differential expression and correlation analyses

The expression levels of lncRNAs were calculated based on FPKM. The number of clean reads mapped to each lncRNA was counted by HTseq V 0.5.4p3. The read counts were standardized among samples to a common value across all sequenced libraries using EdgeR V 2.6.10. Differential analyses of lncRNAs were performed between senescent (HT-15, RT-19, and LT-105) and ripening fruit (RF), between HT-15 and RT-15, between HT-10 and RT-10, between RT-19 and LT-19, between RT-15 and LT-15, and between RT-10 and LT-10, based on a $\log_2$ fold-change threshold of 1. To determine the potential effect of lncRNA on the expression of mRNAs, the correlation coefficient between lncRNAs and mRNAs was calculated by SPSS software (IBM) using the data generated from the 11 samples. The correlation relationship was determined based on Pearson coefficient of 0.6 and false discover rate (FDR) of 0.05.

2.7. Dual-luciferase assay

For analysis of the lncRNA–microRNA interaction, the potential interaction between these microRNAs and lncRNAs was evaluated by psRobot_tar in psRobot with default parameters (Wu et al., 2012). The full-length sequences of pre-microRNA were amplified from genomic DNA using high-fidelity KOD-Plus-Ver.2 DNA Polymerase (Toyobo, Osaka, Japan) and then inserted into a pSAK277 vector (Xue et al., 2018). The microRNA target sequences with a size of 200–300 nt were amplified from the first-strand cDNA and then inserted into the pGreenII Dual-Luciferase microRNA Target Expression Vector (Xue et al., 2018). The process for the introduction of the constructed vectors into *Agrobacterium tumefaciens*, as well as for the suspension, infiltration, and culturing of tobacco plants, were identical to a previous study (Gu et al., 2020).

For analysis of lncRNA–microRNA–mRNA interaction, the pre-microRNAs and their target lncRNAs were individually inserted into the pSAK277 vector and transformed into *A. tumefaciens*. The mRNA was amplified and then inserted into the pGreenII Dual-Luciferase microRNA Target Expression Vector (Xue et al., 2018). The three types of *Agrobacterium* were mixed at a ratio of 4:4:2 (v/v/v). A luminescence assay was conducted using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Firefly luciferase (Luc) and Renilla luciferase (Ren) activities were measured using a Cell Imaging Multi-Mode Reader Cytation 3 (BioTek, Santa Barbara, CA) for at least 10 biological replicates for each assay. All primer sequences are listed in Table S1.

2.8. Estimation of alternative splicing

Alternative splicing events were analyzed using multivariate analysis of transcript splicing and integrative genomics viewer software (Shen et al., 2012). Asprofile v1.0 software (http://ccb.jhu.edu/software/ASprofile/) was used to classify the alternative splicing events into 12 basic types. The number of alternative splicing events was estimated separately in each sample (Sammeth et al., 2008).

3. Results

3.1. Identification and characteristics of lncRNAs

To identify lncRNAs in the pear genome, a total of 1032.60 million clean reads could be assembled to 105826 transcripts. In these transcripts, 6781 had a size less than 200 bp, and 90182 overlapped with the predicted mRNAs. After calculating the FPKM value of each transcript, we found that 2085 transcripts had low level of expression (FPKM $\leq$ 0.5) in all tested samples. Moreover, Pfam-scan, CPC, CNCI, and PLEK analyses found that 3448 transcripts had the potential role of an encoding protein. Thus, the above 102496 transcripts were excluded from the candidate lncRNAs, and the remaining 3330 were identified as lncRNAs (Table S2). These lncRNAs were covered by a total of 218.79 million clean reads, with an average of 19.89 million clean reads in each sample.

Chromosome location showed that these lncRNAs were randomly distributed on all 17 chromosomes (Fig. 1, A) and 279

