

Genome-wide identification of long non-coding RNA targets of the tomato MADS box transcription factor RIN and function analysis

Tongtong Yu¹, David T. W. Tzeng², Ran Li¹, Jianye Chen³, Silin Zhong², Daqi Fu¹,
Benzhong Zhu¹, Yunbo Luo^{1,*} and Hongliang Zhu^{1,*}

¹College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China, ²EG12 Science Centre School of Life Sciences, Chinese University of Hong Kong, Hong Kong 999077, China, and ³State Key Laboratory for Conservation and Utilization of Subtropical Agro-bio resources/Guangdong Key Laboratory for Postharvest Science, College of Horticultural Science, South China Agricultural University, Guangzhou 510462, China

* For correspondence: lyb@cau.edu.cn and hlzhu@cau.edu.cn

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- **Background and Aims** In recent years, increasing numbers of long non-coding RNAs (lncRNAs) have been identified in humans, animals and plants, and several of them have been shown to play important roles in diverse biological processes. However, little work has been performed on the regulation mechanism of lncRNA biogenesis and expression, especially in plants. Compared with studies of tomato MADS-box transcription factor RIPENING INHIBITOR (RIN) target coding genes, there are few reports on its relationship to non-coding RNAs. The aim of the present study was to identify and explore the specific role of RIN target lncRNAs in tomato fruit development and ripening.
- **Methods** lncRNA targets of RIN were identified by chromatin immunoprecipitation sequencing (ChIP-seq) combined with RNA deep sequencing analysis. Six selected lncRNA targets were validated by quantitative real-time PCR, ChIP and electrophoretic mobility shift assays, and we further confirmed differential expression between wild-type and ripening-deficient mutant fruit, and RIN direct binding in the promoter regions. By means of virus-induced gene silencing (VIGS) assays and a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) genome editing strategy, the ripening-related function of a specific target lncRNA (lncRNA2155) was studied.
- **Key Results** We identified 187 lncRNAs as direct RIN targets, which exhibited RIN binding sites in their promoters and showed different expression between the wild-type and *rin* mutant. Six target lncRNAs were shown to bind with RIN directly in their promoters *in vivo* and *in vitro*. Moreover, using CRISPR/Cas9 technology to knock out the locus of the target lncRNA2155 indicated that it delayed fruit ripening in tomato.
- **Conclusions** Collectively, these findings provide new insight into RIN in the transcriptional regulation of lncRNAs and suggest that lncRNAs will contribute to a better understanding of the RIN regulatory network that controls fruit ripening.

Key words: MADS box transcription factor RIN, long non-coding RNAs, chromatin immunoprecipitation, electrophoretic mobility shift assay, fruit ripening, *Solanum lycopersicum*.

INTRODUCTION

Fruits have important nutritious and health roles in the human diet. Several characteristics of the flesh, including colour, flavour, aroma, texture and nutrition, have been studied for their dramatic changes during growth (Osorio *et al.*, 2011). As a tightly coordinated and regulated set of physiological and biochemical processes, the molecular mechanisms of fruit ripening are worthy of study (Giovannoni, 2004). Several well-characterized tomato mutations with impressive effects on ripening can initiate transcription regulation of fruit ripening (Giovannoni, 2007), such as *ripening inhibitor (rin)* (Vrebalov *et al.*, 2002), *nonripening (nor)* (Ng and Tigchelaar, 1977), *colorless ripening (Cnr)* (Manning *et al.*, 2006), *greenripe (Gr)* (Barry and Giovannoni, 2006) and *never ripe (Nr)* (Lanahan *et al.*, 1994). Similarly, transcription factors (TFs) have been identified as major contributory factors for understanding fruit

development and ripening, including TOMATO AGAMOUS-LIKE1 (TAGL1) (Itkin *et al.*, 2009), two FRUITFULL homologues (TDR4/FUL1 and MBP7/FUL2) (Bemer *et al.*, 2012), ETHYLENE RESPONSE FACTOR 6 (ERF6) (Lee *et al.*, 2012) and AP2a (Chung *et al.*, 2010). The MADS-box TF RIPENING INHIBITOR (RIN) has a key role in regulating tomato fruit ripening and has been widely studied in tomato. The *rin* mutant, carrying a mutation in RIN, inhibits numerous ripening-related phenotypes, including loss of the respiratory climacteric and associated ethylene evolution, severely affecting carotenoid accumulation, softening and production of flavour compounds (Vrebalov *et al.*, 2002). Recent work has begun to suggest that *rin* is not a null mutation, but rather it is a gain-of-function mutation that produces a protein that actively represses ripening (Ito *et al.*, 2017). RIN mainly binds to the C-A/T-rich-G (the consensus CARG) motifs and interacts with the promoters of

many ripening-related genes. In total, 241 direct RIN target genes have been identified by chromatin immunoprecipitation (ChIP)-chip and transcriptome analysis (Fujisawa *et al.*, 2013). Transcriptome studies of wild-type and *rin* mutant fruit showed that MADS-RIN activity contributes to the expression of a great number of ripening-related genes, most of which have functionally defined roles, such as various cell-wall-integral and carbohydrate-modifying proteins which help to build the structure of ripening fruit (Zhong *et al.*, 2013); SIUBC32 and PSMD2, which are involved in the ubiquitin-proteasome pathway, are identified as novel direct targets of RIN (Wang *et al.*, 2014). RIN binds directly to the promoter region of MIR172a, implying the potential regulation of microRNA (miRNA) accumulation by RIN (Gao *et al.*, 2015). However, most studies of RIN targets have focused on coding genes, and little is known about the role of RIN in long non-coding RNAs (lncRNAs).

lncRNAs are broadly defined as non-coding RNAs longer than 200 nucleotides in length (Jin *et al.*, 2013). Recent advances suggest that lncRNAs play critical roles in transcriptional and post-transcriptional regulation (Vanwerven *et al.*, 2012), as well as in epigenetic modification, cell differentiation and development (Zofall *et al.*, 2012). Although lncRNAs have received more attention in recent years, research has mainly concerned functional analysis (Bai *et al.*, 2015), and knowledge of the transcriptional regulation mechanism of lncRNAs remains scant.

Here, we report the genome-wide identification of lncRNA targets of RIN using ChIP sequencing (ChIP-seq) combined with transcriptome analysis. A total of 627 lncRNAs were identified to have RIN binding sites and 187 of these lncRNAs were significantly differentially expressed in wild-type and *rin* mutant fruits. Further analysis indicated that RIN binds directly to the promoter regions of several target lncRNAs *in vivo* and *in vitro*. Using a clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated knockout approach, we explored the functional role of a specific target lncRNA2155 in fruit ripening. Overall, our results shed new light on the transcriptional regulation of lncRNAs, and help to better enrich the network of fruit ripening.

MATERIAL AND METHODS

Plant materials and growth conditions

Wild-type tomato (*Solanum lycopersicum* ‘Ailsa Craig’) and *rin* mutant (‘Ailsa Craig’ background) were grown in a glasshouse under standard conditions (16 h under light at 26 °C, 8 h in the dark at 20 °C), with regular addition of fertilizer and lighting. To collect fruits, they were tagged at anthesis, and harvested at the immature green (IM), mature green (MG), breaker (BR), BR+3, pink (PK) and red-ripe (RR) stages based on days post-anthesis (dpa), respectively. Immediately upon harvest, the pericarp was manually dissected, frozen in liquid nitrogen, and stored at –80 °C until use. Seeds of 35S-driven overexpression of RIN in tomato were kindly provided by Prof. Guozheng Qin (Institute of Botany, CAS, Beijing, China). Wild-type MicroTom (*S. lycopersicum* ‘Micro Tom’) were also planted for virus-induced gene silencing (VIGS) and CRISPR/Cas9 transgenic lines.

ChIP-seq data analysis

Sequencing reads were mapped to the tomato genome available at the Genome Project (Tomato Gene Consortium, 2012) using Bowtie2 (http://solgenomics.net/organism/Solanum_lycopersicum/genome) (Langmead *et al.*, 2009). The raw sequencing data were deposited in the National Center for Biotechnology Information Sequence Read Archive, which are publicly available (<http://www.ncbi.nlm.nih.gov/sra/>) under accession number SRA053345. To estimate the number of uniquely mapped reads, we used SAMtools (Sequence Alignment Map format) for mapping score as a filter (samtools view –bq 1). For peak calling, data were analysed using the program MACS2 (Zhang *et al.*, 2008), and 32 243 peaks were obtained. MACS2 provided the false-discovery rate for each peak, and we used 0.01 as the cut-off threshold. Using these criteria, we obtained 23 594 significantly enriched and overlapping peaks from two RIN ChIP-seq replicates. RIN binding motifs were then predicted by using MEME-ChIP. We extracted the sequences 250 bp upstream and downstream of the peak summits for MEME-ChIP enrichment analysis, which used the programs MEME and DREME to identify sequence motifs (Machanick and Bailey, 2011).

