New Phytologist

The transcriptional regulatory network mediated by banana (*Musa acuminata*) dehydration-responsive element binding (MaDREB) transcription factors in fruit ripening

Jian-Fei Kuang[1,2], Jian-Ye Chen[1], Xun-Cheng Liu[3], Yan-Chao Han[1], Yun-Yi Xiao[1], Wei Shan[1], Yang Tang[1], Ke-Qiang Wu[3], Jun-Xian He[2] and Wang-Jin Lu[1]

[1]State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources/Guangdong Provincial Key Laboratory of Postharvest Science of Fruits and Vegetables, College of Horticultural Science, South China Agricultural University, Guangzhou 510642, China; [2]School of Life Sciences and State Key Laboratory of Agrobiotechnology, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong SAR, China; [3]Key Laboratory of South China Agricultural Plant Molecular Analysis and Genetic Improvement, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China

Authors for correspondence:
Wang-Jin Lu
Tel: +86 020 8528 5523
Email: wjlu@scau.edu.cn

Jun-Xian He
Tel: +852 3943 6299
Email: jxhe@cuhk.edu.hk

Received: *29 January 2016*
Accepted: *16 November 2016*

New Phytologist (2017) **214:** 762–781
doi: 10.1111/nph.14389

Key words: banana, chromatin immunoprecipitation coupled DNA sequencing (ChIP-Seq), dehydration-responsive element binding (DREB), fruit ripening, transcriptional regulation.

Summary

- Fruit ripening is a complex, genetically programmed process involving the action of critical transcription factors (TFs). Despite the established significance of dehydration-responsive element binding (DREB) TFs in plant abiotic stress responses, the involvement of DREBs in fruit ripening is yet to be determined.
- Here, we identified four genes encoding ripening-regulated DREB TFs in banana (*Musa acuminata*), *MaDREB1*, *MaDREB2*, *MaDREB3*, and *MaDREB4*, and demonstrated that they play regulatory roles in fruit ripening.
- We showed that MaDREB1–MaDREB4 are nucleus-localized, induced by ethylene and encompass transcriptional activation activities. We performed a genome-wide chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) experiment for MaDREB2 and identified 697 genomic regions as potential targets of MaDREB2. MaDREB2 binds to hundreds of loci with diverse functions and its binding sites are distributed in the promoter regions proximal to the transcriptional start site (TSS). Most of the MaDREB2-binding targets contain the conserved (A/G)CC(G/C)AC motif and MaDREB2 appears to directly regulate the expression of a number of genes involved in fruit ripening. In combination with transcriptome profiling (RNA sequencing) data, our results indicate that MaDREB2 may serve as both transcriptional activator and repressor during banana fruit ripening.
- In conclusion, our study suggests a hierarchical regulatory model of fruit ripening in banana and that the MaDREB TFs may act as transcriptional regulators in the regulatory network.

Introduction

Fruit ripening is a complex, genetically programmed process that is characterized by dramatic changes in color, texture, flavor, aroma and nutritional components (Giovannoni, 2004). Fruits are generally characterized as belonging to two physiological groups, climacteric and nonclimacteric, based on the presence or absence of the climacteric rise in respiration and of autocatalytic ethylene production. In climacteric fruits such as banana (*Musa acuminata*) and tomato (*Solanum lycopersicum*), ethylene is essential for normal fruit ripening as blocking either the biosynthesis or perception of this phytohormone prevents ripening (Seymour *et al.*, 2013). In contrast, the ripening of nonclimacteric fruits such as strawberry (*Fragaria* spp.) and citrus (*Citrus sinensis*) fruits does not absolutely require ethylene, and the nature of the triggers for ripening in these fruits remains to be elucidated (Liu *et al.*, 2015b).

The ripening processes in both fruit types encompass several stages, such as fruit development, maturation, ripening and finally senescence, which are modulated by external and internal cues, including phytohormones and expression of developmental genes such as transcription factors (TFs) (Adams-Phillips *et al.*, 2004; Bapat *et al.*, 2010). Hundreds of TFs have been identified with differential expression during fruit development and many of them show expression changes during the ripening process (Alba *et al.*, 2005). Among these, tomato *RIPENING-INHIBITOR* (*RIN*), *NON-RIPENING* (*NOR*), and *COLORLESS NON-RIPENING* (*CNR*), which encode MCM1/AGAMOUS/DEFICIENS/SRF (MADS), NAM/ATAF1,2/CUC2 (NAC), and SQUAMOSA PROMOTER BINDING

PROTEIN (SBP) TFs, respectively, are critical for tomato ripening (Giovannoni, 2007). Mutations in these genes resulted in similar nonripe fruit phenotypes such as inhibition of most ripening-related genes, lack of climacteric ethylene production and inability to respond to exogenous ethylene (Vrebalov *et al.*, 2002; Giovannoni, 2004; Manning *et al.*, 2006). Furthermore, TFs control fruit ripening by directly regulating the expression of ripening-related genes. For example, the *Lycopersicon esculentum* homeobox 1 (LeHB1) was found to bind the promoter of the ethylene biosynthetic gene *Lycopersicon esculentum 1-aminocyclopropane-1-carboxylate oxidase 1* (*LeACO1*) in tomato to regulate fruit ripening (Lin *et al.*, 2008). The ripening regulator RIN governs fruit ripening through transcriptional regulation of genes involved in lycopene accumulation, ethylene production, chlorophyll degradation, and many other physiological processes (Fujisawa *et al.*, 2013). Other ripening-regulating TFs such as Ethylene Response Factors (ERFs) and EIN3-like (EILs) were proposed to modulate fruit ripening via regulation of cell wall-modifying genes (Yin *et al.*, 2010). In spite of this large number of TFs identified, only a handful of them have been characterized as being involved in a transcriptional regulatory mechanism associated with fruit ripening to date.

As a single TF may control the expression of many target genes through specific binding to the conserved *cis*-acting elements in the promoters of the respective target genes, the genome-wide identification of *in vivo* TF targets is critical for a full understanding of transcriptional regulation (Nakashima *et al.*, 2009). Chromatin immunoprecipitation coupled with high-throughput DNA sequencing (ChIP-Seq) or microarray (ChIP-chip) can provide high-resolution spatial and temporal information about the interactions between TFs and DNA on a genome-wide scale (Gilchrist *et al.*, 2009; Heyndrickx *et al.*, 2014). In plants, by using ChIP-Seq or ChIP-chip technologies, the complex transcriptional regulatory networks mediated by several ripening-associated TFs have recently been identified, including tomato RIN (Fujisawa *et al.*, 2013), FRUITFULL homologs FUL1 and FUL2 (Fujisawa *et al.*, 2014). However, no such transcriptional regulatory mechanisms have been revealed for TFs associated with fruit ripening in banana.

Dehydration-responsive element binding (DREB) proteins belong to the plant-specific APETALA2/ethylene-responsive element binding factor (AP2/ERF) family of TFs, which are characterized by the conserved AP2/ERF DNA-binding domain consisting of 60–70 amino acid residues (Yamaguchi-Shinozaki & Shinozaki, 1994). The first group of DREB proteins were identified by using yeast one-hybrid screens to screen for the *trans*-factors of the dehydration-responsive element (DRE) present in a set of drought- and cold-inducible gene promoters (Yamaguchi-Shinozaki & Shinozaki, 1994; Stockinger *et al.*, 1997). Further studies indicated that these DREB TFs are indeed implicated in plant responses to cold and drought tolerance (Agarwal *et al.*, 2006; Thomashow, 2010; Lata & Prasad, 2011). In *Arabidopsis thaliana*, *c.* 12−20% of cold-induced genes are modulated by three members of the DREB subfamily, C-repeat Binding Factor 1 (AtCBF1), AtCBF2 and AtCBF3 (Fowler & Thomashow, 2002; Van Buskirk & Thomashow, 2006). In addition, DREB proteins have been reported to function in some developmental processes. For example, in melon (*Cucumis melo*), CMe-DREB1 was found to activate the transcription of *Cucumis melo 1-aminocyclopropane-1-carboxylatesynthase 2* (*CMe-ACS2*) through binding to the GCCGAC sequence (Mizuno *et al.*, 2006), implying that DREB-like TFs control ethylene action by regulating ethylene biosynthesis. Furthermore, a recent study has reported that transient expression of *EgCBF3* from oil palm (*Elaeis guineensis*) in tomato fruits affects the expression of genes related to ethylene and abscisic acid biosynthesis and fruit ripening (Ebrahimi *et al.*, 2015). All these studies suggest that the DREB TFs are not only critical regulators of plant responses to stress but also important for plant development such as fruit ripening.