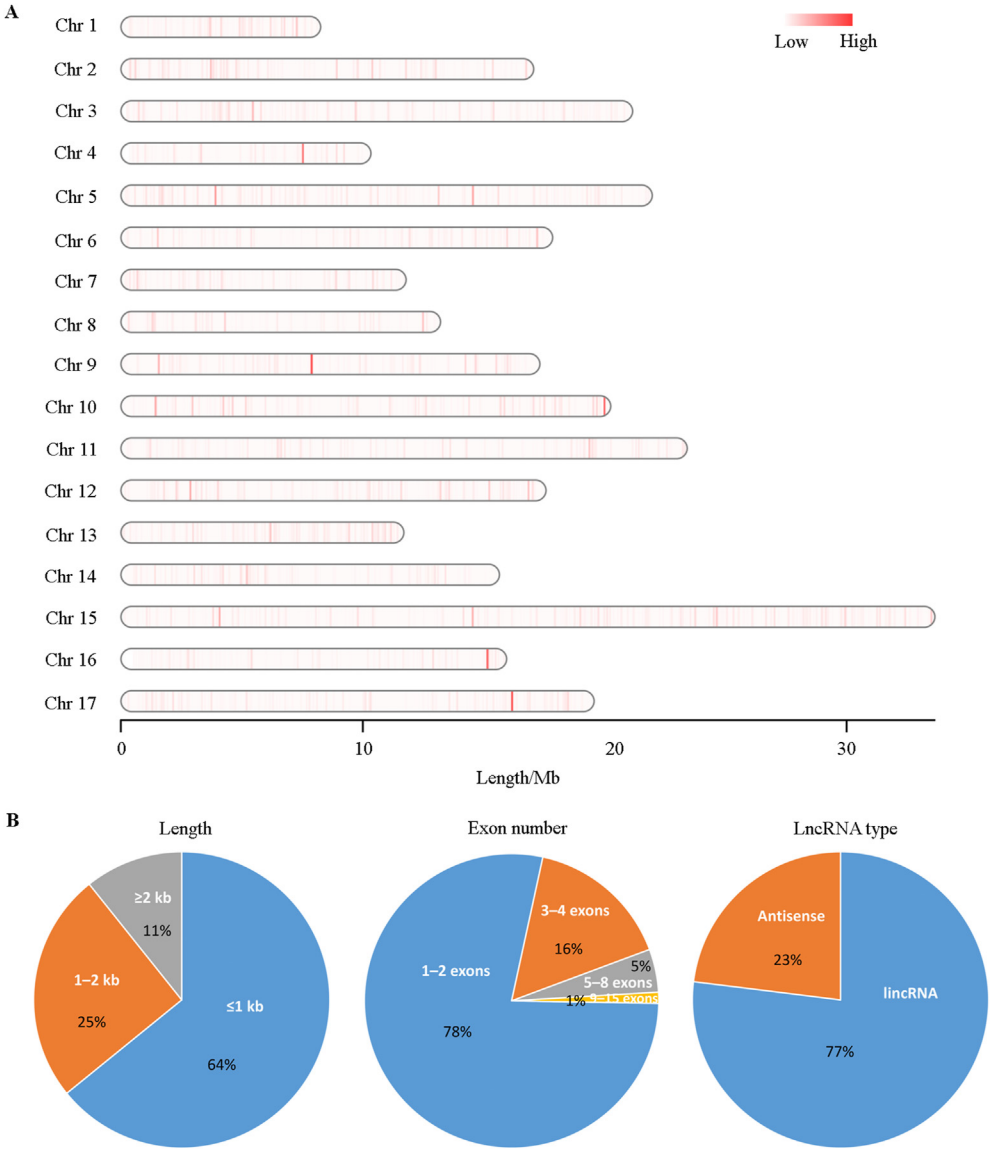

Fig. 1 Chromosome location (A) and characteristics (B) of the identified lncRNAs in pear

With the transition from white to red, the density of lncRNA in each chromosome was gradually increased. Low and high represent 0 and 0.54 lncRNAs per 100 kb, respectively. LincRNA represents the location of lncRNA within the intergenic region. Antisense represents the derivation of lncRNA from antisense.

scaffolds (Table S2). These lncRNAs ranged from 201 bp to 7902 bp in length (Table S2). Of these, over two-thirds had a length less than 1 kb, a quarter had a range of 1–2 kb, and only 11% had a length more than 2 kb (Fig. 1, B). The exon number of these lncRNAs ranged from 1 to 15 (Table S2). Over three-quarters of these lncRNAs had 1 or 2 exons, and only 1% had 9–15 exons (Fig. 1, B). Moreover, over three-quarters of these lncRNAs were located within the intergenic region. Only 23% derived from antisense, and none derived from the intronic region (Fig. 1, B; Table S2).

3.2. *Identification of lncRNAs responsive to fruit senescence under high- or low-temperature conditions*

To identify the lncRNAs responsive to fruit senescence, differential expression analysis was conducted between the senescent (HT-15, RT-19, and LT-105) and ripening fruit (RF). In the detected 3330 lncRNAs, 795 were upregulated, but 827 were downregulated in RT-19 compared to RF (Table S3); 1958 were upregulated, but 594 were downregulated in HT-15 compared to RF (Table S4); and 705 were upregulated, but 1 144 were downregulated in LT-105 compared to RF (Table S5). Notably, 200 upregulated and 173 downregulated lncRNAs commonly existed in the three sets of differential expression analyses (Fig. 2, A). Therefore, these 373 lncRNAs were responsive to fruit senescence in pear.

To isolate the lncRNAs responsive to fruit senescence under high-temperature conditions, differential expression analysis was conducted between high-temperature-induced senescent fruit (HT-15/HT-10) and its control fruit (RT-15, RT-10). The results showed that 1947 lncRNAs were upregulated but 662 lncRNAs were downregulated in HT-15 compared to RT-15 (Table S6), and