RNA-seq analysis

Clean reads were obtained by RNA-seq and deposited in the National Center for Biotechnology Information Sequence Read Archive (<http://www.ncbi.nlm.nih.gov/sra/>) under accession number SRP106775. The program FastQC (v.0.11.3) was used to check whether the quality of reads was credible for further analyses. All clean reads were then mapped to the tomato reference genomes (SGN release SL2.50; ftp://ftp.sgn.cornell.edu/tomato_genome) combined with tomato lncRNAs from our data using TopHat (v.1.4.6), and fragments were assigned to genes by the feature count and count program. Reads per kilobase per million mapped reads (RPKM) was used to express the expression value. Fold changes were calculated by using \log_2 (RPKM RIN RNAi / RPKM AC). lncRNAs were considered as differentially expressed under the threshold of \log_2 fold change > 1 , $P < 0.05$.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from fruit samples using Trizol reagent prepared in our lab (Zhu *et al.*, 2015). RNA integrity was validated by 1.5 % (v/v) agarose gel electrophoresis. Genomic DNA was removed from the extracted total RNA by DNase I (TaKaRa, Shiga, Japan). RNA concentration and purity were measured using a NAS-99 spectrophotometer (ATC Gene, NJ, USA). A 2- μ g aliquot of total RNA was reverse transcribed via cDNA synthesis using a TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (Trans, Beijing, China) with random primer. Quantitative real-time PCR (qRT-PCR) was performed with a Bio-Rad CFX96 real-time PCR detection system in standard mode using SYBR Green Supermix (Bio-Rad, CA, USA). The following PCR programme was used: 95 °C for 2 min, followed

by 39 cycles of 95 °C for 15 s and 60 °C for 30 s. Samples were normalized against the *Actin* gene (Solyc03g078400), and relative gene expression values were measured using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method. Three biological replicates were included and each independent sample was performed in triplicate. Oligonucleotide primers used are listed in [Supplementary Data Table S1](#).

Chromatin immunoprecipitation

The ChIP assay was performed as described by [Wang et al. \(2014\)](#). The pericarp of the fruit tissue was sliced and fixed with 1 % formaldehyde under vacuum, and then subjected to nuclear isolation. The chromatin was sheared to an average length of approximately 500 bp by sonication. A small aliquot of the sonicated chromatin was reversibly cross-linked, and was used as the input DNA control. The remaining chromatin sample was centrifuged and the supernatant was diluted 10-fold in ChIP dilution buffer and pre-cleared by incubation with Dynabeads (Millipore, Waltham, MA, USA) for 1 h at 4 °C. The monoclonal anti-FLAG antibody (Sigma, St Louis, MO, USA) and pre-immune serum IgG (negative control) were used. DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen, Dusseldorf, Germany). ChIP assays were repeated with three biological replicates. The immunoprecipitated DNA was analysed by qPCR using the primers detailed in [Table S1](#).

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was carried out according to the method of [Han et al. \(2016\)](#) with some modifications. The full-length RIN was amplified from tomato cDNA, fused in frame to glutathione *S*-transferase (GST) and expressed in *Escherichia coli* BM Rosetta (DE3) cells by induction with 0.5 mM IPTG (isopropyl β -D-thiogalactoside) for 4 h at 16 °C. The recombinant protein was purified with Glutathione-Sepharose 4B (GE Healthcare, NJ, USA). An EMSA was performed using the Electrophoretic Mobility Shift Assay kit from Thermo Fisher Scientific (Waltham, MA, USA). Probes containing one or more CARG boxes which were derived from the promoter regions of lncRNAs were labelled with biotin using the DNA 3' End Biotinylation kit (Thermo Fisher Scientific). The competitor was the same unlabelled DNA fragment, and the mutant control was the probe with an ANNNNNNNT sequence instead of CARG box. The probes were incubated with the fusion protein for 25 min. After cross-linking, the biotin-labelled probes on the membrane were detected by using the chemiluminescence reagents provided with the EMSA kit. The probes are listed in [Table S1](#).

Protein extraction and western blot

Protein extraction was performed according to [Ma et al. \(2016\)](#). In brief, fruit samples were placed in extraction buffer containing 10 % TCA/acetone and centrifuged at 4 °C, the supernatant was removed, and the precipitate was then mixed

with 80 % MeOH and 0.1 M ammonium acetate, and centrifuged. The supernatant was discarded and the tubes were washed with 80 % acetone, centrifuged and the supernatant was again discarded. Then phenol/SDS solution [Tris-phenol, pH 8.0; SDS buffer (30 % sucrose, 2 % SDS, 0.2 M Tris pH 8.0, 5 % β -mercaptoethanol)] was added (1: 1, v/v) to extract protein. After washing in MeOH and 80 % acetone, the protein was finally suspended in SDS buffer (0.5 M Tris pH 7.0, 1.4 % SDS) and stored at -80 °C until use. Protein concentration was determined by the Bradford method (Solarbio, Beijing, China) using bovine serum albumin as standard. For immunoblotting, proteins were separated by SDS-PAGE (12 % acrylamide gels) and electrotransferred to Immobilon-P PVDF membranes (Millipore, MMAS, USA). The membrane was blocked in 5 % skimmed milk for 2 h at room temperature. An anti-FLAG monoclonal antibody (Sigma, MO, USA) was used. Membranes were washed with 0.05 % Tween 20 in Tris-buffered saline three times and then reacted with the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP) at a dilution of 1: 10 000. An enhanced chemiluminescence kit (Absin, Beijing, China) was used for detection after incubation with the HRP-conjugated secondary antibody (B&M, Beijing, China).

Virus-induced gene silencing

VIGS assays were carried out on MicroTom fruit using tobacco rattle virus (TRV) following the method of [Fu et al. \(2005\)](#). The primers used for the lncRNA and *phytoene desaturase* (*PDS*) gene fragments were designed by the VIGS tool (<http://solgenomics.net/tools/vigs>). Specific cDNA fragments were amplified and inserted into pTRV2 vectors which were transferred to *Agrobacterium* strain GV3101, with pTRV1, pTRV2 and pTRV2 fragments respectively (*PDS* and lncRNA cDNA). After culturing the bacteria at 28 °C overnight, cultures were harvested and resuspended in infiltration buffer (10 mM MgCl₂, 200 μ M acetosyringone) to a final OD₆₀₀ of 1.0. Vectors pTRV1 and pTRV2 (empty or with insert) were mixed at a 1: 1 ratio and incubated at room temperature for 3 h. *Agrobacterium* was infiltrated into the carpodium of fruits with a 1-mL syringe. Tomato fruits infiltrated with pTRV1 and pTRV2 were used as controls. Each inoculation was carried out three times, and on each occasion six different plants were infiltrated. When the VIGS phenotype was visible, different sections of tomato fruits were collected and stored at -80 °C. Primers for this analysis are listed in [Table S1](#).

Selection of target sequences and vector construction of pYLCRISPR/Cas9

CRISPR-P (<http://cbi.hzau.edu.cn/crispr/>) was used to select three specific single guide RNAs (sgRNAs) for targeting lncRNA2155 ([Table S2](#)). Selection criteria aimed to maximize editing efficiency: GC content in target sites was > 40 % and target sequences avoided more than four consecutive T nucleotides as RNA polymerase III would recognize such regions as a termination signal. The RNA Folding program (<http://mfold.ma.albany.edu/?q=mfold/RNA-Folding-Form2.3>) was used to ensure that

no more than 5 bp were between the target and sgRNA sequences because sgRNA secondary structure greatly influenced editing efficiency. The primers used for this analysis are listed in Table S1.

DNA extraction and mutation analysis of transgenic lines

DNA was extracted from 100 mg fresh frozen leaves using a hi-DNA secure plant kit (Tiangen, Beijing, China). Primers were designed to contain the target sites and were used for PCR amplification of T₀ and T₁ transgenic lines. PCR products were directly sequenced or cloned into the pEasy-T1 (TransGen Biotech, China) vector and mutations were then identified. The sequences of the mutants were analysed by DSDecode (<http://dsdecode.scgene.com/>). Primers for this analysis are listed in Table S1.

Statistical analysis

GraphPad Prism 6.0 was used for all statistical analyses. Statistical significance was calculated with Student's *t* test, and *P* values less than 0.05 were considered significantly different, while *P* values less than 0.01 were considered highly significant. For three or more data sets, statistical significance was calculated by Duncan's test. Statistically significant differences (*P* < 0.05) are indicated by different lowercase letters in figures.