Banana is one of the most important fruit crops grown in the countries of the tropics and is consumed all over the world (D'Hont *et al.*, 2012). Being typical climacteric fruits, bananas have a short shelf life once they have started to ripen, as a result of rapid deterioration of peel color and pulp firmness, resulting in severe postharvest losses, ranging from *c.* 20 to 80% (Tadesse, 2014). At one time, chemical treatments were widely applied to reduce postharvest losses, but this method has been discouraged or even dispensed with in recent years because of economic, environmental and health concerns. Therefore, understanding the molecular mechanisms underlying banana ripening could offer safer and more effective approaches to ensure predictable ripening and good quality of fruit. Many genes related to banana ripening have been isolated and characterized, such as those encoding starch phosphorylase (Mota *et al.*, 2002), xyloglucan endotransglycosylase (XET) (Lu *et al.*, 2004), alcohol acyltransferase (Beekwilder *et al.*, 2004), and members of the 1-aminocyclo-propane-1-carboxylate (ACC) synthase and ACC oxidase families (Huang *et al.*, 2006; Inaba *et al.*, 2007). However, how these genes are regulated is not clear. Recently, several ripening-induced TFs, including MADS, NAC, ERF, LATERAL ORGAN BOUNDARIES DOMAIN (LBD) and BTF2-like transcription factors, synapse-associated proteins and DOS2-like proteins (BSD), have been identified in banana fruit, shedding light on their roles in controlling fruit ripening via interaction with or transcriptional regulation of genes involved in ethylene biosynthesis and signaling, or other ripening-related genes (Elitzur *et al.*, 2010; Choudhury *et al.*, 2012a; Shan *et al.*, 2012; Xiao *et al.*, 2013; Ba *et al.*, 2014a,b). Nevertheless, the regulatory network mediated by these TFs has not yet been uncovered.

In this study, we demonstrated that four DREB TFs from banana, MaDREB1–MaDREB4, are important regulators of fruit ripening in banana. MaDREB1 to MaDREB4 are nucleus-localized proteins and possess transcriptional transactivation abilities. *MaDREB1–MaDREB4* transcripts are induced by ethylene and accumulated during fruit ripening. Genome-wide ChIP-Seq in combination with RNA sequencing (RNA-Seq) analyses revealed that MaDREB2 directly binds to genes involved in fruit ripening and several other processes. The identification of the potential target genes and DNA-binding sites of MaDREB2 provides important insights into the

transcriptional regulatory mechanisms underlying fruit ripening in banana.

Materials and Methods

Plant material and treatments

Pre-climacteric banana (*Musa acuminata*) fruits of the Cavendish cultivar at 75–80% maturation (*c.* 110−120 d after anthesis) were obtained from a local commercial plantation (Xianghui Farm, Zengcheng District) near Guangzhou, China. Three postharvest treatments, namely ethylene-induced ripening (100 μl l^{-1} ethylene for 18 h), 1-methylcyclopropene (1-MCP)-delayed ripening (0.5 μl l^{-1} 1-MCP for 18 h; 1-MCP is a competitive inhibitor of ethylene action), and a combined treatment of ethylene and 1-MCP (100 μl l^{-1} ethylene followed by 0.5 μl l^{-1} 1-MCP for 18 h), and an untreated control (natural ripening), were employed to create different ripening regimes, and sampling procedures followed those described previously (Shan *et al.*, 2012). Fruit ripening was assessed in terms of ethylene production, fruit firmness and peel color according to Shan *et al.* (2012). All experiments were conducted with three biological replicates.

RNA extraction and gene cloning

Total RNA was extracted using the hot borate method described by Wan & Wilkins (1994). Total RNA extract was treated with DNaseI (Promega, Madison, WI, USA), and the resulting DNA-free RNA was used as the templates for reverse transcription PCR (RT-PCR). According to our transcriptome profiling results (Supporting Information Notes S4), nine *DREB* genes (termed *MaDREB1*−*MaDREB9*) were identified and they were then cloned by RT-PCR. The IDs of these nine genes are: *MaDREB1*, *GSMUA_Achr9G04630_001*; *MaDREB2*, *GSMUA_Achr5G28050_001*; *MaDREB3*, *GSMUA_Achr6G32780_001*; *MaDREB4*, *GSMUA_Achr11G24820_001*; *MaDREB5*, *GSMUA_Achr9G12950_001*; *MaDREB6*, *GSMUA_Achr4G19660_001*; *MaDREB7*, *GSMUA_Achr3G01650_001*; *MaDREB8*, *GSMUA_Achr3G04360_001*; *MaDREB9*, *GSMUA_Achr4G19660_001*. The primers used in this study are listed in Table S1.

RNA-Seq experiments

RNAs from unripe (0 d after ethylene treatment) and ripe (5 d after ethylene treatment) banana fruits were extracted separately from three randomly selected individual fruits (as one biological replicate). Three biological replicates were used for each type of fruit. Before the pooling of three biological intra-samples, the individual total RNA quantity and concentration were measured on a Bioanalyzer 2100 and RNA6000 Nano Lab Chip Kit (Agilent, Palo Alto, CA, USA) with an RNA integrity number (RIN) > 8.0. Total mRNA was isolated with oligo(dT) cellulose, fragmented and reverse-transcribed with random primers. Second-strand cDNA was synthesized using DNA polymerase I and RNase H. Then the cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Hilden, Germany), underwent end repair and dA-tailing, and were ligated to Illumina adapters (Illumina, San Diego, CA, USA, all rights reserved). The ligation products were size-fractioned by agarose gel electrophoresis, and fragments were excised for PCR amplification. The amplified fragments were sequenced using Illumina HiSeq™ 2000 by Gene Denovo Co. (Guangzhou, China).

For the assembly library, raw reads were filtered to remove those containing adaptors and reads with > 5% unknown nucleotides. Low-quality reads were also removed, in which the percentage of low *Q*-value (≤10) base was higher than 20%. *De novo* transcriptome assembly was performed using Trinity (released on 10 November 2013) with default parameters (Grabherr *et al.*, 2011). The DEseq software was used to identify differentially expressed genes in pair-wise comparisons (Anders & Huber, 2010), and the results of all statistical tests were corrected for multiple testing with a Benjamini–Hochberg false discovery rate (FDR) < 0.01. To annotate the unigenes, we used a standalone version of the National Center for Biotechnology Information (NCBI) Blastx program against the Arabidopsis protein database at The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org), Michigan State University (MSU) Rice genome annotation and the NCBI nonredundant protein (Nr) database (http://www.ncbi.nlm.nih.gov) and The Banana Genome Hub (http://banana-genome.cirad.fr/) using the Blastx algorithm with an *E*-value cut-off of 10^{-5} and extracting only the top hit for each sequence.

Quantitative real-time PCR (qRT-PCR) analysis

Synthesis of first-strand cDNA and all qRT-PCR analyses were performed as described previously (Chen *et al.*, 2011b). *ribosomal protein4* (*MaRPS4*) was selected as a reference gene in accordance with the findings of our previous study on the selection of reliable reference genes under different experimental conditions (Chen *et al.*, 2011b). qRT-PCR reactions were normalized with the C_t (cycle threshold) value of the reference gene. Relative expression levels of target genes were calculated with the formula $2^{-\Delta\Delta CT}$. Three independent biological replicates were used in the analysis.

Promoter activity assays

Genomic DNA was extracted from banana leaves using the DNeasy Plant Mini Kit (Qiagen). The promoters of *MaDREB1* and *MaDREB2* were isolated using the Genome Walker Kit (Clontech, Palo Alto, CA, USA) with nest PCR according to the manufacturer's instructions.

The promoter activity assays were performed both in tomato fruits and in tobacco (*Nicotiana tabacum*) BY-2 protoplasts. For the assays in tomato fruits, the *MaDREB1/2* promoter regions (~2 kb) were amplified by PCR and the PCR products were ligated into the corresponding sites of *pBI121* in place of the deleted cauliflower mosaic virus (CaMV) 35S promoter. The resultant constructs *MaDREB1/2 pro:GUS* and the control

vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Before the GUS activity assays, transformed fruits were sliced into discs and treated with or without 10 mM ethrel. Histochemical staining of GUS activity was performed following a previously described protocol (Ishida *et al.*, 2007).