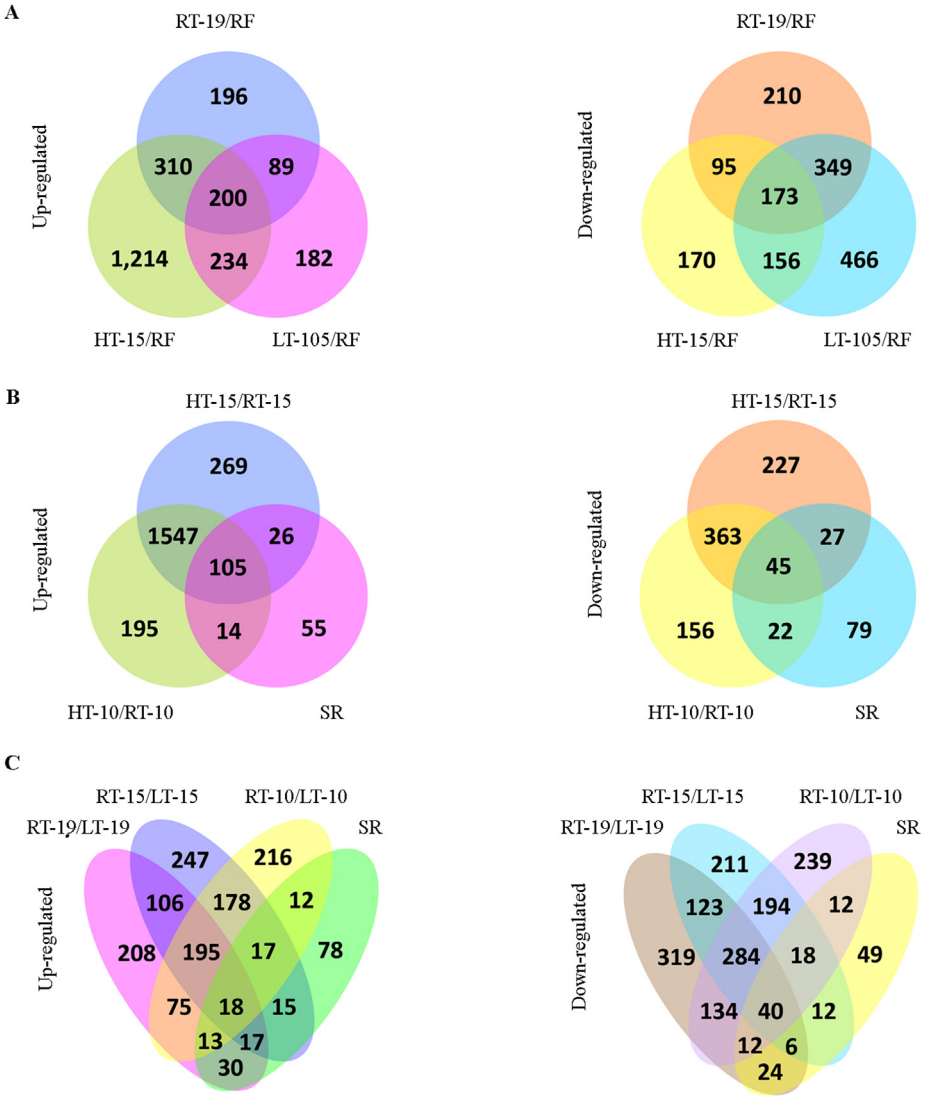

Fig. 2 Identification of lncRNAs responsive to fruit senescence under high- or low-temperature conditions

(A) Differential analysis was conducted between RF and senescent fruit (RT-19/HT-15/LT-105) to identify lncRNAs responsive to fruit senescence. (B) Differential analysis was conducted between HT-15 and RT-15 (HT-15/RT-15) and between HT-10 and RT-10 (HT-10/RT-10). (C) Differential analysis was conducted between RT-19 and LT-19 (RT-19/LT-19), between RT-15 and LT-15 (RT-15/LT-15), and between RT-10 and LT-10 (RT-10/LT-10). SR is the abbreviation for senescence-related.

1861 lncRNAs were upregulated but 586 lncRNAs were downregulated in HT-10 compared to RT-10 (Table S7). A total of 1652 upregulated and 408 downregulated lncRNAs commonly existed in these two sets of differential expression analysis (Fig. 2, B), indicating that these lncRNAs were responsive to high-temperature conditions. In the 2060 differentially expressed lncRNAs (DELs), 105 upregulated and 45 downregulated lncRNAs overlapped with the lncRNAs responsive to fruit senescence (Fig. 2, B). Therefore, these 150 DELs were responsive to fruit senescence under high-temperature conditions.

Similarly, to isolate the lncRNAs responsive to fruit senescence under low-temperature conditions, differential expression analysis was conducted between room-temperature-induced senescent fruit (RT-19/RT-15/RT-10) and its control fruit with low-temperature treatment (LT-19/LT-15/LT-10). As a result, 662 lncRNAs were upregulated, but 942 lncRNAs were downregulated in RT-19 compared to LT-19 (Table S8); 793 lncRNAs were upregulated, but 888 lncRNAs were downregulated in RT-15 compared to LT-15 (Table S9); and 724 lncRNAs were upregulated, but 933 lncRNAs were downregulated in RT-10 compared to LT-10 (Table S10). A total of 213 upregulated and 324 downregulated lncRNAs commonly existed in these three sets of differential expression analysis (Fig. 2, C), indicating that these lncRNAs were responsive to the low-temperature condition. In these DELs, 18 upregulated and 40 downregulated lncRNAs overlapped with the lncRNAs responsive to fruit senescence (Fig. 2, C). Therefore, these 58 DELs are responsive to fruit senescence under low-temperature conditions. In addition, qRT-PCR validation showed that the expression profiles of nine randomly selected lncRNAs were positively correlated to those identified from the transcriptome data ($P < 0.05$; Fig. S1). This result indicates that the qRT-PCR results are consistent with the transcriptome data.