RESULTS

Mapping of binding sites of RIN in the promoter regions of lncRNAs

The whole genome of binding sites of RIN in tomato lncRNA promoter regions were detected by using the ChIP-seq approach (Zhong *et al.*, 2013). ChIP-seq of transcripts from 'Ailsa Craig' (AC) fruits was performed in two biological replicates. A total of 32 243 peaks were obtained (Fig. 1A). To identify immuno-enriched regions, we made use of the MACS2 software program (Zhang *et al.*, 2008). MACS2 generated a list of 23 594 enriched peaks from high-throughput ChIP-seq data (Fig. 1A). We extracted the sequence of 250 bp up- and downstream of the peak summits to perform MEME-ChIP enrichment analysis, which used the programs MEME and DREME to identify sequence motifs. The ChIP-seq expression score (ES) of the ChIPed DNA relative to the input DNA, defined as signal enrichment for the region, localized to the upstream regulatory region of the corresponding lncRNAs, was used to discard RIN binding sites with very low enrichment using a threshold ES > 3. We defined the promoter region as 2 kb upstream of the lncRNA start site. Using these criteria, we identified 928 of the 23 594 enriched peaks which localized to a transcriptional regulatory region. The 928 binding sites were mapped to 627 lncRNAs. Of these lncRNAs, 362 had one binding site in the promoter region, 232 had two, 30 had three and three had four (Fig. 1B). Overall, on the basis of the presence of information on the genomic positions of the RIN binding sites, we identified 627 candidate target lncRNAs that carried one or more RIN binding sites in the transcriptional regulatory region (Table S3).

ChIP-seq combined with transcriptome analysis to identify target lncRNAs regulated by RIN

To further identify target lncRNAs regulated by RIN directly, we screened the candidate target lncRNAs together with lncRNA expression profiling (Zhu *et al.*, 2015), using the threshold \log_2 fold change > 1, *q* < 0.05. Of the 627 potential target lncRNAs, 75 were included in the set of down-regulated lncRNAs in the *rin* mutant compared with AC (breaker stage of fruits). Similarly, 112 lncRNAs were up-regulated (Fig. 1A, C; Table S4). The remaining 112 down-regulated and 378 up-regulated lncRNAs were not selected as direct RIN targets which suggested that they were not directly regulated by RIN at the transcriptional level. Six lncRNAs were chosen based on their high ChIP-seq enrichment score for further analysis, and qRT-PCR validation of these selected target lncRNAs indicated that four were down-regulated and two were up-regulated in the *rin* mutant compared with AC (WT) (Fig. 2). The *rin* mutation caused fusion of adjacent truncated RIN and MC genes (RIN-MC), and the functions of the RIN-MC fusion gene in the *rin* mutant are largely unknown (Zhong *et al.*, 2013). To identify the functions of RIN, we reanalysed ChIP-seq data and RNA-seq data of the *rin* knockout mutant (AC 35S::*RIN* RNAi in AC background) compared with AC (breaker+5 stage) (Li *et al.*, 2018). In total, 56 positively and 78 negatively regulated target lncRNAs were identified in comparison with the wild-type (AC) with AC 35S::*RIN* RNAi (Fig. S1, Table S5). For our selected six lncRNAs, four (lncRNA2155, lncRNA1780, lncRNA2250, lncRNA609) were in the overlap group of RIN positively regulated targets (Fig. S1). The other two (lncRNA443, lncRNA3197) had very low expression at the Breaker+5 stage, so they could not be detected by RNA-seq of AC and AC 35S::*RIN* RNAi.

To better detect RIN target lncRNAs, the overlaps between RIN binding sites (ChIP peaks) and differential expression of transcriptome analysis in the promoter regions of lncRNA2155, lncRNA1780 and lncRNA3197 were manually visualized using the Integrative Genomics Viewer (IGV) genome browser (Fig. 3). The reliability of these RNA-seq analyses was verified by differential expression of *RIN* in AC and AC 35S::*RIN* RNAi, *rin* and *rin* 35S::*RIN-MC* RNA fruits (Fig. S2).

RIN binds to the promoters of six target lncRNAs in vivo

To test the hypothesis that RIN could regulate the six target lncRNAs as shown previously, we performed ChIP *in vivo* and investigated if the promoter region (2000 bp upstream region starting from the lncRNA start site) contains the C-A/T-rich-G (CARG box) motif, which is known as the MADS box protein target (Ito *et al.*, 2008). The qPCR amplified region was confirmed by the FUZZNUC program with one or more CARG boxes (Table S6). Chromatin from 35S-driven RIN-overexpressing tomato fruit picked 3 d after the breaker stage was immunoprecipitated with anti-FLAG monoclonal antibody and pre-immune mouse IgG was set as the negative control (Fig. S3). The promoter region of *E8*, a known RIN binding sequence (Ito *et al.*, 2008), was tested with RIN enrichment by ChIP-qPCR as a positive control and *Actin* was used as negative control, which supported the reliability of the detection and

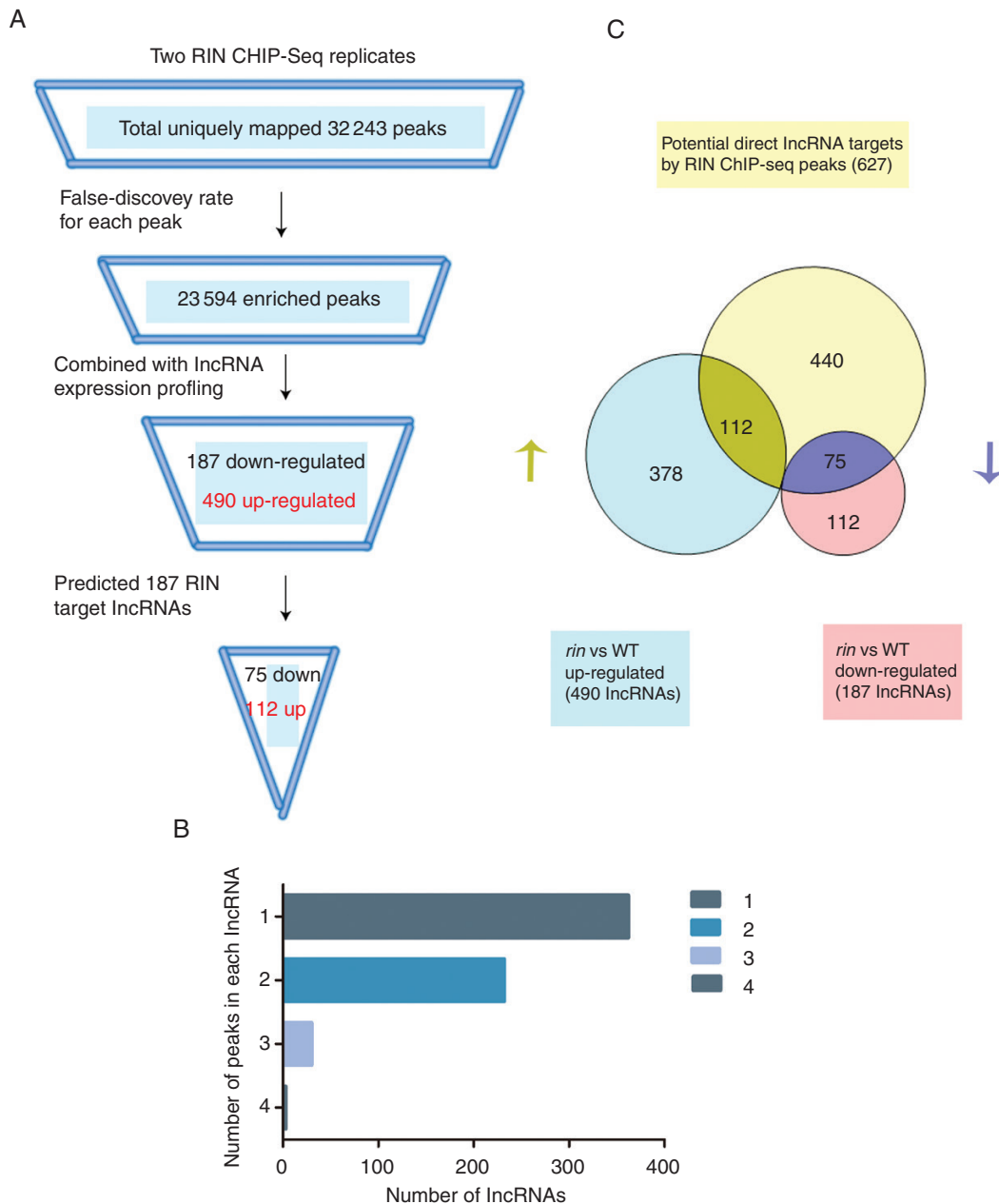


FIG. 1. Genome-wide identification of RIN target lncRNAs. (A) Schematic of identification target lncRNAs by RIN. The sequencing reads were mapped to the tomato genome and estimated 32 243 uniquely mapped reads. Then 23 594 significantly enriched and overlapping peaks were identified based on a 0.01 cut off threshold criterion. Combining these peaks with lncRNAs significant up- and down-regulated in ripening mutant tomato, RIN target lncRNAs were identified (112 up-regulated and 75 down-regulated). (B) The number of ChIP-seq peaks detected in each target lncRNA. (C) Venn diagram of potential direct RIN target lncRNAs selected based on ChIP-seq peaks and lncRNAs positively and negatively regulated by RIN. The overlapping area indicates the number of lncRNAs that are direct targets by RIN.