For the assays in tobacco BY-2 protoplasts, the *MaDREB1/2* promoter regions were inserted into the *pUC-GFP* vector by replacing the CaMV 35S promoter to generate the *MaDREB1/2pro::GFP* constructs, and then were transfected into tobacco BY2 protoplasts as described previously (Shan *et al.*, 2012). Transfected protoplasts were subjected to 0 (control) or 0.8 mM ethrel treatment for 14 h at 23°C. The GFP signal was detected before and after ethylene treatment using a fluorescence microscope (Zeiss Axioskop 2 Plus; Zeiss, Thornwood, NY, USA).

Subcellular localization

Subcellular localization assays were conducted in both tobacco BY2 protoplasts and tobacco (*Nicotiana benthamiana*) leaves. The coding sequences of *MaDREB* genes without a stop codon were amplified by PCR and subcloned into the *pUC-GFP* vector and *pBE* binary vector, respectively, in frame with the GFP sequence, resulting in *35S::MaDREB-GFP* vectors under the control of the CaMV 35S promoter. For transient expression in tobacco BY2 protoplasts, the fusion constructs and the control GFP vector (*pUC* frame) were transformed into protoplasts from tobacco BY2 suspension cell culture according to Shan *et al.* (2012). For the localization assay in tobacco leaves, *A. tumefaciens* strain GV3101 carrying the construct of interest was infiltrated into the abaxial air space of 4- to 6-wk-old plants, using a needleless 2-ml syringe. GFP fluorescence was observed under a fluorescence microscope (Zeiss Axioskop2 Plus). All the transient expression assays were repeated at least three times.

Transactivation assay of MaDREBs in yeast and tobacco BY-2 protoplasts

For the transactivation assay in yeast, the coding regions of *MaDREB1*−*MaDREB4* were each subcloned into the *pGBKT7* vector (Clontech). The constructs were then transformed into the AH109 yeast strain using the lithium acetate method. The *pGBKT7-53*+*pGADT7-T* and *pGBKT7* plasmids were used as positive and negative controls, respectively. The transcriptional activation activities and quantification of transformants were performed based on the yeast protocols handbook (Clontech).

For the transactivation assay in tobacco BY2 protoplasts, the coding sequence of *MaDREB* genes without the stop codon was cloned into the reconstructed GAL4-DBD vector as the effector (Hao *et al.*, 2010). The double reporter vector includes a GAL4 DNA consensus binding site derived from the yeast GAL4 gene upstream of luciferase (GAL4-LUC) and an internal control Renilla (REN) driven by the 35S promoter. GAL4-LUC contains five copies of the GAL4-binding element and minimal TATA region of CaMV 35S, and these sequences are located in front of the LUC. Both the effector and reporter plasmids were co-transfected into BY-2 protoplasts. The LUC and REN luciferase activities were assayed using dual luciferase assay kits (Promega). At least six assays were included for each pair of effector and reporter.

Preparation of MaDREB2 polyclonal antibody and protein immunoblot analysis

The complete coding region of *MaDREB2* was subcloned into the *pET-28a(+)* vector and was then introduced into *Escherichia coli* BL21 (DE3) for expression. Fusion proteins were expressed in BL21 (DE3) cells with induction by 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich) for 6 h at 37°C. The His-MaDREB2 fusion proteins were purified using Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose (Qiagen, Hilden, Germany) according to the manufacturer's manual. The antibody was prepared by immunizing rabbits with the His-MaDREB2 fusion protein, and then affinity purified. The antibody titer was measured using an enzyme-linked immunosorbent assay (ELISA) according to Chen *et al.* (2011a). For protein immunoblot analysis, proteins from banana fruits after ethylene treatment were extracted with phenol and purified by ammonium acetate-methanol precipitation, and protein immunoblot analyses were performed with the purified antibodies at 1 : 1000 dilution and visualized with enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Chromatin immunoprecipitation

To cross-link the genomic DNA and proteins in fruit tissue, sliced fruit pulp from unripe (0 d after ethylene treatment) and ripe (5 d after ethylene treatment) stages were submerged in 1% formaldehyde and vacuumed for 10 min. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M and applying a vacuum for an additional 5 min. After rinsing with sterile water, fruit tissues were frozen in liquid nitrogen. Subsequently, the chromatin was isolated and sonicated as described previously (Chen *et al.*, 2014).

ChIP-Seq libraries were sequenced on the Illumina HiSeq2000 platform. Sequence reads that failed the CASAVA quality filter were eliminated. Sequence reads were mapped to the unmasked *M. acuminata* genome (http://banana-genome.cirad.fr/content/download-dh-pahang) using the SOAPaligner (v.2) program (Li *et al.*, 2009). A maximum of two mismatches and no gaps were allowed. ChIP-Seq results were visualized using the Integrative Genomics Viewer (IGV) (Robinson *et al.*, 2011). The mapped reads were applied to MACS (Zhang *et al.*, 2008) with default parameters to identify enriched regions. A cut-off fold enrichment of 20 was used to finalize the peak list. For the genomic distribution of MaDREB2-binding peaks relative to gene structure, we divided the genome into five regions: the 2-kb region upstream of the transcription start site (TSS), 5′ untranslated regions (UTRs), exons, introns, and the region from the 3′ end of the gene to 1 kb downstream of it. The frequency of binding

peaks in each of these five regions was calculated. If a peak was located within 5 kb upstream of a gene and 1 kb downstream of another gene, the peak was counted in both regions. Peaks outside these regions were not included and peaks existing within 5 kb upstream of two different genes were counted twice. To identify *in vivo* MaDREB2-binding motifs, DNA sequences of binding peaks were applied to Multiple Em for Motif Elicitation (MEME)-ChIP with a maximum motif length of 10. The motifs identified by MEME-ChIP were further analyzed by comparing the frequencies of the motifs in the binding peaks to those in the whole *M. acuminata* genome (http://banana-genome.cirad.fr/content/download-dh-pahang).

For ChIP-qPCR assays, the isolated ChIP DNA was used as a template for PCR amplification. Enrichment folds of MaDREB2-bound DNA fragments were calculated by comparing the samples with immunoglobulin G (IgG) antibody. The data are presented as mean ± SD of three biological replicates.

Gene ontology (GO) analysis

Transcripts were first searched against the Nr protein database using local BLASTX with an *E*-value cut-off of 10^{-5}. Then the BLAST2GO program (http://www.geneontology.org) was used to obtain the gene ontology (GO) annotation according to biological process (Conesa *et al.*, 2005). Enrichment analysis was performed using BiNGO 2.3 (Maere *et al.*, 2005).

Electrophoretic mobility shift assay (EMSA)

The putative DNA-binding domain of MaDREB2 (nucleotide sequence from 1 to 210 bp) was cloned into *pMAL-c2x* bacterial expression vectors (New England Biolabs, Beverly, MA, USA) and expressed in *E. coli* BL21 (DE3). Purified recombinant proteins were used for EMSA along with the biotin-labeled fragments of MaDREB2 promoters. EMSA was performed using the Lightshift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions.

Results

Isolation of the *DREB* transcription factors from banana fruit

Based on our RNA-Seq results and gene cloning by RT-PCR, we isolated nine full-length *DREB* cDNAs from ripe banana fruit and designated them *MaDREB1* to *MaDREB9*. These cDNAs were predicted to encode proteins of 296, 266, 318, 260, 339, 318, 330, 250 and 318 amino acids (aa), with calculated molecular weights of 32.90, 29.62, 34.94, 28.60, 36.79, 33.75, 36.43, 28.42 and 33.75 kDa and isoelectric points (*p*I) of 4.84, 4.58, 5.14, 4.54, 5.94, 9.16, 5.23, 9.33 and 9.16, respectively. Sequence alignments of MaDREB1−MaDREB9 with the DREB-containing proteins from *A. thaliana*, rice (*Oryza sativa*), and soybean (*Glycine max*) revealed a conserved AP2/ERF domain, which is capable of binding DNA via the DRE/C-repeat (CRT)-box and is present in most DREB-type TFs (Fig. S1). In addition, two key amino acids (valine and glutamic acid) at the 14th and 19th residues within the AP2/ERF domain are conserved in most MaDREBs (Fig. S1).

Phylogenetic analyses using the DREB sequences from banana, rice, maize (*Zea mays*) and *A. thaliana* revealed six DREB clades, namely A-1, A-2, A-3, A-4, A-5, and A-6 (Fig. S2), which is in agreement with previous findings (Zhao *et al.*, 2013). Banana MaDREB1−MaDREB4 are clustered within the A-2 clade, with *A. thaliana* AtDREB2A−AtDREB2C, and MaDREB5, MaDREB7 and MaDREB8 falling into the A-6 clade, while MaDREB6 and MaDREB9 are placed in the A-1 clade (Fig. S2). These data indicate that MaDREB1−MaDREB9 from banana fruit are members of the DREB family of TFs.