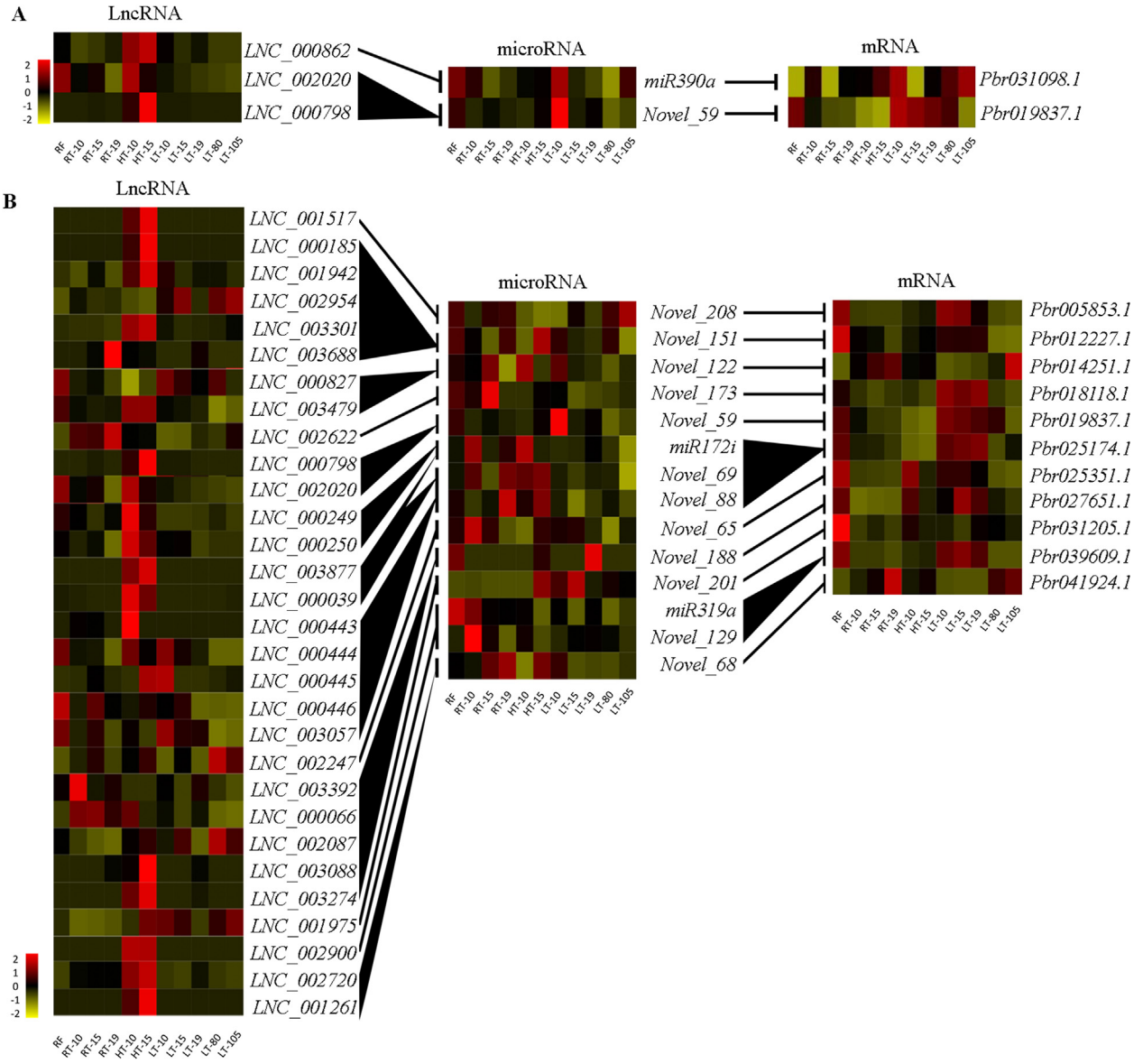

Fig. 3 A diagram of the lncRNA–microRNA–mRNA pathway during fruit senescence under high- or low-temperature conditions

(A) During fruit senescence under high-temperature conditions, two high-temperature-responsive mRNAs, *Pbr031098.1* and *Pbr019837.1*, are the targets of *miR390a* and *Novel_59*, respectively. However, *LNC_000862* could interact with *miR390a* to disrupt the action of *miR390* on *Pbr031098.1* expression. Similarly, two lncRNAs could interact with *Novel_59* to enhance *Pbr019837.1* expression. (B) During fruit senescence under low-temperature conditions, nine low-temperature-responsive mRNAs are the targets of nine microRNAs, and both *Pbr025174.1* and *Pbr039609.1* are the targets of at least two microRNAs. Notably, these 14 microRNAs could target at least one lncRNA, leading to the disruption of these microRNAs on the expression of their target mRNAs. For heatmap, RPKM value is standardized by comparison to the mean of all 11 samples of each lncRNA/microRNA/mRNA. The $\log_2$ standardization was used to calculate the color bar (from −2 to 2). RF is the fruit collected at ripening stage. RT-10, RT-15, and RT-19 are the fruit collected at 10, 15, and 19 days after treatment under room-temperature condition, respectively. HT-10 and HT-15 are the fruit collected at 10 and 15 days after treatment under high-temperature condition, respectively. LT-10, LT-15, LT-19, LT-80, and LT-105 are the fruit collected at 10, 15, 19, 80, and 105 days after treatment under low-temperature condition, respectively.

3.3. *Correlation analysis between DELs and DEGs*

Presently, we determined that 202 DEGs were involved in fruit senescence under high-temperature conditions (Gu et al., 2020), and 150 DELs were responsive to fruit senescence under high-temperature conditions. To explore the role of these DELs in fruit senescence, a correlation analysis was conducted between these DELs and DEGs. The result showed that only 82 DELs were correlated with all 202 DEGs (*FDR* < 0.05; Table S11). Notably, both DELs and DEGs in 175 of the 2 807 lncRNA–mRNA pairs were located on a chromosome (Table S11). The DELs positively correlated to the mRNAs in 164 lncRNA–mRNA pairs, while the