suggested that the ChIP-qPCR data warranted further study (Fig. S4). We detected RIN binding to the following six targets, lncRNA2155, lncRNA1780, lncRNA3197, lncRNA2250, lncRNA609 and lncRNA443 (Fig. 4). Intriguingly, the relative enrichment of these lncRNAs by the anti-FLAG antibody compared with IgG was quite different. The relative enrichment of lncRNA2250, lncRNA609 and lncRNA443 was low, while that of lncRNA3197, lncRNA1780 and lncRNA2155 was much

higher. The relative enrichment determined by ChIP qPCR was consistent with the ChIP-seq enrichment score. These data suggested that RIN exhibited differential binding ability to the promoter fragments of these lncRNAs and suggested that the ChIP-seq data were accurate. Taken together, these data clearly demonstrated that RIN binds to the promoter regions of the six target lncRNAs *in vivo*, which suggested a direct regulation of lncRNAs in tomato fruit by RIN.

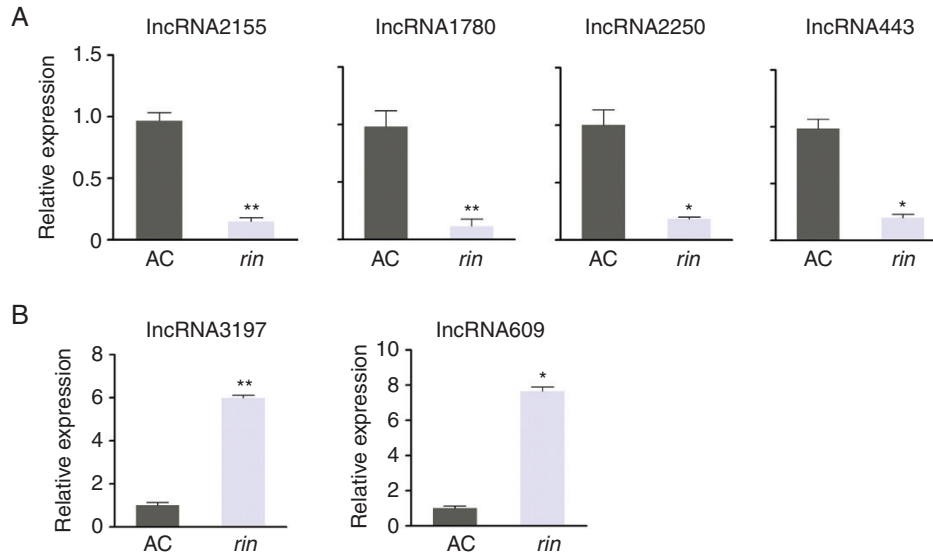


FIG. 2. qRT-PCR validation of six target lncRNAs. lncRNAs down-regulated (A) and up-regulated (B) in *rin* according to RNA-seq data were quantified. Actin expression values were used as the internal reference. The relative level of lncRNA transcripts was normalized to that in 'Ailsa Craig' (AC) fruits where the amount was arbitrarily assigned a value of 1. Error bars indicate \pm s.d. of three biological replicates, each measured in triplicate. Asterisks indicate a significant difference as determined by Student's *t*-test (* $P < 0.05$; ** $P < 0.01$).

RIN binds to the promoters of six target lncRNAs *in vitro*

We further carried out EMSAs to detect if RIN could bind to the promoters of six target lncRNAs *in vitro*. Recombinant RIN protein with GST was purified (Fig. S5). Previous studies have shown that RIN can bind the CARG box of DNA sequences (Fujisawa *et al.*, 2013). The probes were designed to contain one or more CARG-box motifs in the promoter region of lncRNAs (Table S1). The results indicated that RIN can bind to the six target lncRNAs (Fig. 5). To determine if the binding was specific, unlabelled CARG-box probes (cold probes) and mutant probes were added. The binding bands weakened when the amount of cold probes was increased, suggesting that the unlabelled CARG-box probes can competitively bind with RIN. By contrast, the binding of GST-RIN to the CARG-box was not decreased when the mutant probes were added, highlighting the binding specificity of RIN to the target lncRNAs *in vitro*. (Fig. 5). In addition, the fact that RIN bound strongly to the CARG boxes of lncRNA2155, lncRNA1780 and lncRNA3197 (Fig. 5) strengthened the result of ChIP qPCR. These data collectively indicated that RIN bound to six lncRNA targets both *in vivo* and *in vitro*, and that the binding ability might be positively related to the ChIP-seq enrichment score.

Silencing of a novel ripening-related *RIN* target lncRNA

Six lncRNAs were identified as direct targets of RIN, some of which might be involved in tomato fruit ripening. To test this prediction, a VIGS method was used to silence these six target lncRNAs. VIGS of the *PDS* gene was used as a positive control. Among the target lncRNAs, we found that silencing of lncRNA2155 resulted in obvious phenotypes in tomato fruit. Compared with TRV-control fruit (Fig. 6A), a clear phenotype, orange and partly yellow fruit skin, could be detected in

TRV-PDS and TRV-lncRNA2155 fruits (Fig. 6B, C). mRNA levels were measured by qRT-PCR. The results showed that expression of PDS and lncRNA2155 were reduced by approximately 80 % and 65 %, respectively, in the yellow part of tomato fruits compared with the TRV-control fruits (Fig. 6D). The results suggested that transient silencing of lncRNA2155 could affect fruit ripening. In addition, lncRNA2155 was expressed in all major tissues during tomato growth and development (Fig. 6E), although the abundance of lncRNA2155 mRNA was relatively higher in fruits than in roots, stems and leaves. In particular, the mRNA level of lncRNA2155 was highest at the pink stage of ripening (Fig. 6E). Moreover, it has recently been reported that several lncRNAs could function as protein-coding genes. lncRNA2155 does not seem to encode a protein and *RIN* was used as a positive control (Data Fig. S6).

Generation of stable loss-of-function lncRNA2155 mutants using the CRISPR/Cas9 gene-editing system

To further investigate the function of lncRNA2155 in tomato fruit ripening, we generated transgenic mutants using CRISPR/Cas9 gene-editing technology. Three target sites were designed for lncRNA2155 (Fig. 7A; Table S2), and two CRISPR/Cas9 CR-lncRNA2155 mutant lines were detected from first-generation transgenic plants (T_0), including chimeric and heterozygous mutants. CR-lncRNA2155-1 was a chimeric mutant with a 58-bp deletion on one allele and CR-lncRNA2155-2 was a heterozygous mutant with a 8-bp deletion on one allele (Fig. S7). Compared with WT fruits, the ripening period of the two mutants was much longer. In addition, we selected two putative off-targets to verify the specific knockout of lncRNA2155 in modified T_0 plants and no mutation was found in any putative off-target site (Table S2).

To further confirm that lncRNA2155 can promote tomato fruit ripening, we identified homogenous mutants with a 58-bp