Expression patterns of *MaDREB* genes during banana fruit ripening

To reveal the possible roles of *MaDREB1* to *MaDREB9* in fruit ripening, we determined their expression profiles in banana pulp under different ripening regimes, including natural, ethylene-induced, 1-MCP-delayed ripening, and a combination of 1-MCP with ethylene treatment (Fig. 1a). As shown in Fig. 1(b), the naturally ripening fruits started to trigger significant ethylene production at 15 d of storage, which reached a maximum at 19 d and thereafter declined. Fruit firmness and hue angle continuously decreased as fruit ripening progressed. By contrast, application of ethylene accelerated ripening, while 1-MCP treatment delayed fruit ripening, as indicated by the changes in ethylene production, fruit firmness and hue (*h*) value (Fig. 1b). When ethylene and 1-MCP treatments were combined, ethylene production peaked at 28 d, which coincided with decreases in fruit firmness and *h* value (Fig. 1b). Consistent with the transcriptome data, transcripts of *MaDREB1* to *MaDREB9* were highly induced by ethylene treatment (Figs 2a, S3). In particular, the expression of *MaDREB1* to *MaDREB4* showed a consistent pattern with the evolution of ethylene, namely relatively low levels at the preclimacteric stage but high levels at the ripening stage (Fig. 2a). By contrast, *MaDREB5* to *MaDREB9* showed highest expression before the peak of ethylene production in naturally ripening bananas (Fig. S3). These results suggest that *MaDREB5* to *MaDREB9* may play less important roles than *MaDREB1* to *MaDREB4* in fruit ripening, and indeed they might play other roles in harvested banana fruit.

To further confirm that the expression of *MaDREB* genes is induced by ethylene, we performed two additional experiments. First, *MaDREB1* and *MaDREB2* promoters were fused with a GUS reporter gene (Fig. 2b) and were then transiently expressed in tomato fruits with or without ethrel treatment. As expected, *MaDREB1* and *MaDREB2* promoter-driven *GUS* was found to be strongly expressed in the fruits treated with ethrel, but not in untreated fruits (Fig. 2c), which is very different from the pattern of *CaMV35Spro::GUS*, which showed ubiquitous expression in the fruits regardless of ethrel treatment (Fig. 2c). In the second experiment, we performed a protoplast transient assay using a GFP reporter driven by the *MaDREB1/2* promoters (*MaDREB1/2pro::GFP*). In agreement with previous

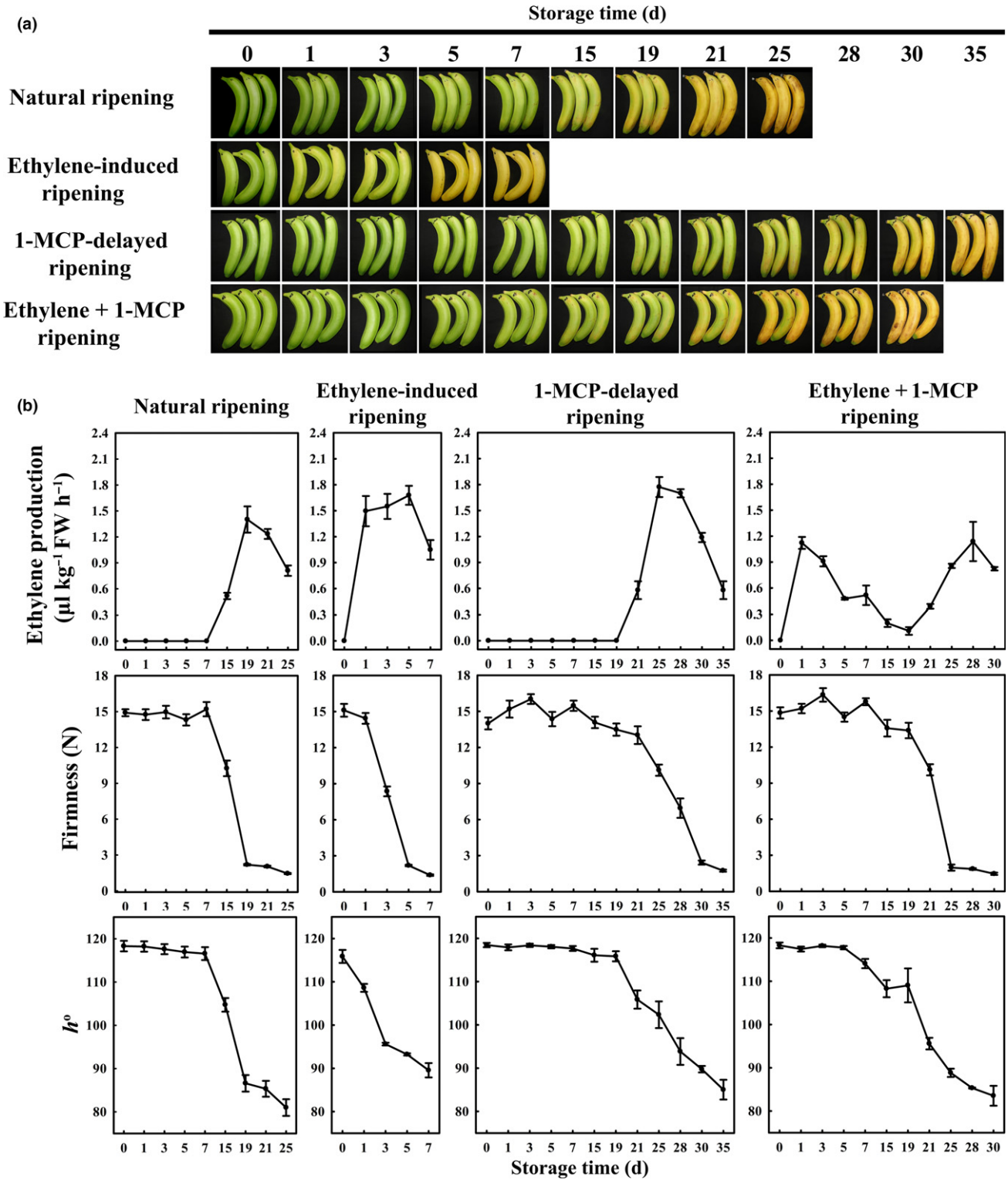

Fig. 1 Photographs of bananas with four different ripening characteristics, consisting of natural (control), ethylene-induced, 1-methylcyclopropene (1-MCP)-delayed, and a combination of 1-MCP + ethylene-treated ripening (a), and changes in ethylene production, fruit firmness and *h* value (b) during ripening. Each value indicates the mean ± SE of three biological replicates.

observations, GFP signals were only observed in the protoplasts transfected with *MaDREB1/2pro::GFP* after ethylene treatment (Fig. 2d). On the basis of all these expression data, we reasoned that *MaDREB1* to *MaDREB4* may play more important roles in fruit ripening than other *DREB* genes and hence they were selected for further study.