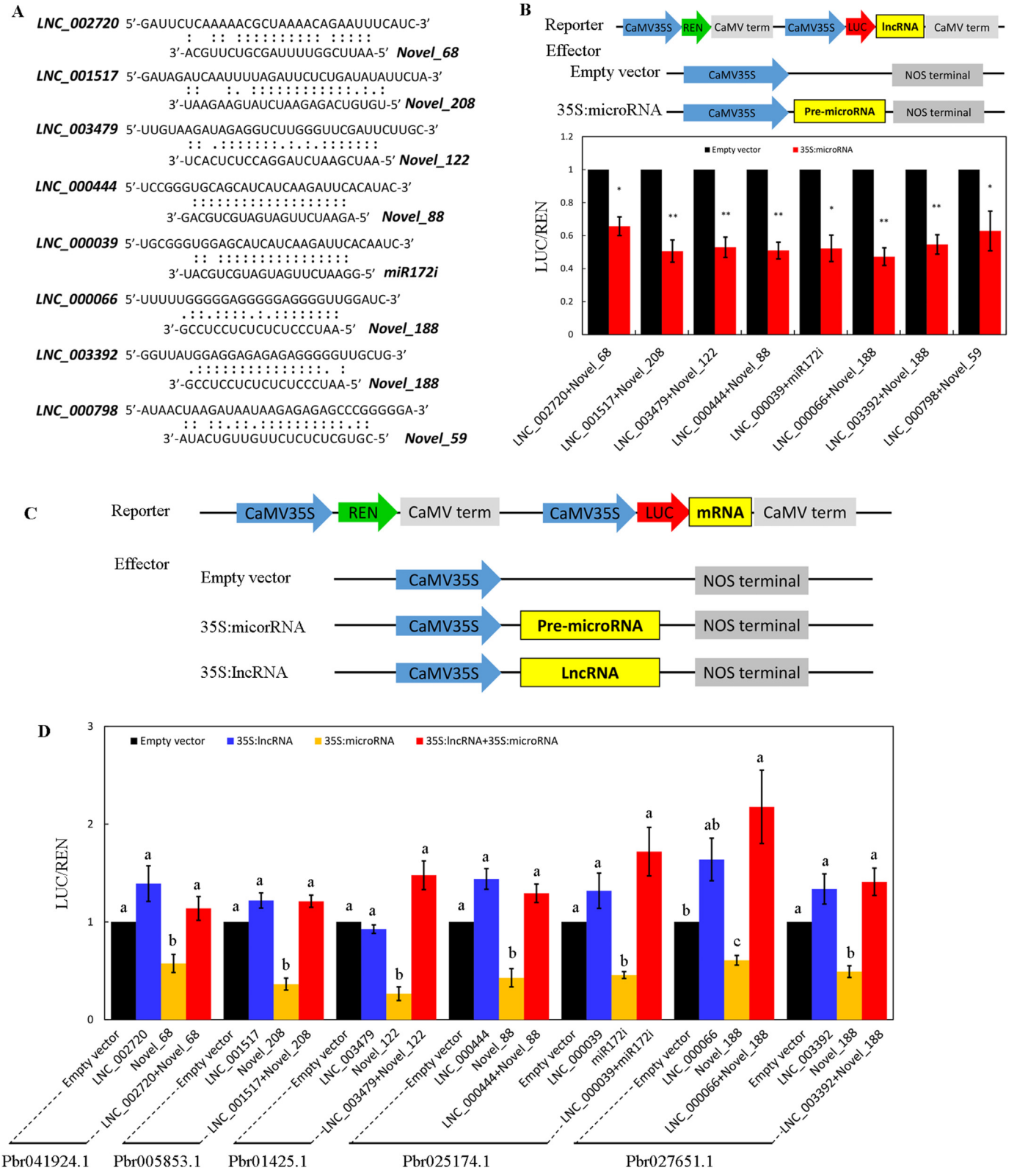

Fig. 4 Validation of the predicted lncRNA–microRNA and lncRNA–microRNA–mRNA interactions

(A) Predicted interaction between microRNAs and their target lncRNA sequences. (B) Dual-luciferase assay of lncRNA–microRNA interactions. Means and standard errors were calculated using the Student's *t*-test. Single and double asterisks indicate significant differences at *P*-value < 0.05 and *P*-value < 0.01, respectively. (C) Construction of reporter and effector vectors. (D) Dual-luciferase assay of lncRNA–microRNA–mRNA interactions. Means and standard errors were calculated using the Student's *t*-test. The different lowercase letters indicate a level of significance at $P < 0.05$.

DELs negatively correlated to the mRNAs in the remaining 11 pairs. However, the distance between DEL and DEG in any pair was larger than 10 kb, inferring that *cis*- or *trans*-regulation of these isolated DELs on the expression of its correlated mRNAs hardly occurred.

Similarly, we determined that 530 DEGs were involved in fruit senescence under low-temperature conditions (Gu et al., 2020), and 48 DELs were responsive to fruit senescence under low-temperature conditions. To explore the role of these DELs in fruit senescence, a correlation analysis was conducted between these DELs and DEGs. Only 24 DELs could correlate to 522 DEGs (*FDR* < 0.05; Table S12). Notably, both DELs and DEGs in 238 of the 4780 lncRNA–mRNA pairs located on a chromosome. DELs positively correlated with DEGs in 224 lncRNA–mRNA pairs and negatively correlated to DEGs in the remaining 14 pairs. However, the distance between DELs and DEGs in any pair was also larger than 10 kb, indicating that high-temperature- and low-temperature-responsive DELs could hardly influence the expression of their correlated mRNAs through *cis*- or *trans*-regulation, during fruit senescence.

3.4. *Prediction of lncRNA-microRNA interactions*

In a previous study, four microRNAs (*miR390a*, *miR156x*, *Novel_59*, and *Novel_81*) were involved in fruit senescence under high-temperature condition by microRNA–mRNA interactions (Gu et al., 2020), but it is unknown whether these microRNAs also interact with the lncRNAs responsive to high-temperature conditions. To test this hypothesis, the potential interaction between these microRNAs and lncRNAs was evaluated. As a result, no lncRNA was predicted as the target of *miR156x* or *Novel_81*. In contrast, the lncRNA, *LNC_000862*, was predicted as the target of