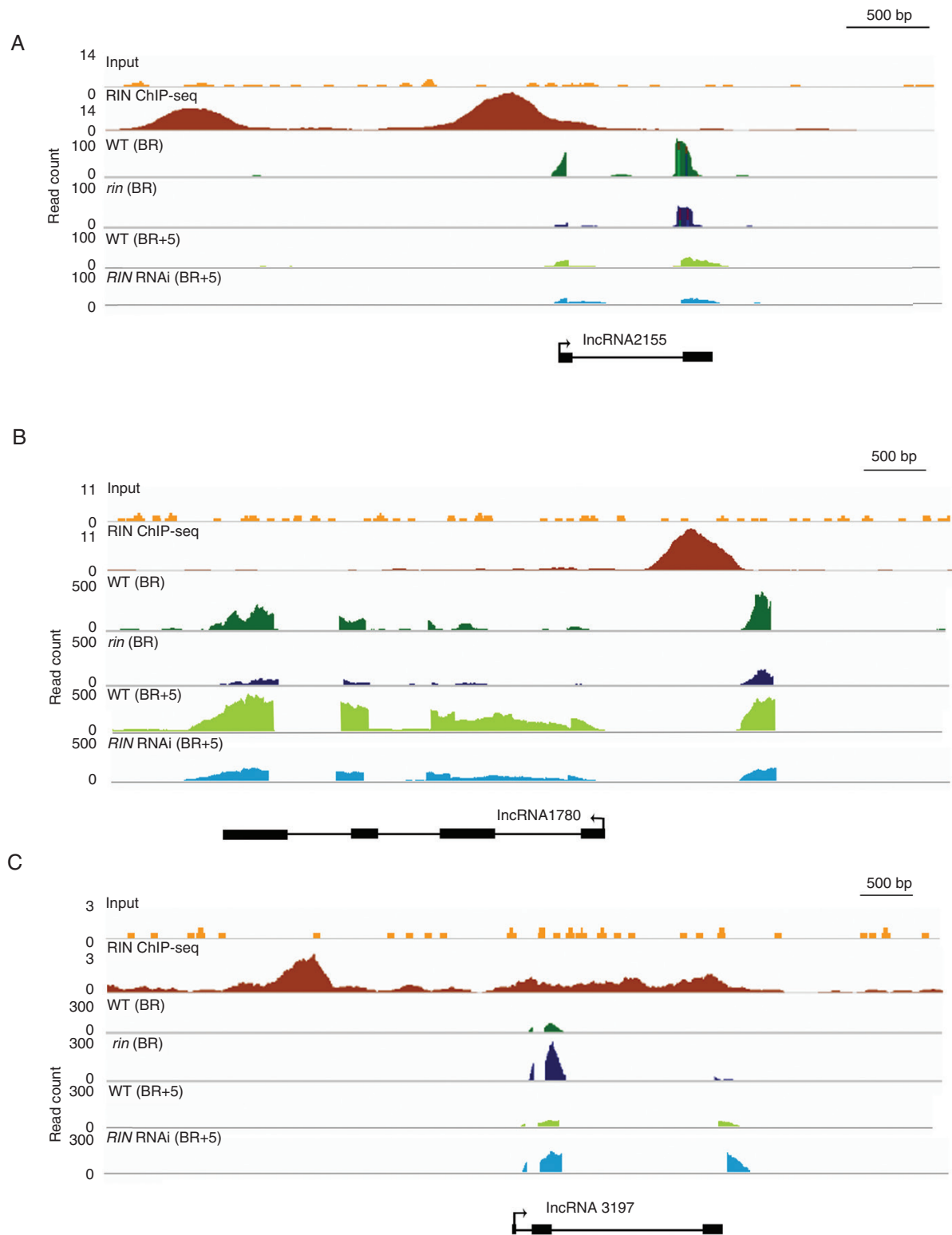


FIG. 3. Visual verification of the ChIP-seq peaks and transcriptome analysis. A histogram of three selected genomic regions of IncRNA2155 (A), IncRNA1780 (B) and IncRNA3197 (C). The three tracks represent calls for significantly enriched regions of RIN public ChIP-seq data, RNA-seq in comparison with the breaker (BR) stage of wild type and *rin* fruits, and the BR+5 stage of wild type and AC *RIN* RNAi fruits; statistically significant peaks were observed. The corresponding gene organization in exons (boxes) and introns (lines) is also shown. The arrow indicates the sense of transcription.

deletion and 65-bp deletion at both alleles of the IncRNA2155 locus in the second generation (T_2) of CR-IncRNA2155-1 and CR-IncRNA2155-2 plants (Fig. 7B, C). Fruit ripening time was

much slower in the CR-IncRNA2155 mutants than in the WT (Fig. 7D). A comparison of WT and IncRNA2155 mutant fruits at different stages of ripening revealed that fruit colour change

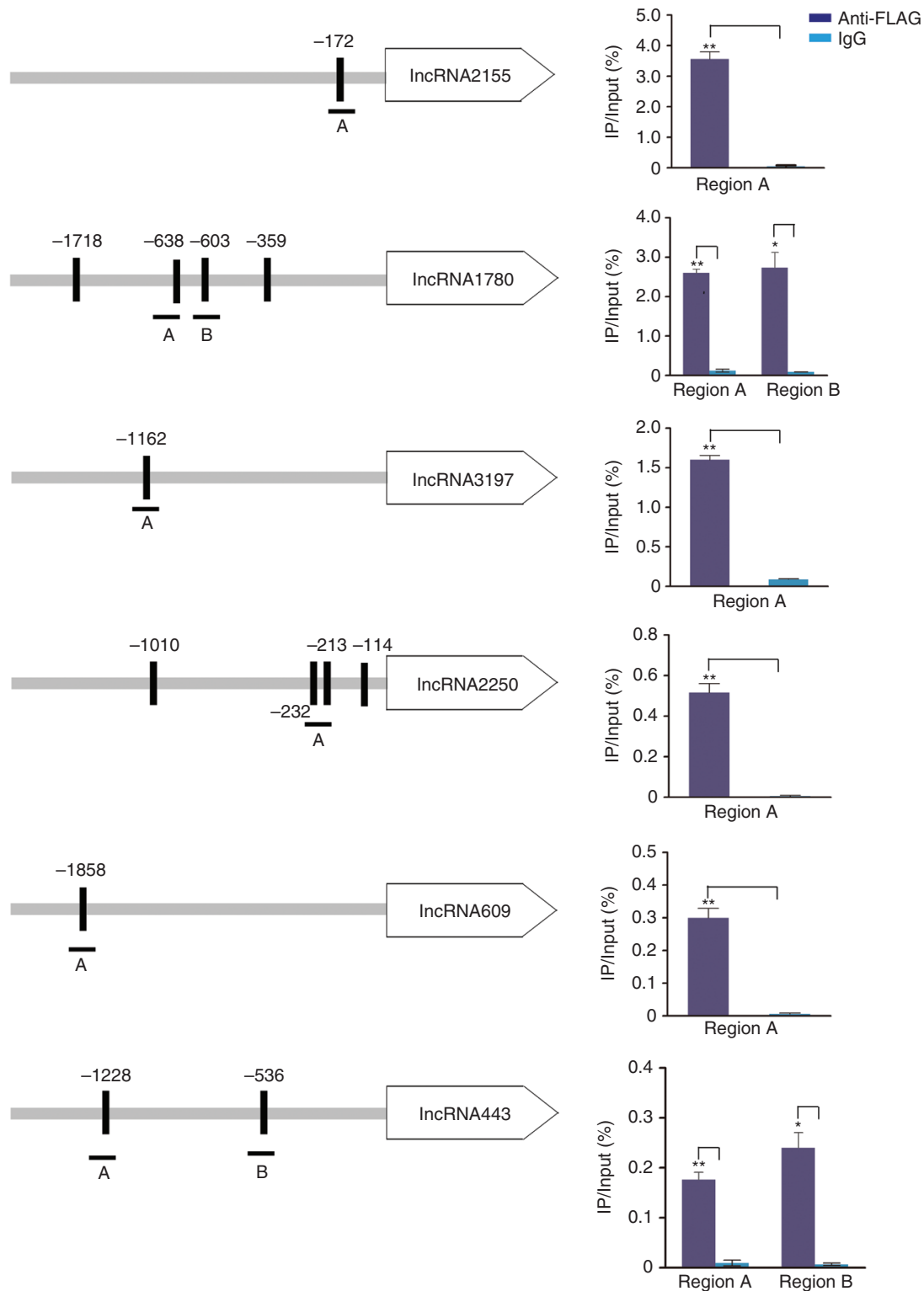


FIG. 4. RIN directly binds to the promoter regions of six lncRNAs as revealed by chromatin immunoprecipitation. ChIP qPCR shows the binding of RIN to the promoter regions of six lncRNAs. The promoter structures of the target genes are presented. Boxes represent CArG box elements and numbers indicate the position of these motifs. Fragments with upper-case letters A or B represent the regions used for ChIP qRT-PCR. Values are the percentage of DNA fragments that co-immunoprecipitated with anti-FLAG antibody (blue bars) or non-specific antibodies (pre-immune mouse IgG; cyan bars) relative to the input DNAs. * $P < 0.05$, ** $P < 0.01$, difference from IgG, by Student's t -test.