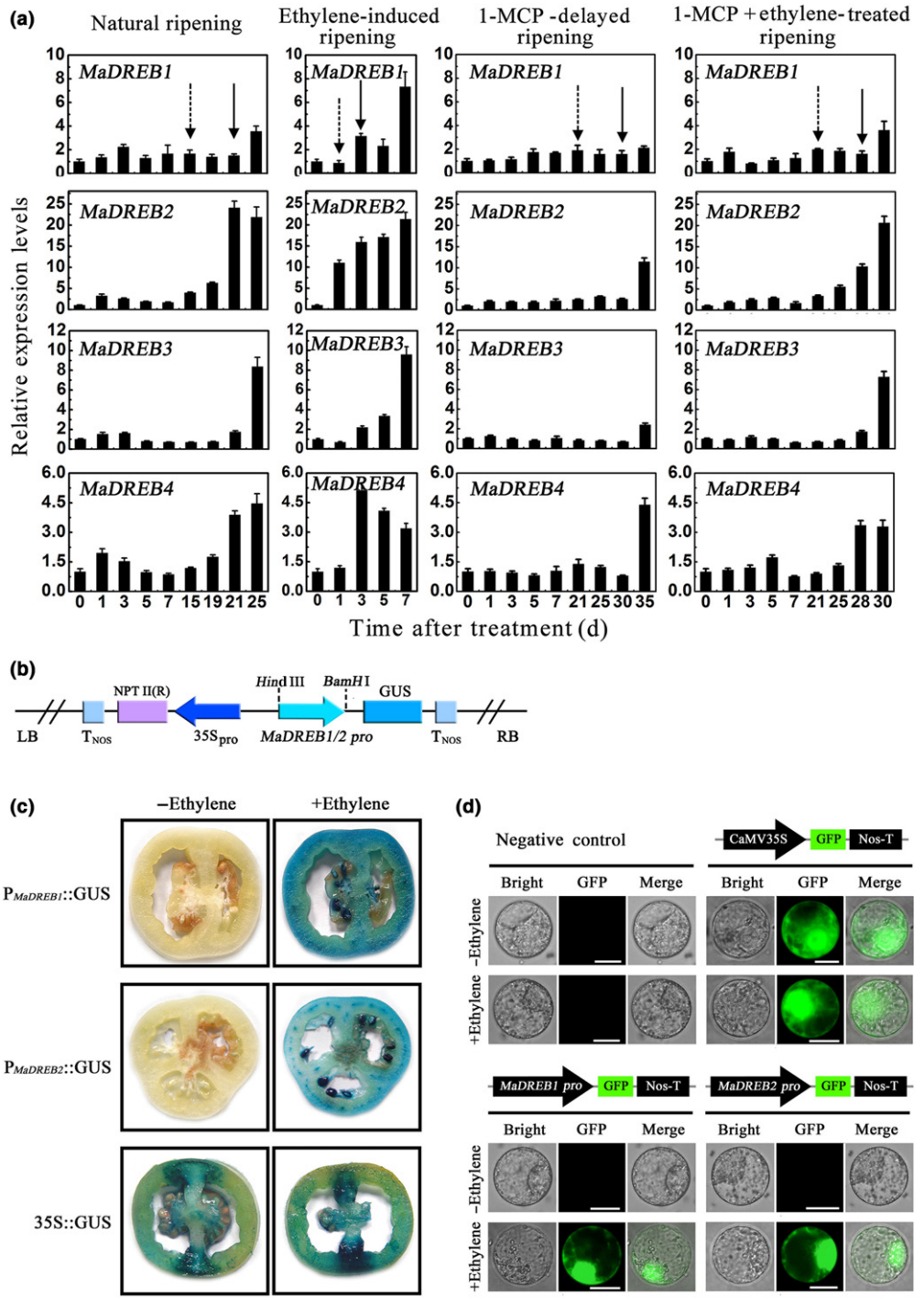

Fig. 2 Expression analysis of *Musa acuminata dehydration-responsive element binding* (*MaDREB*) genes in banana fruit. (a) qRT-PCR analysis of *MaDREB1–MaDREB4* expression in pulp during four ripening treatments: natural (control), ethylene-induced, 1-methylcyclopropene (1-MCP)-delayed, and a combination of 1-MCP and ethylene-treated ripening. The expression level of each gene was expressed as a ratio relative to that at harvest time (0 d of natural ripening bananas), which was set at 1. Each value represents the mean ± SE of three biological replicates. Dashed arrow, the time-point at which ethylene production began to increase; solid arrow, the peak of ethylene production in each treatment. (b) Schematic map of *MaDREB1/2pro-GUS*. LB, left border of T-DNA; RB, right border of T-DNA; T_{NOS}, terminator of *nos* (nopaline synthase) gene. (c) Detection of *MaDREB1/2* promoter activities transiently expressed in tomato fruit using a histological GUS assay. *MaDREB1/2* promoters were amplified by PCR and then subcloned into the *pBI121* vector in place of the cauliflower mosaic virus (CaMV) 35S promoter. The resulting promoter-GUS fusion constructs were transiently expressed in tomato fruit using *Agrobacterium tumefaciens*-mediated transformation. After the incubation of transformed fruits for 24 h, GUS staining was detected in fruits treated with or without 10 mM ethrel. (d) Detection of *MaDREB1/2* promoter activities transiently expressed in BY-2 protoplasts using a GFP assay. GFP reporter constructs containing the *MaDREB1/2* promoters (*MaDREB1/2pro*-GFP) and the CaMV 35S promoter (*35Spro*-GFP; positive control) were transiently transfected into tobacco BY-2 protoplasts using a modified polyethylene glycol (PEG) method and tested for ethylene induction. Nontransfected protoplasts were used as a negative control. After incubation for 12 h, GFP fluorescence was observed by fluorescence microscopy. Bars, 25 μm.

Subcellular localization and transcriptional activation activity of MaDREBs

To examine their subcellular localization in plants, MaDREB1−MaDREB4 were each fused to GFP and transiently expressed in tobacco BY-2 protoplasts. Fluorescence microscopy analysis showed that the four MaDREBs were all localized exclusively in the nuclei (Fig. 3a). Similar results were obtained in tobacco leaf epidermal cells (Fig. 3b).

To investigate the transcriptional activities of MaDREBs, a GAL4-responsive reporter system in yeast was used. Yeast transformants harboring the pGBKT7-53 + pGADT7-T and pGBKT7-MaDREB1 to pGBKT7-MaDREB4 constructs grew well in the SD/-Trp/-His/-Ade medium, and showed expression of α-galactosidase activity, whereas the yeasts transformed with the pGBKT7 vector alone did not (Fig. 3c). Protein truncation analysis indicated that the C-terminal parts of MaDREB1−MaDREB4 have transcriptional activation activity as evidenced by α-galactosidase expression (Fig. 3d).

The transcriptional activities of MaDREBs were further confirmed *in vivo* using tobacco BY-2 protoplasts. Consistent with the observations in yeast, all four of the pBD-MaDREBs could strongly activate the transcription of the *LUC* reporter gene in tobacco BY-2 cells as compared with the negative control (Fig. 3e), and the strongest activity was found for MaDREB3, with ~14-fold higher LUC/REN activity relative to the control (Fig. 3e). Collectively, these results indicate that MaDREB1−MaDREB4 are nucleus-localized proteins and possess transcriptional activation activities.

Genome-wide identification of MaDREB2-binding regions in banana fruits

To further investigate the regulatory roles of MaDREBs in fruit ripening, the genome-wide DNA-binding sites of MaDREB2 were surveyed using a ChIP-Seq approach. Before the ChIP-Seq experiments, we raised a polyclonal antibody against MaDREB2 (Fig. S4a–c) and detected MaDREB2 expression in response to ethylene treatment. The results showed that MaDREB2 protein was induced by ethylene and fruit ripening (Fig. S4d), consistent with its mRNA expression pattern (Fig. 2a). Moreover, MaDREB2 antibody is able to recognize the MaDREB2 recombinant protein, but not the remaining three MaDREB proteins (Fig. S5), confirming the specificity of the antibody.

For the ChIP experiment, cross-linked protein−DNA complexes from unripe or ripe banana fruit tissues were immunoprecipitated with the polyclonal MaDREB2 antibody. An IgG antibody was used as a negative control. To ensure data reliability, three biologically independent immunoprecipitated samples were used for library construction and high-throughput sequencing (Fig. S6). A total of 1961, 1347 and 1175 peaks were obtained from the three biological replicates, respectively (Table S2). Comparative data analysis of the three biological replicates revealed 697 overlapping peaks, which were considered the high-confidence MaDREB2-binding regions and were used for further analysis (Fig. 4a; Notes S1). Six randomly selected MaDREB2-binding peaks from the 697 peaks were successfully validated by ChIP-qPCR (Fig. S7), which indicated a good fidelity of the ChIP-Seq experiments. MaDREB2 bound to various genomic segments, including promoters, 5′- and 3′-UTRs, exons, and introns (Fig. 4b). Notably, the DNA-binding sites of MaDREB2 were predominantly (68%) located within the 2-kb region upstream of the TSS and with the highest distribution in the region adjacent to the TSS (Fig. 4b,c), consistent with MaDREB2 being a typical TF with DNA-binding ability and gene regulatory activity. Additionally, the percentage of MaDREB2 chromatin-association sites on each chromosome was proportional to chromosomal sizes, with 6–10% distribution from chromosome 1 to 11, suggesting that MaDREB2 does not exhibit a preference for a specific chromosome (Fig. 4d). Functional classification of the MaDREB2-binding genes using GO analysis showed that MaDREB2-binding genes are involved in various biological processes (FDR < 0.001) including signal transduction, cellular response to organic substance, single organism signaling, signaling, hormone-mediated signaling pathway, cellular response to hormone, endogenous and chemical stimuli, cell communication and intracellular signal transduction (Fig. 4e; Notes S2).