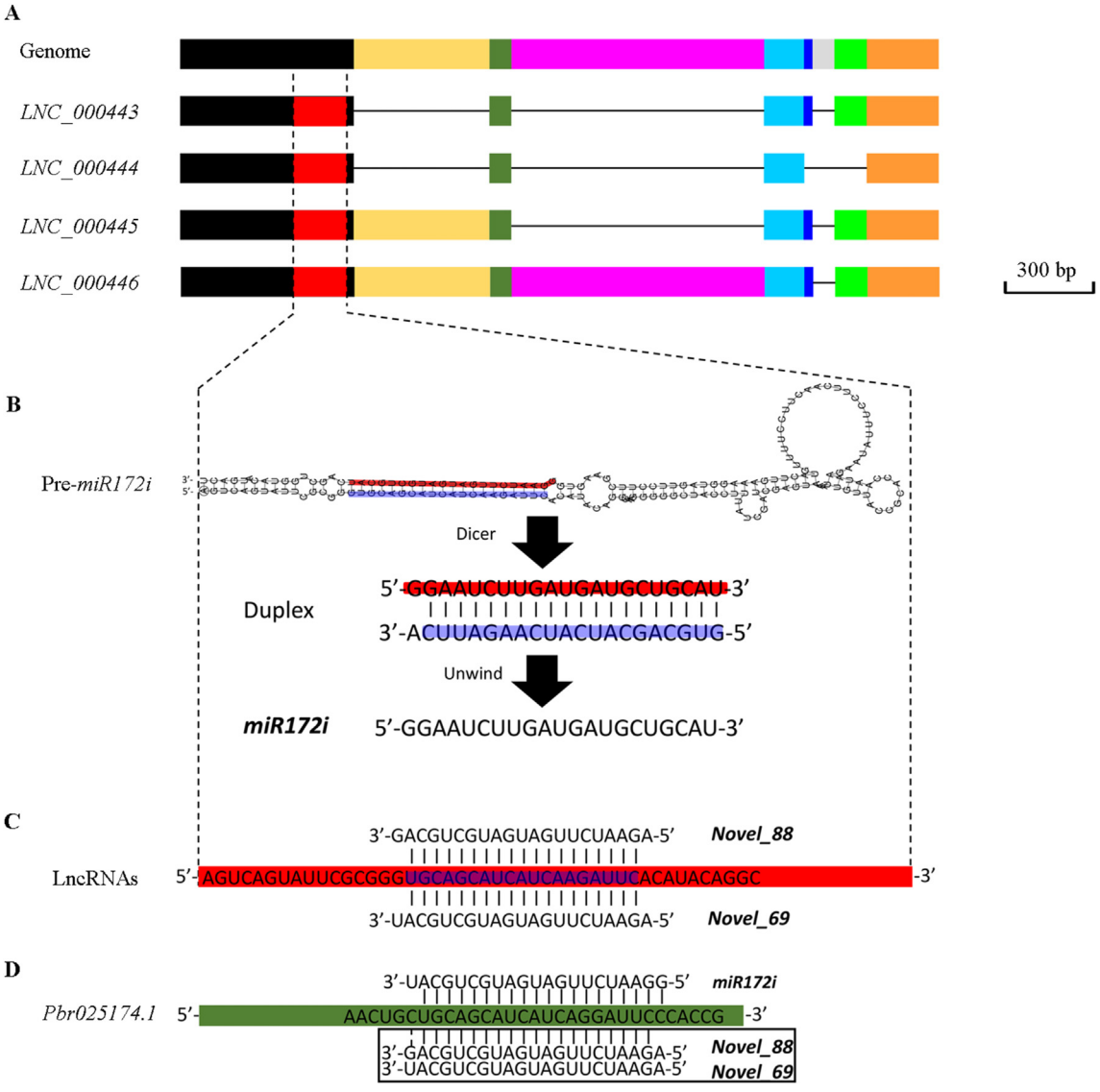

Fig. 5 Generation of *miR172i* from *Novel_69*/*Novel_88*-target lncRNAs

(A) Four lncRNAs derived from alternative splicing events of a genome fragment. (B) Generation of *miR172i* from four lncRNAs. (C) *Novel_69* and *Novel_88* bind to pre-*miR172i* sequences. (D) *miR172i*, *Novel_69*, and *Novel_88* could bind to the coding sequences of *Pbr025174.1*.

miR390a, and two lncRNAs, *LNC_002020* and *LNC_000798*, were the target of *Novel_59* (Fig. 3, A). In these three lncRNAs, only *LNC_000862* was responsive to high-temperature conditions but not fruit senescence (Fig. 3, A). This result indicates that *LNC_000862* may be involved in fruit senescence under high-temperature conditions by lncRNA–microRNA interaction.

Similarly, it was determined that 23 microRNAs were involved in fruit senescence under low-temperature conditions by microRNA–mRNA interactions (Gu et al., 2020). To determine whether these microRNAs also interact with the lncRNAs responsive to low-temperature conditions, we evaluated the potential interactions and found that 30 lncRNAs were the targets of 14 of the 23 microRNAs (Fig. 3, B). In these lncRNAs, *LNC_000249* was responsive to low-temperature conditions but not fruit senescence, while *LNC_ 000827*, *LNC_000445*, and *LNC_002247* were responsive to fruit senescence but not low-temperature conditions. Moreover, *LNC_002622* was responsive to fruit senescence under low-temperature conditions, while the remaining 25 lncRNAs were neither responsive to fruit senescence nor low-temperature conditions. Therefore, *LNC_000249* and *LNC_002622* may be involved in fruit senescence under low-temperature conditions by lncRNA–microRNA interactions.

3.5. *LncRNA–microRNA–mRNA interaction*

To validate the potential lncRNA–microRNA interactions, eight pairs of lncRNA and microRNA were selected for the dual-luciferase assay (Fig. 4, A). The lncRNA fragment was inserted into the 3′-flanking of *LUC* and then used as the reporter, and the microRNA was inserted into the pSAK277 vector to construct a 35S-driven microRNA (35S : microRNA) and used as an effector. *LUC* activities in the leaves infiltrated with any 35S:microRNA were lower than in the leaves infiltrated with the empty vector (Fig. 4, B), suggesting that these eight lncRNAs interact with the corresponding microRNAs involved in fruit senescence under high- or low-temperature conditions.

To determine whether these lncRNAs function as ceRNAs to disrupt microRNA–mRNA interaction, the six microRNAs (*miR172i*, *Novel_68*, *Novel_208*, *Novel_122*, *Novel_88*, and *Novel_188*), which have been reported to interact with the mRNAs involved in fruit senescence under high- or low-temperature conditions (Gu et al., 2020), were selected for lncRNA–microRNA–mRNA cascade analysis. The microRNA target lncRNA was inserted into the pSAK277 vector to construct a 35S-driven lncRNA (35S:lncRNA) and then used as an effector, while the microRNA target mRNA was inserted into the 3′-flanking of *LUC* and used as