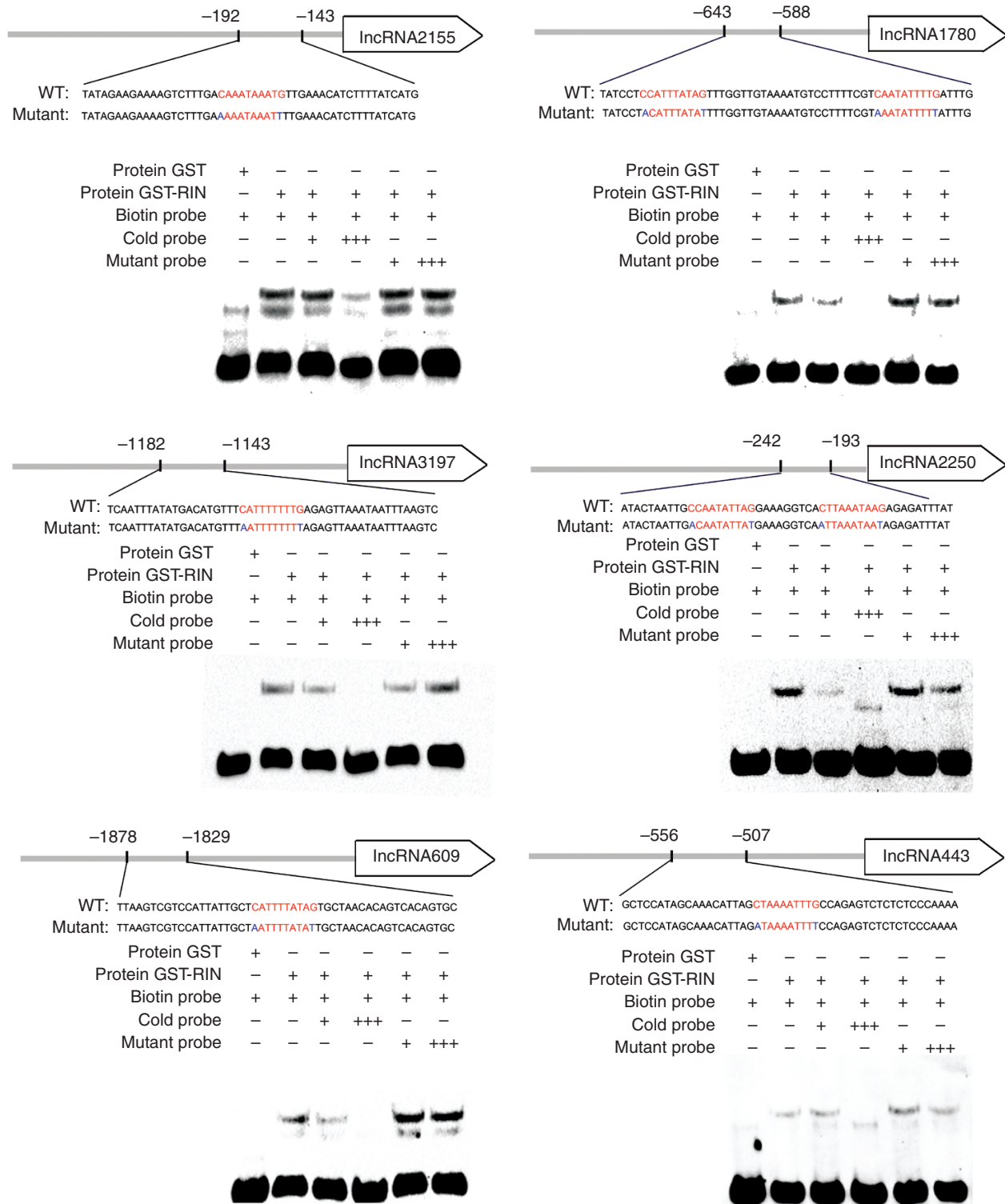


FIG. 5. Electrophoretic mobility shift assay of RIN binding to the promoter regions of six target lncRNAs. Binding of RIN to the promoters of six target lncRNAs containing a RIN binding motif (GARg-box element). The sequences of the wild-type probes containing the GARg-box were labelled with biotin. Competition for RIN binding was performed with 100x and 1000x cold probes containing the wild-type CARg-box (CNNNNNNNNG) or mutated CARg-box (ANNNNNNNNT), respectively. The symbols - and + represent absence or presence, respectively, and +++ indicates increasing amounts.

in the mutant was postponed for approx. 14 d compared to the WT fruits. To elucidate how lncRNA2155 regulated fruit ripening, we performed qRT-PCR of several key genes associated

with fruit ripening from WT and CR-lncRNA2155 fruits at 35 dpa (three biological replicates for each sample) (Fig. 7E). The down-regulated genes included *nor*, *Cnr* and *RIN* that

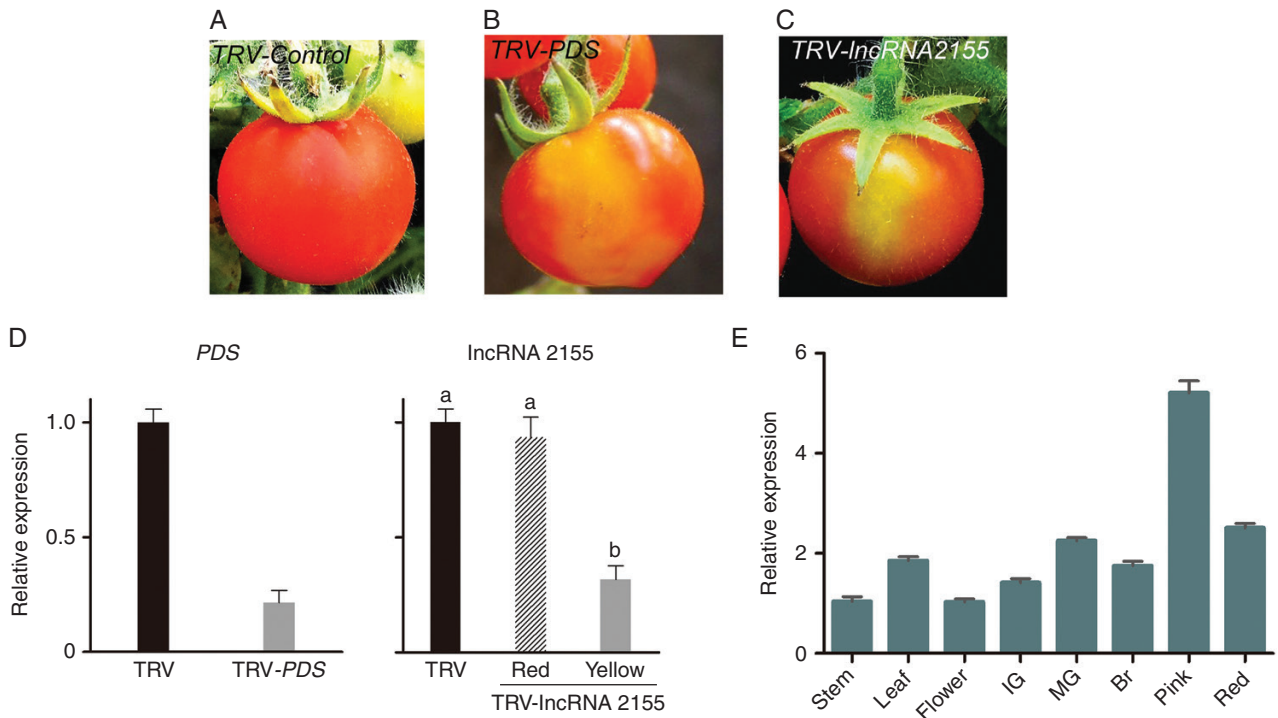


FIG. 6. Compared with TRV control tomato fruit (A), virus-induced gene silencing (VIGS) of lncRNA2155 tomato fruits infiltrated with TRV-PDS (B) and TRV-lncRNA2155 (C) revealed partial ripening. (D) qRT-PCR analysis of *PDS* transcript in TRV control and TRV-PDS tomato fruits (yellow sections). (E) lncRNA2155 expression in major organs analysed by qRT-PCR. *Actin* was the internal control. Error bars indicate \pm s.d. of three biological replicates. Different lowercase letters indicate values with significant differences.

encoded TFs, and *ACS2*, *ACS4*, *phytoene synthase (PSY1)* and *ζ-carotene desaturase (ZDS)* associated with ethylene and lycopene biosynthesis. Overall, the silencing of lncRNA2155 clearly resulted in a delayed maturity phenotype. These data demonstrated that lncRNA2155 delays fruit ripening and might play an essential role in the regulation of tomato fruit ripening.

DISCUSSION

In this study, we identified 627 lncRNAs as significantly enriched in *RIN* in a genome-wide study through ChIP-seq analysis, 187 of which were differentially expressed between wild-type and the *rin* mutant (Fig. 1A; Table S4). *RIN* was further shown to bind with the promoter regions of several target lncRNAs *in vivo* and *in vitro*. Using CRISPR/Cas9-mediated lncRNA2155 deletion in wild-type tomato fruits, we found that lncRNA2155 inactivation delayed fruit ripening by modulating the expression of ripening-related genes. Our data revealed the transcriptional regulation of lncRNAs in plants and demonstrated a unique role for lncRNA in fruit ripening, providing a new biological resource and a novel means to regulate fruit ripening.