Identification of MaDREB2-binding motifs

To gain more insights into the DNA-binding properties of MaDREB2 in fruit ripening, *de novo* motif prediction was performed on the basis of MaDREB2-binding regions identified in our ChIP-Seq analysis using the Multiple Em for Motif Elicitation (MEME) program. This analysis resulted in the identification of three abundant motifs (Fig. 5; Notes S3). The first motif was represented by (A/G)CC(G/C)AC, which was present in 1240 sites (72.31%) and was confirmed at the *Expansin A7*, *Mitogen-Activated Protein Kinase10* (*MAPK10*), *myeloblastosis* (*MYB*) and *starch synthesis1* loci (Fig. 5a). This DNA-binding sequence differs slightly from the previously reported DRE/CRT motif ((A/G)CCGAC), showing a G or C nucleotide at position 4 compared with a G nucleotide at the same position in the DRE/CRT motif. The second enriched motif comprised CCAAT(C/A)AC(A/G)A with 53 predicted target regions (7.03%) such as *Tre-2/Bub2/Cdc16* (*TBC*), *ribonuclease 2*, and genes encoding a vacuolar protein and a hypothetical protein (Fig. 5b). The third motif, (G/T)G(G/A/C)(A/T/C)CC(C/A)(A/G)C(A/T), was enriched in 44 sites (6.31%) including *14-3-3*, *ADP-ribosylation factor GTPase-activating protein* (*AGD6*) and genes encoding hypothetical protein loci (Fig. 5c). Remarkably, a consensus motif embedded within extended motif 3 showed some similarity with motif 1 (Fig. 5c), suggesting ACCCAC as a conserved sequence bound by MaDREB2.

Expression analysis of potential MaDREB2 target genes during fruit ripening

To investigate the expression patterns of putative MaDREB2-regulated genes during fruit ripening, we performed comparative transcriptome sequencing (RNA-Seq) of unripe and ripe banana fruits. A total of 7867 differentially expressed genes were

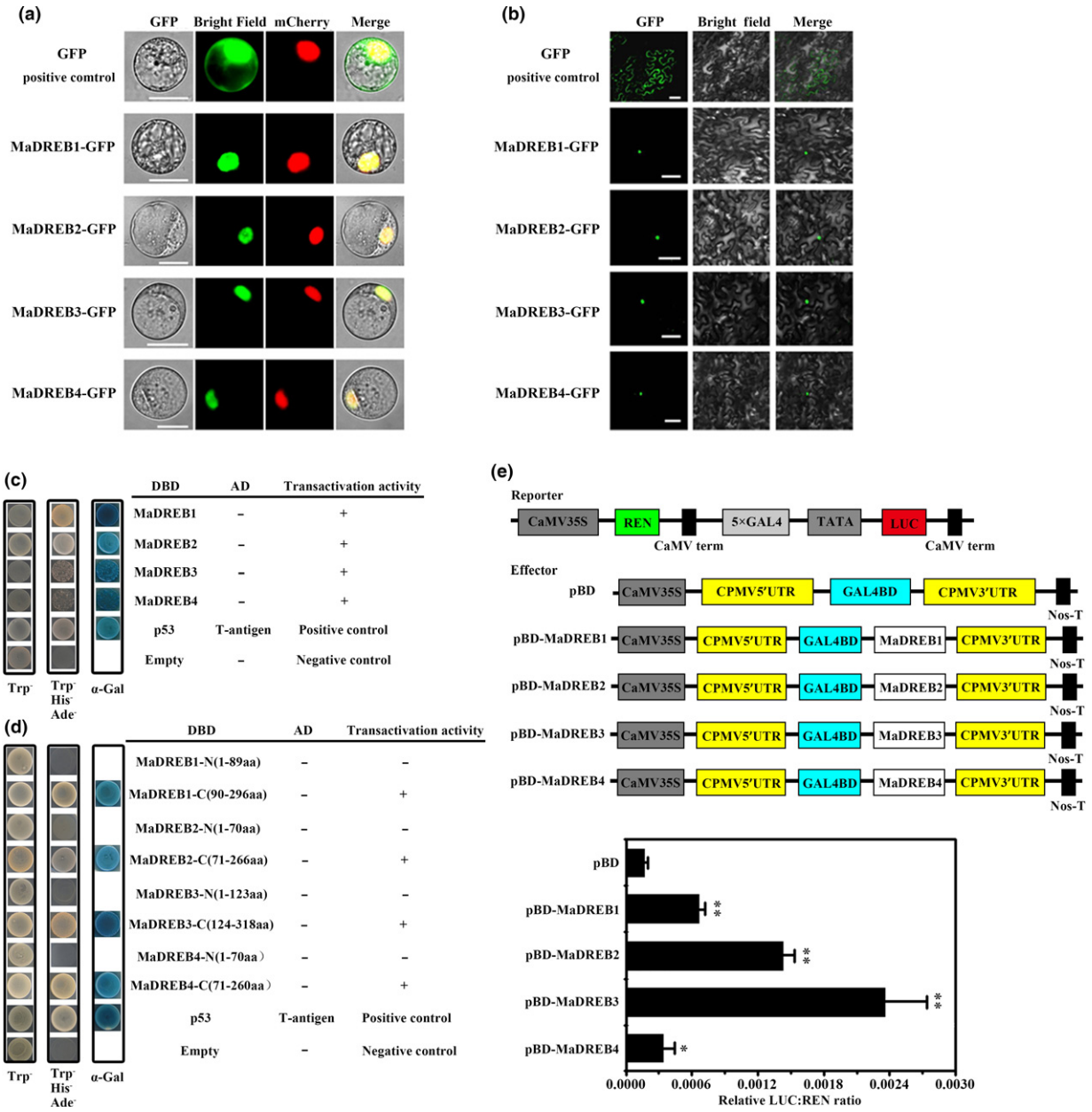

Fig. 3 Subcellular localization and transactivation activities of *Musa acuminata* dehydration-responsive element binding 1 (MaDREB1)–MaDREB4 proteins. (a) Subcellular localization of MaDREBs in tobacco BY-2 protoplasts. Protoplasts were transiently transformed with *35S:MaDREB-GFP* constructs or *35S:GFP* vector using a modified polyethylene glycol (PEG) method. GFP fluorescence was observed with a fluorescence microscope. *VirD2NLS-mCherry* was included in each transfection to serve as a control for successful transfection, as well as for nuclear localization. Images were taken in a dark field for green fluorescence, while the cell outlines were photographed in a bright field. Bars, 25 μm. (b) Subcellular localization of MaDREBs in tobacco leaves. Leaves were transiently transformed with *MaDREB-GFP* constructs or the GFP vector through *Agrobacterium tumefaciens* transfection. GFP fluorescence was observed with a fluorescence microscope. Images were taken in a dark field for green fluorescence, while the cell outlines were photographed in a bright field. Bars, 50 μm. (c) Transactivation activation activity assay of MaDREB1–MaDREB4 in yeast cells. The coding regions of *MaDREB1–MaDREB4* were cloned into the *pGBKT7* (GAL4-DNA binding domain, GAL4-DBD) vector to create the respective *DBD-MaDREB* constructs. (d) Truncation analysis of activation domains of MaDREB1–MaDREB4 for transcriptional activation. C- and N-terminal derivatives of *MaDREB1–MaDREB4* were fused with the *pGBKT7* vector to create the respective *DBD-MaDREB* constructs. The numbers in brackets indicate the amino acid residues of the truncated polypeptides. (c, d) All the constructs including the positive control (p-53 + T-antigen) and negative control (pGBKT7) were transformed into yeast strain AH109. Yeast clones transformed with different constructs were grown on SD plates without tryptophan (-Trp) or without tryptophan, histidine, and adenine (-Trp/-His/-Ade) but containing 125 μM aureobasidin A (AbA) for 3 d at 30°C. Transcription activation was monitored by detecting yeast growth and the expression of α-galactosidase (α-Gal) activity. (e) Transcriptional activation activity assay of MaDREB1–MaDREB4 in tobacco BY-2 protoplasts. Plasmid combinations of dual Renilla (RNE)/luciferase (LUC) reporters and effectors were co-transfected into BY-2 protoplasts. The protoplasts were incubated for 16 h, and the transactivation ability of MaDREB1–MaDREB4 is indicated by the ratio of LUC : REN. Each value represents the mean of six biological replicates, and vertical bars represent the SE. Significant difference (Student's *t*-test): *, $P < 0.05$; **, $P < 0.01$. CPMV, Cowpea mosaic virus; LUC, firefly luciferase; REN, renilla luciferase; UTR, untranslated regions.