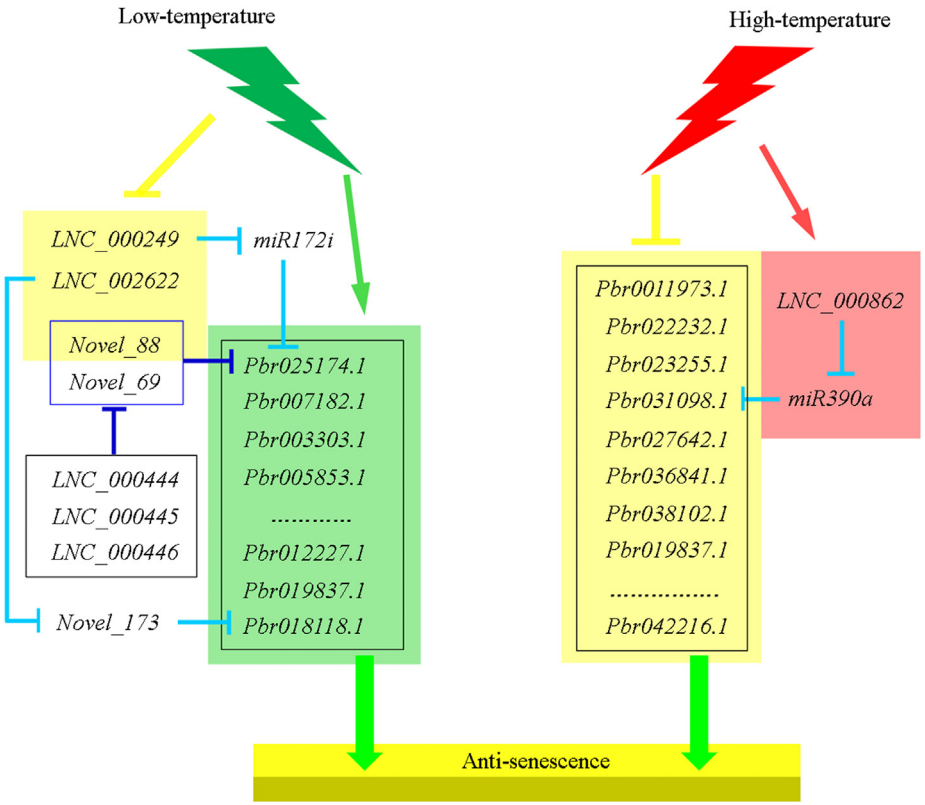

Fig. 6 A diagram of lncRNA–microRNA–mRNA networks regulating fruit senescence under high- and low-temperature conditions
During fruit senescence under high-temperature conditions, high-temperature induces *LNC_000862* expression. *LNC_000862* interacts with *miR390a* to disrupt the *miR390a–Pbr031098.1* interaction. During fruit senescence under low-temperature conditions, low-temperature similarly induces the expression of two lncRNAs, *LNC_000249* and *LNC_002622*. The lncRNAs, microRNAs, and mRNAs with green/red and yellow backgrounds are positively and negatively responsive to high- or low-temperature conditions, respectively. In contrast, the lncRNAs and microRNAs without any background are not responsive to high- or low-temperature conditions.

the reporter (Fig. 4, C). The dual-luciferase assay showed an almost identical profile of *LUC* activity in all lncRNA-microRNA-mRNA groups (Fig. 4, D). For instance, *LUC* activity in the leaves infiltrated with the 35S : LNC_000039 was similar to the activity in the leaves infiltrated with the empty vector, but higher than the activity in the leaves infiltrated with the 35S : miR172i (Fig. 4, D). Notably, *LUC* activity was increased in the leaves co-infiltrated with 35S : miR172i and 35S : LNC_000039 compared to the leaves infiltrated with 35S:miR172i (Fig. 4, D). These results indicate that *LNC_000039* can interact with *miR172i* but not its target mRNA (*Pbr025174.1*), and that this interaction disrupts the *miR172i*–*Pbr025174.1* interaction. Therefore, these lncRNAs are involved in fruit senescence under high- or low-temperature conditions by lncRNA–microRNA–mRNA interactions.

3.6. Derivation of miR172 from Novel_88/Novel_69 target lncRNAs

In a previous study, we predicted that *Pbr025174.1* was the target of *miR172i*, *Novel_88*, and *Novel_69*, and confirmed that both *miR172i* and *Novel_88* interact with *Pbr025174.1* (Gu et al., 2020). Herein, we found that *LNC_000443*, *LNC_000444*, *LNC_000445*, and *LNC_000446* were the targets of both *Novel_88* and *Novel_69* (Fig. 3, B) and confirmed that *Novel_88* interacts with *LNC_000444* (Fig. 4, B). These four lncRNAs, *LNC_000443*, *LNC_000444*, *LNC_000445*, and *LNC_000446*, were derived from the alternative splicing events of a genome fragment (Fig. 5, A) and contained pre-*miR172i* sequences (Fig. 5, B). Notably, the *Novel_88*/*Novel_69* target sequences were included in the pre-*miR172i* sequences (Fig. 5, C).

Sequence analysis showed that the guanine (G) located at the 5′-terminal of the *miR172i* sequences was replaced by adenine (A) in both *Novel_88* and *Novel_69* sequences and the uracil (U) located at the 3′-terminal of the *miR172i* sequences was replaced by guanine (G) in the *Novel_88* sequences (Fig. 5, D). Moreover, in the expression pattern, *miR172i* was positively correlated to *Novel_69* (Pearson coefficient = 0.741 and *P*-value = 0.009) but was not correlated to *Novel_88* (Pearson coefficient = 0.235 and *P*-value = 0.487). These results suggest that *Novel_69*, but not *Novel_88*, is likely involved in the cleavage of the pre-*miR172i* hairpin to generate mature *miR172i*.

4. Discussion

4.1. LncRNAs have lower expression than mRNAs in pear fruit

LncRNAs are transcripts with low protein-coding potential and long exon sequences (> 200 bp; Liu et al., 2015; Quinn and Chang, 2016). Based on these features, 3330 lncRNAs were isolated from pear fruit. These lncRNAs derived from the intergenic region and antisense region were randomly distributed on all 17 chromosomes and 279 scaffolds. Moreover, we found that the average level of lnRNAs expressed at each stage was lower than that of mRNAs (Fig. S2, A), and identical results were detected between the lncRNAs and mRNAs responsive to fruit senescence and both high- and low-temperature conditions (Fig. S2, B–D). Previously, lncRNAs were proposed to act *in cis* at a low level of expression but *in trans* at a high level of expression (Kung et al., 2013; Yang et al., 2014b). Because over 90% of lncRNAs had a lower level than the average level in any stage, most lncRNAs in pear fruit would function *in cis* rather than *in trans*. However, the average level of 82 lncRNAs expressed in fruit under high-temperature treatment (HT-10 and HT-15) was similar to their correlated 202 mRNAs involved in fruit senescence under high-temperature conditions, while the average expression levels of these lncRNAs in the controls of the high-temperature treatment (RT-10 and RT-15) were lower than the 202 mRNAs (Fig. S2, E). The expression levels of these lncRNAs and mRNAs were at least 2.4-fold and at most 0.25-fold, respectively, in fruits under high-temperature treatment compared to the control, suggesting that high-temperature can induce a global upregulation of these lncRNAs and downregulation of the 202 mRNAs. Similarly, the average level of 24 lncRNAs expressed in fruit under low-temperature treatment (LT-10, LT-15, and LT-19) was similar to the 528 correlated mRNAs involved in fruit senescence under low-temperature conditions, while the average expression level of these lncRNAs in the control of the low-temperature treatment (RT-10, RT-15, and RT-19) was lower than that of the 522 mRNAs (Fig. S2, F). The expression level of these lncRNAs and mRNAs was at least 1.9-fold higher in fruit under low-temperature treatment than in the control, suggesting that low-temperature can induce a global upregulation of these lncRNAs and correlated mRNAs. Taken together, lncRNAs had a lower expression than mRNAs in pear fruit, and this trend at the global level was not altered by high- or low-temperature conditions.