As an important member of non-coding RNA, lncRNA can regulate gene expression at transcriptional, post-transcriptional and epigenetic levels and plays an important functional role in animals and plants (Feng et al., 2006). Although lncRNA has received increasing attention in recent years, research in plants is far behind that in humans and animals (Zhu and Wang, 2012). In recent years, the function of lncRNA in tomato has

received greater attention (Jun et al., 2015). Several lncRNAs act as competing endogenous target mimics (eTMs) for tomato miRNAs which participated in tomato yellow leaf curl virus infection (Wang et al., 2015). lncRNA314 is predicted to be involved in tomato fruit ripening because it is highly expressed in fruits at the breaker ripening stage and is suppressed in different ripening mutants (Wang et al., 2016). lncRNA16397-GRXs is demonstrated to play a role in the response of tomato to *Phytophthora infestans* infection, which provided candidates for breeding to enhance biotic stress resistance in tomato (Cui et al., 2017). Studies also report that plant lncRNAs exert regulatory functions similar to those in humans and animals (Zhang and Chen, 2013). Consistent with this, recent study has showed that protein 53 (p53) can bind to the promoters of some large intervening non-coding RNAs (lincRNAs) and all of these lincRNAs are significantly induced in p53 cells (Guttman et al., 2009). In addition, approximately half resided in the cluster associated with p53-mediated DNA damage response, confirming the validity of the functional inference and suggesting that lincRNAs which are directly bound and regulated by TFs could show functions associated with TFs (Guttman et al., 2009).

Our results showed that six selected target lncRNAs were significantly differentially expressed in the *rin* mutant compared with AC by qRT-PCR analysis and further validated to bind with *RIN* *in vivo* and *in vitro* by ChIP-qPCR and EMSA. For our selected six lncRNAs, it is noticeable that only four (lncRNA2155, lncRNA1780, lncRNA2250, lncRNA609) are in the overlap group of *RIN* target lncRNAs by ChIP-seq and

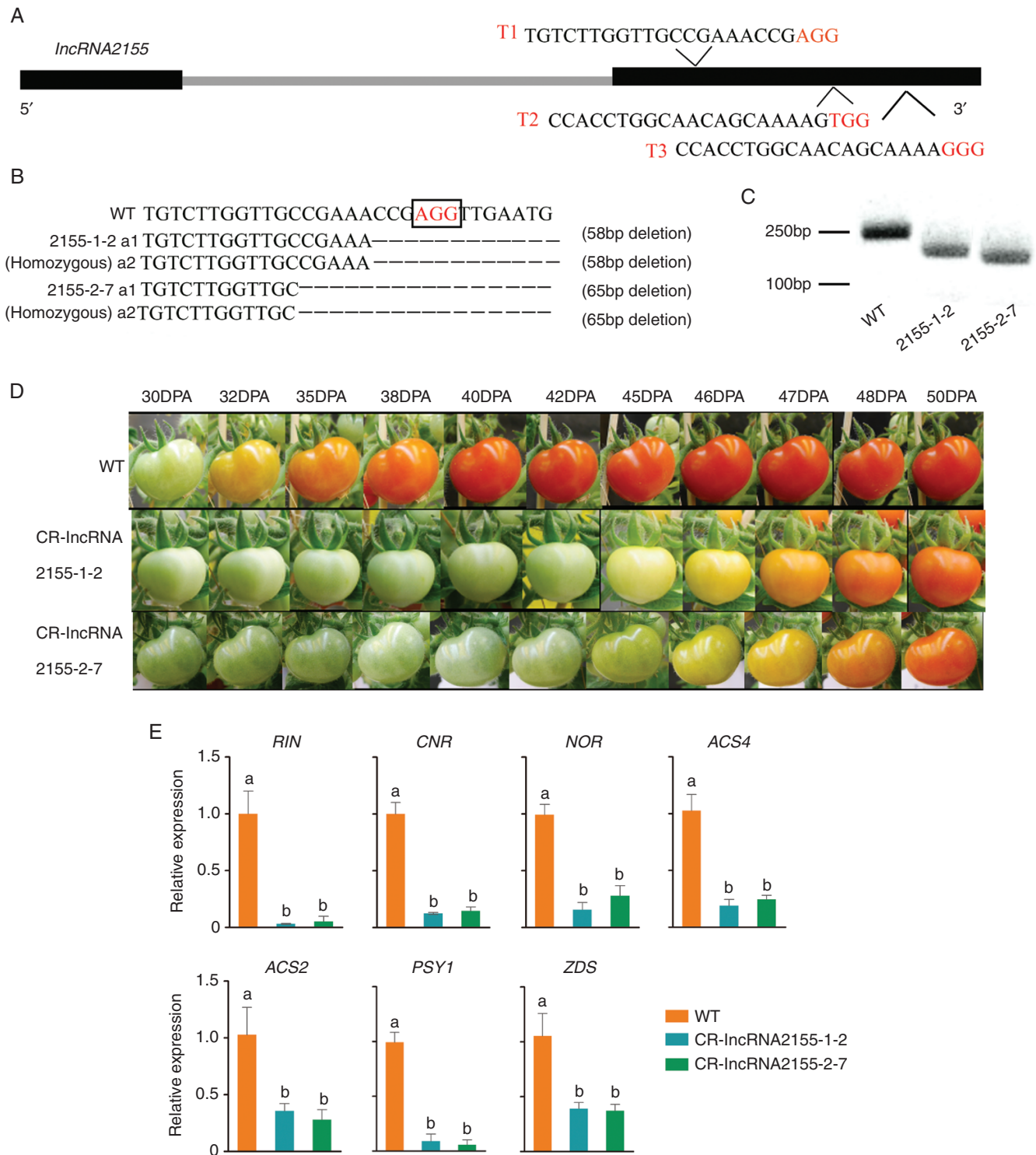


FIG. 7. Loss of *IncRNA2155* function greatly delayed tomato fruit ripening. (A) Three targets in the *IncRNA2155* genome sequence. (B) Editing type analysis of CR-*IncRNA2155-1-2* and CR-*IncRNA2155-2-7*. (C) DNA large deletions are validated in CR-*IncRNA2155* by PCR. (D) Compared with WT, the CR-*IncRNA2155* mutants show delayed fruit ripening. (E) qRT-PCR revealed different expression of ripening-related genes in CR-*IncRNA2155* and WT. Error bars indicate \pm s.d. of three biological replicates, each measured in triplicate. Different lower-case letters indicate statistically significant differences based on an ANOVA followed by Duncan's test ($P < 0.05$).

RNA-seq of AC and AC *RIN* RNAi fruits, and the other two (*IncRNA443*, *IncRNA3197*) could not be detected by RNA-seq of AC *35S::RIN* RNAi and AC fruits (Fig. S1). One of the

reasons for this may be that the stage of fruits used for the two RNA-seqs are BR and BR+5, respectively, and the *IncRNAs* showed decreased expression levels at the BR+5 stage relative