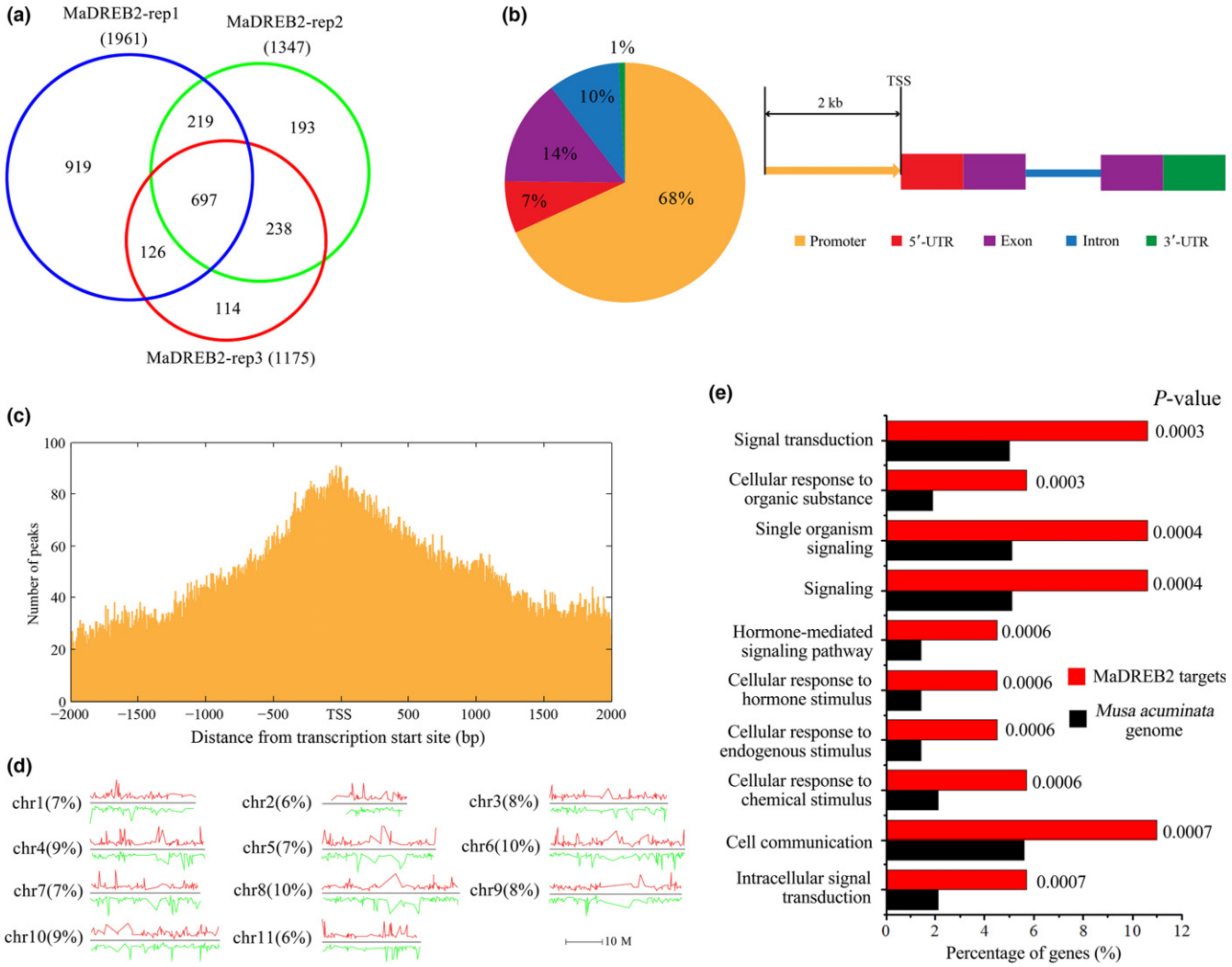

Fig. 4 Genome-wide distribution of *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2) associated sites. (a) Chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) using three biological replicates revealed 697 high-confidence MaDREB2-binding peaks, which were shared in three replicates. (b) Distribution of MaDREB2 association sites in different regions of annotated genes. (c) MaDREB2 association sites are highly enriched in the region proximal to the transcriptional start sites. (d) Distribution of MaDREB2 association sites across the 11 *M. acuminata* chromosomes. ChIP-Seq peaks indicate the MaDREB2 association sites. The numbers in brackets indicate the percentage of ChIP-Seq peaks from each chromosome. (e) Gene ontology (GO) categorization of MaDREB2-binding genes with a likely role in fruit ripening. Enriched GO categories of MaDREB2 compared with all *M. acuminata* genes are shown. A false discovery rate (FDR) cut-off was implemented on the basis of a *P*-value < 0.001. Numbers indicate *P*-values. For GO term details, see Supporting Information Notes S2.

identified in unripe and ripe banana with a cut-off fold change of 1.5 (*P*-value < 0.05). Of the 7867 genes, 3208 (40.8%) were ripening-activated and 4659 (59.2%) were ripening-repressed (Notes S4). Interestingly, a comparison with the RNA-Seq data from a previous study in banana (Asif *et al.*, 2014) showed that the top 50 up-regulated genes associated with fruit ripening in their data set were also found in our RNA-Seq data set with similar expression changes.

To determine the expression of MaDREB2-bound genes during fruit ripening, we merged our ChIP-Seq and RNA-Seq data. Of the 697 MaDREB2-bound candidate genes, 81 (11.6%) were in the up-regulated group and thus could be considered as the direct targets positively regulated by MaDREB2. In contrast, 115 genes (16.5%) fell into the down-regulated group and therefore might be direct targets negatively regulated by MaDREB2 (Fig. 6a). Although MaDREB2 was well characterized as a transcriptional activator of fruit ripening (Fig. 3c-e), the data obtained here indicate that MaDREB2 may also act as a transcriptional repressor. From the positively and negatively regulated direct targets of MaDREB2, we have listed the top 50 up- or down-regulated MaDREB2 targets during fruit ripening (Tables 1, 2), which have a general association with fruit ripening, stress/defense responses, transcriptional regulation and post-translational modifications (Fig. 6b).

MaDREB2 directly regulates genes for cell wall modification and aroma production

To identify additional targets of MaDREB2 that are involved in banana ripening, the genomic regions of banana were searched