4.2. Two channels of lncRNAs affecting mRNA expression during pear fruit senescence

In eukaryotes, lncRNA affects mRNA expression either *in cis* or *in trans* through a number of mechanisms (Liu et al., 2015; Quinn and Chang, 2016). It has been reported that lncRNA can influence the transcript of the nearest mRNA (< 10 kb; Sánchez et al., 2014; Yang et al., 2016), which may result from enhancer lncRNAs (Natoli and Andrau, 2012; Lam et al., 2014), a lncRNA-directed RNA–DNA triplex (Sun et al., 2013b; Wahba and Koshland, 2013), the RdDM pathway (Kim and Sung, 2012; Wierzbicki, 2012), or polydimerzation, phosphorylation, or chromatin modification of upstream (Shamovsky et al., 2006; Csorba et al., 2014; Wang et al., 2014a). In this study, we found that 134 lncRNAs correlated to all 202 mRNAs involved in fruit senescence under high-temperature conditions, and 48 lncRNAs correlated to 528 mRNAs involved in fruit senescence under LT conditions. However, these lncRNAs were far from positively correlated mRNAs, indicating that *cis*- or *trans*-regulation was hardly occurred during fruit senescence under high- or low-temperature conditions.

Moreover, lncRNA can produce 19- to 24-nucleotide small RNAs, thereby being involved in post-transcriptional gene silencing and transcriptional gene silencing (Liu et al., 2012; Bologna and Voinnet, 2014; Kim et al., 2014). In a previous study, a total of 25 microRNAs (24-nucleotide) were involved in fruit senescence under high- or low-temperature conditions (Gu et al., 2020). Of these 27 microRNAs, only miR172 derived from lncRNAs (*LNC_000443*, *LNC_000444*, *LNC_000445*, and/or *LNC_000446*). Although *miR172i* is neither responsive to low-temperature nor fruit senescence, its target, *Pbr025174.1*, is involved in fruit senescence under low-temperature conditions (Gu et al., 2020). Changes in the expression level of *miR172i* may be associated with the process of fruit senescence under low-temperature

conditions. Therefore, *LNC_000443*, *LNC_000444*, *LNC_000445*, and/or *LNC_000446* may be involved in fruit senescence under low-temperature conditions by producing *miR172i*.

In plants, lncRNAs can also act as ceRNAs to bind microRNA, thereby influencing the expression of mRNAs that share the same or similar microRNA response elements (Franco-Zorrilla et al., 2007; Zhang et al., 2018; Jiang et al., 2019). Herein, 33 lncRNAs were predicted as targets of 16 microRNAs involved in fruit senescence under high- or low-temperature conditions. The dual-luciferase assay confirmed the prediction of lncRNA–microRNA interactions, and these interactions relieved the action of microRNAs on repressed expression of their target mRNAs. These results indicated that these 33 lncRNAs are involved in fruit senescence under high- or low-temperature conditions through lncRNA–microRNA–mRNA interactions.

4.3. *LncRNA–microRNA–mRNA networks regulate fruit senescence under high- or low-temperature conditions*

Senescence has been widely studied in leaves and fruit. *WRKY54*, *WRKY70*, and *VND-INTERACTING2* (a NAM/ATAF1/2/CUC2 transcription factor) are the negative regulators of leaf senescence in *Arabidopsis thaliana* (Yang et al., 2011; Besseau et al., 2012), as well as *OsTZF1* (a CCCH-tandem zinc finger protein) in rice (Jan et al., 2013). In annual tomato, silencing *ACS2* (an ethylene biosynthesis gene) delays fruit ripening and senescence (Xie et al., 2006), and overexpressing *FYFL* (a *MADS-box* gene) delays leaf senescence and fruit ripening (Xie et al., 2014). In perennial fruit trees, fruit senescence is negatively correlated to the expression patterns of energy-related genes in litchi (Wang et al., 2013) and may be mediated by histone deacetylase (*HD2*) and ethylene responsive factor (*ERF1*) in longan (Kuang et al., 2012). In pear, transcriptome analysis showed that 951 genes correlate to fruit senescence, and of these, 77 genes are involved in ethylene-induced fruit senescence (Xu et al., 2019). Subsequently, we found that 202 and 538 mRNAs are involved in fruit senescence under high- and low-temperature conditions, respectively, and 24 of these mRNAs are the targets of 25 microRNAs (Gu et al., 2020). However, the expression pattern of these microRNAs hardly correlated with their target mRNAs, which may result from multiple targets for one microRNA. These targets should comprise mRNAs and ceRNAs.

LncRNA is one type of ceRNA that disrupts miRNA–mRNA interactions (Huang et al., 2016; Li et al., 2017). In this study, 33 lncRNAs were predicted as ceRNAs involved in fruit senescence under high- or low-temperature conditions. Of these lncRNAs, one and two were responsive to high and low-temperature conditions, respectively. As seen in Fig. 6, during fruit senescence under high-temperature conditions, the expression of *Pbr031098.1* is influenced by *LNC_000862–miR390a* interaction. During fruit senescence under low-temperature conditions, the expressions of *Pbr025174.1* and *Pbr018118.1* were influenced by *LNC_000249–miR172* and *LNC_002622–Novel_173* interactions, respectively. These results suggest that both high- and low-temperature affect fruit senescence by lncRNA–microRNA–mRNA networks. Interestingly, *Pbr031098.1*, *Pbr025174.1*, and *Pbr018118.1* were associated with the accumulation of anti-senescent compounds, suggesting that *LNC_000862*, *LNC_000249*, and *LNC_002622* are involved in temperature-affected fruit senescence by influencing the accumulation of anti-senescent compounds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.hpj.2022.08.004.

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