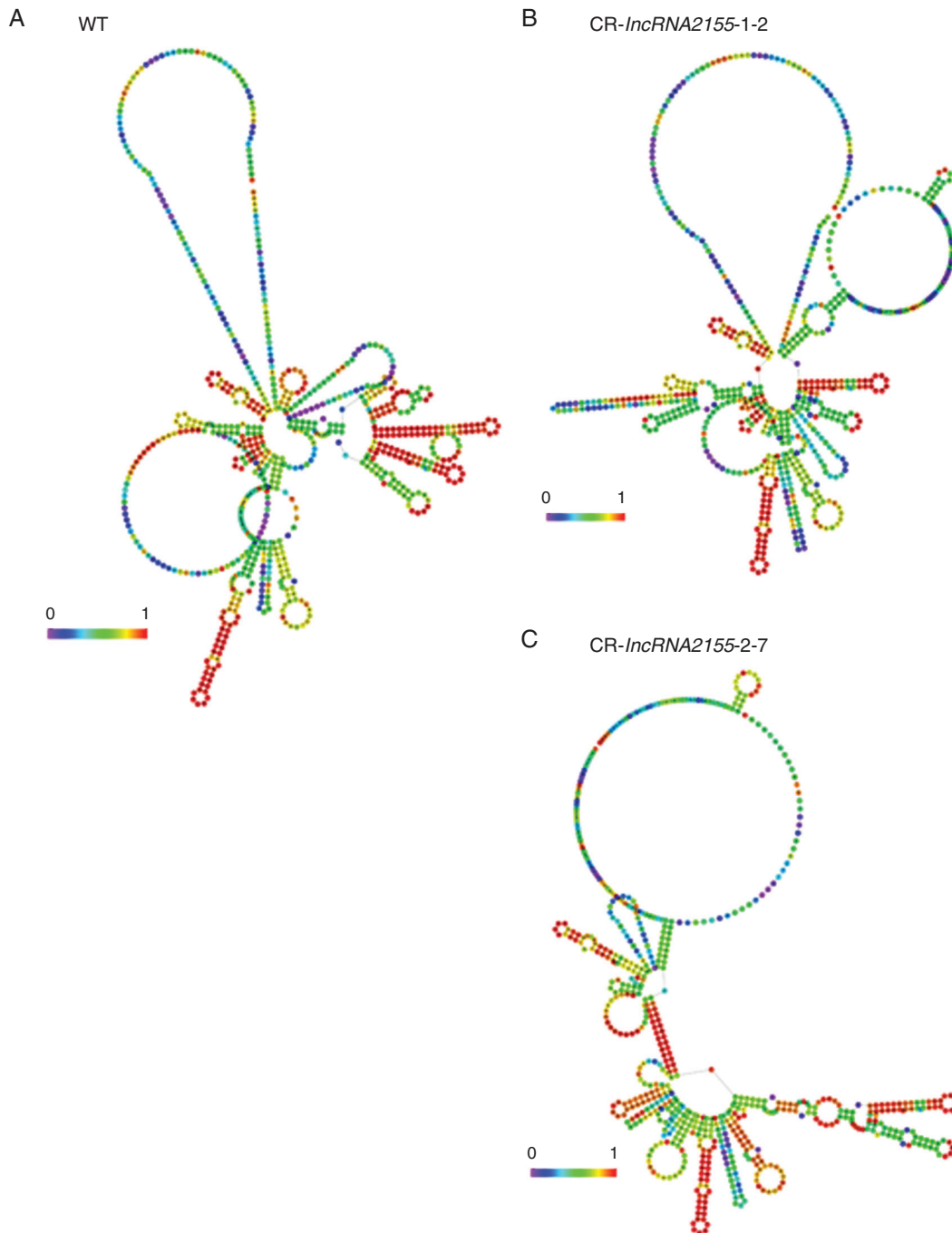


FIG. 8. Secondary structure of WT (A), CRlncRNA2155-1-2 (B) and CRlncRNA2155-2-7 (C).

to the levels at the BR stage. The result suggested that fruit stage plays an important role for RNA-seq analysis

In recent decades, the transcription factor RIN has been recognized as a key component in tomato fruit ripening (Ng and Yanofsky, 2001) and affects many physiological and biochemical processes and metabolic pathways (Vrebalov et al., 2002). RIN regulates the expression of numerous genes participating in all primary ripening pathways, such as ethylene biosynthesis, ethylene perception, cell-wall metabolism, carotenoid accumulation, lycopene accumulation, chlorophyll degradation

and other ripening-related TFs (Martel et al., 2011). RIN target genes have been identified as ripening-related characters such as phenotypes and in ethylene production (Fujisawa et al., 2011). In total, 1046 RIN binding sites were detected by ChIP-chip in which ChIPed DNA samples are hybridized with the microarray which have probes designed from the 2-kb upstream regions of all tomato predicted coding genes (Fujisawa et al., 2011, 2013). Although there has been increasingly interest in downstream targets regulated by RIN, few studies have used non-coding RNAs, especially lncRNAs. A total of 32 243 peaks

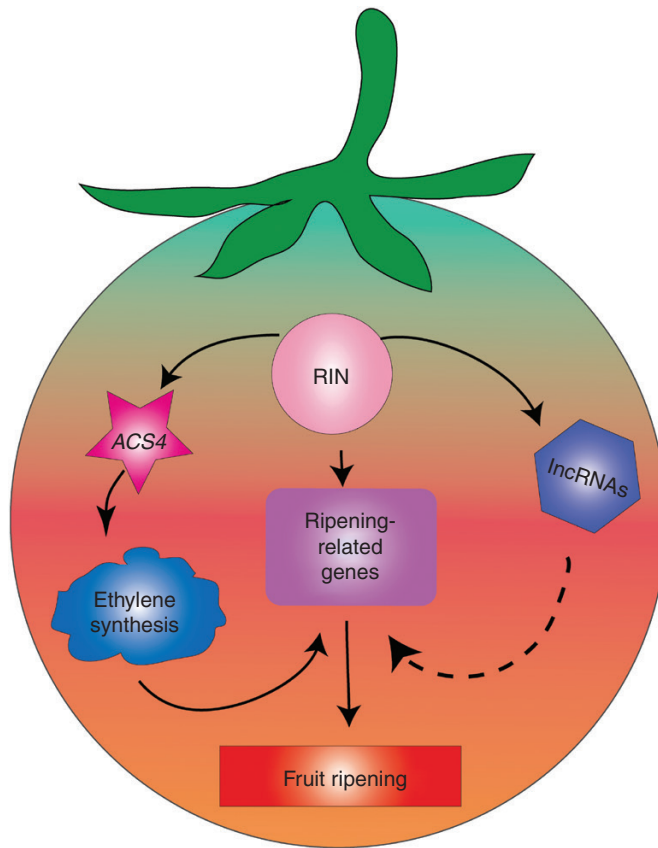


FIG. 9. Schematic representation of the proposed model for a regulatory mechanism of tomato fruit ripening. Full-line arrows indicate RIN regulates fruit ripening by ethylene via *ACS4* and ripening-related target genes. Dotted-line arrow indicates that the regulatory mechanism of lncRNAs during fruit ripening is unclear.

were obtained by ChIP-seq analysis, including promoter regions of both coding and non-coding genes in our study. Six target lncRNAs that were shown to be regulated by RIN directly were analysed for potential function in fruit ripening by VIGS assay. Of these targets, transient silencing of lncRNA2155 in tomato fruit appeared to promote a different skin colour. The yellow did not change to orange, indicating that lncRNA2155 might be involved in the regulation of tomato fruit ripening (Fig. 6). The CRISPR/Cas9 system was used to eliminate lncRNA2155 and investigate its specific role in fruit ripening. The results clearly demonstrated that loss function of lncRNA2155 can generally extend fruit shelf life. Two independent homozygous transgenic mutants (CR-lncRNA2155-1 and CR-lncRNA2155-2) were detected in the T_1 generation. Moreover, the two lncRNA2155 mutants with 58-bp and 65-bp deletions exhibited delayed fruit ripening of more than 10 d compared to the time of the colour change in WT fruits. The mutant fruits remained orange at 50 dpa (Fig. 7D). In addition, the mRNA abundance of some key fruit ripening-related genes was up-regulated or down-regulated significantly in lncRNA2155 mutant fruits in comparison to the WT, implying that RIN target lncRNAs might influence fruit ripening by regulating those ripening-related genes directly or indirectly (Fig. 7E). In addition, the secondary structure of CR-lncRNA2155-1-2 and CR-lncRNA2155-2-7 was

significantly changed compared with WT (Fig. 8). Because the secondary structure of lncRNA has an important role in function (Li et al., 2016), the dramatic changes in structure suggested that they can affect their functions. In tomato fruit, it has been established that RIN is involved in regulating fruit ripening with a series of TFs and target genes (Giovannoni et al., 2017). In the present study, we suggest a new network model of tomato fruit ripening (Fig. 9). In this model, RIN changes initial ethylene production by regulating *ACS4*, which is responsible for the ethylene-independent transcriptional regulatory pathway (Ito et al., 2017), and affects fruit ripening by regulating the expression of many coding and non-coding targets, especially lncRNAs. Given the diverse and complex functions of these regulatory networks, further investigations of how target lncRNAs influence fruit ripening would improve our understanding of the mechanism of regulatory networks of fruit ripening by lncRNAs in plants.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Table S1: Primers used in this study. Table S2: Characteristics of CR-lncRNA2155. Table S3: List of lncRNAs with RIN enrichment in promoter regions by ChIP-seq data. Table S4: List of potential direct lncRNA targets with RIN. Table S5: List of lncRNA targets expressed differentially in AC RNA RNAi and AC. Table S6: Predicted RIN binding motifs within the 2000-bp upstream region of lncRNAs analysed in this study. Fig. S1: Venn diagram of potential target lncRNAs by RIN compared with ChIP-seq and RNA-seq. Fig. S2: Visual verification of the ChIP-seq peaks and RNA-seq expression of RNA. Fig. S3: Western blot showing the specificity of the anti-FLAG monoclonal antibody used for ChIP assay. Fig. S4: Preparation and validation of ChIPed DNA samples recovered with the anti-FLAG antibodies. Fig. S5: Gel electrophoresis and western blot demonstrating antibody for EMSA. Fig. S6: Protein-coding potential in the lncRNA2155 transcript. Fig. S7: CR-lncRNA2155 mutants were generated using the CRISPR/Cas9 system in the T_0 generation of transgenic tomato plants.

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