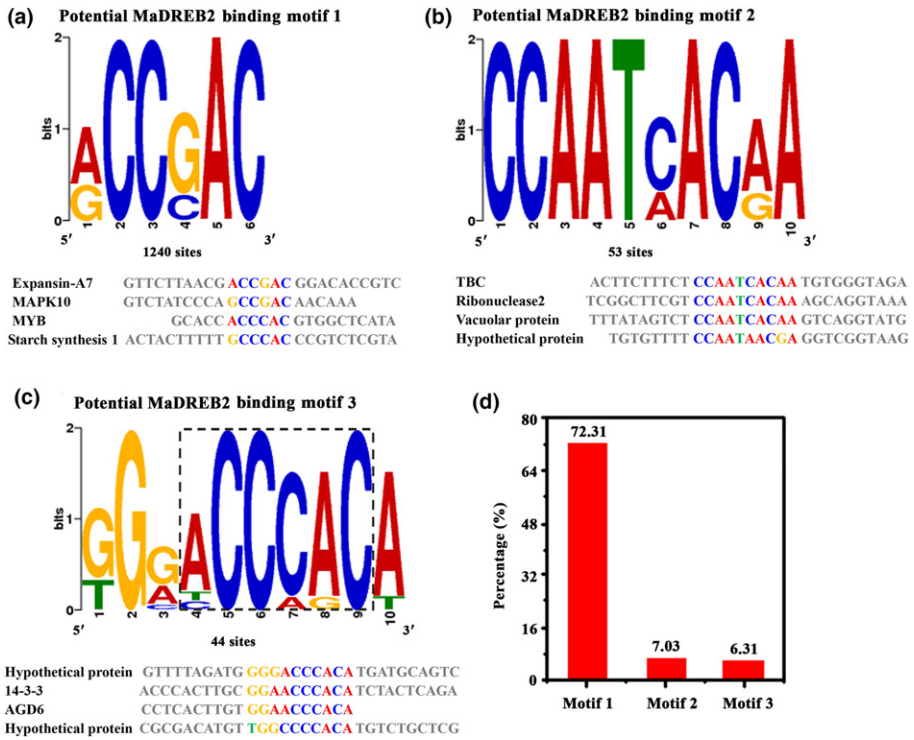

Fig. 5 Binding motif analysis in the target regions of *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2). (a) The potential DNA-binding motif 1 of MaDREB2 analyzed according to chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) results. The binding sequences in the *Expansin A7*, *Mitogen-Activated Protein Kinase10* (*MAPK10*), *myeloblastosis* (*MYB*) and *starch synthesis 1* loci are shown by colored letters. The sequence logo was derived from the 1240 binding sites. (b) The potential DNA-binding motif 2 of MaDREB2 analyzed according to ChIP-Seq results. The binding sequences in *Tre-2/Bub2/Cdc16 (TBC)*, *Ribonuclease 2*, *vacuolar protein* and *hypothetical protein* loci are shown by colored residues. The sequence logo was derived from 53 binding sites. (c) The potential DNA-binding motif 3 of MaDREB2 analyzed according to ChIP-Seq results. The binding sequences in *hypothetical proteins*, *14-3-3*, and *ADP-ribosylation factor GTPase-activating protein* (*AGD6*) loci are shown as colored letters. The sequence logo was derived from 44 binding sites. A potential motif 1 embedded within motif 3 is boxed. (d) The relative proportion of three binding motifs distributed in the target genes.

for a possible MaDREB2-binding motif 1 ((A/G)CC(G/C)AC). We found that *c.* 23 504 genes contained at least one motif in their promoter region within 1 kb in front of the start code (ATG) (Notes S5), which might be the potential targets of MaDREB2. The two most significant features of banana fruit ripening are softening and aroma evolution. Several reported cell wall modification- and aroma production-related genes, such as *Expansin 1* (*MaEXP1*), *MaEXP3*, *MaEXP5*, *xyloglucan endotransglycosylase3* (*MaXET3*), *MaXET7*, *alcohol dehydrogenase1* (*MaADH1*) and *pyruvate decarboxylase* (*MaPDC*) (Mbéguié-A-Mbéguié *et al.*, 2009; Yang *et al.*, 2011), contain MaDREB2-binding motif 1 (Fig. 7a), and we analyzed the *in vivo* binding of MaDREB2 to these genes by ChIP-qPCR. The results showed that MaDREB2 bound strongly to the promoters of *MaEXP1*, *MaEXP3*, *MaEXP5*, *MaXET3*, *MaXET7*, *MaADH1* and *MaPDC* (Fig. 7b). Furthermore, the binding of MaDREB2 to four selected targets (*MaEXP1*, *MaXET7*, *MaADH1* and *MaPDC*) was confirmed by EMSA (Fig. S8). The expression of all these genes increased as fruit ripening progressed (Fig. 7c).

MaDREB2 binds to its own promoter

Based on our ChIP-Seq data set, we found that *MaDREB2* itself was a target of MaDREB2 (Notes S1; Fig. 8a). To validate whether MaDREB2 binds to its own promoter, we utilized the ChIP-qPCR assay. As shown in Fig. 8b, the *MaDREB2* promoter was greatly enriched in chromatins precipitated with the MaDREB2 antibody compared to the IgG antibody. Next, the binding of MaDREB2 to its promoter was verified by an *in vitro* EMSA. As shown in Fig. 8c, recombinant MaDREB2 was able to bind to its promoter fragment and caused a mobility shift of the labeled probe. Addition of the unlabeled promoter fragments competed for the binding of the labeled probe in a dose-dependent manner, indicating that the binding of MaDREB2 to its own promoter is specific. Collectively, these data indicate that MaDREB2 directly binds to its own promoter.

Discussion

The AP2/ERF family of TFs are divided into four groups, the AP2, ERF, DREB and Related to ABI3/VP (RAV) subfamilies (Nakano *et al.*, 2006; Lata & Prasad, 2011). Recently, 265 and 318 *AP2/ERF* genes have been identified in *M. acuminata* and *Musa balbisiana,* respectively. Recent studies have demonstrated that ERFs function as key regulators of fruit ripening and senescence (Yin *et al.*, 2010; Kuang *et al.*, 2012; Xiao *et al.*, 2013), as nearly 40 *ERF* genes showed significantly differential expression

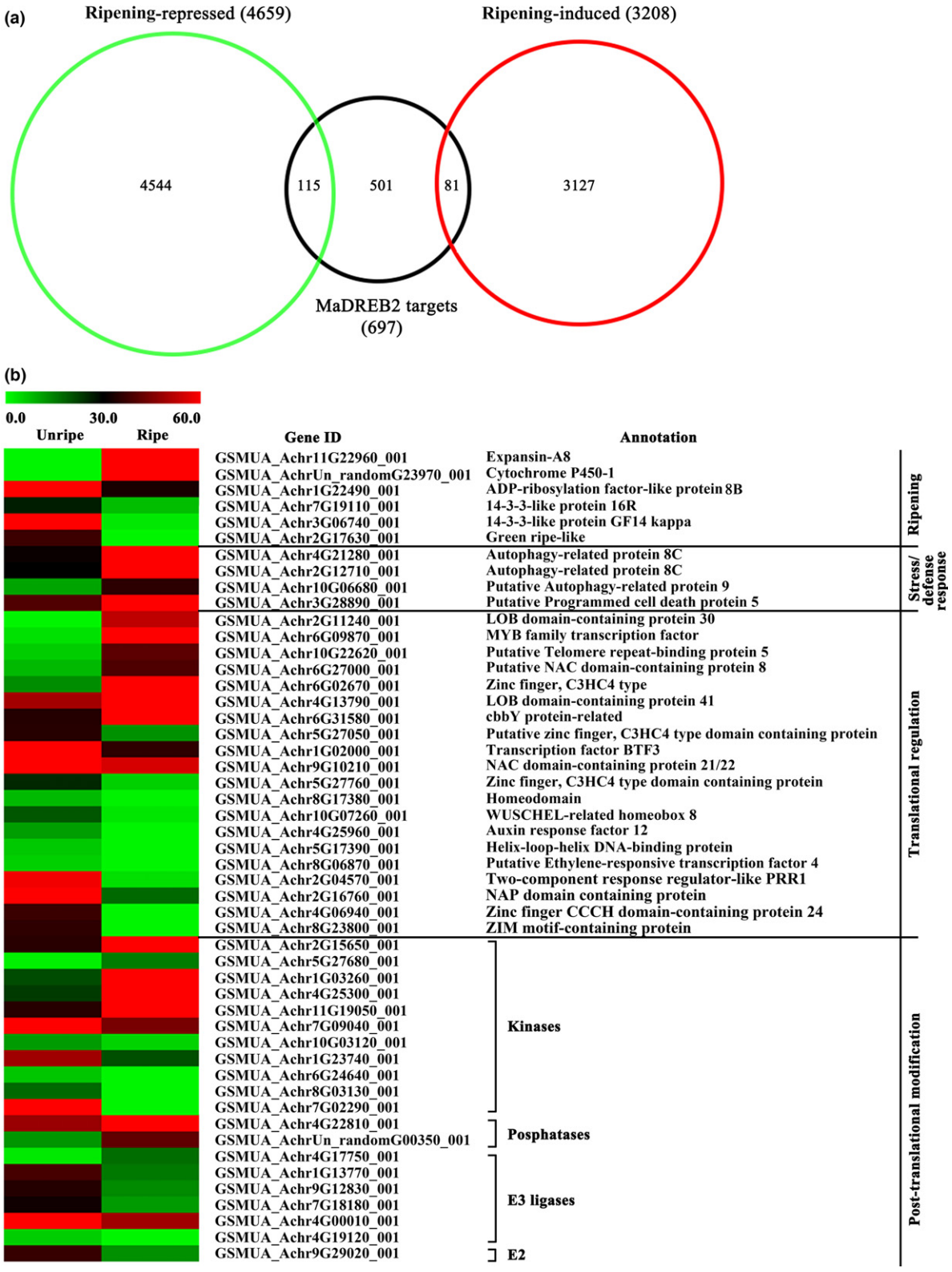

Fig. 6 Expression profiles of *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2)-binding genes. (a) Venn diagram showing the overlap between genes bound by MaDREB2 from chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) and the genes differentially expressed during banana fruit ripening determined by RNA sequencing (RNA-Seq). (b) Differentially expressed genes that encode proteins associated with ripening, stress/defense response, transcriptional regulation and posttranslational modifications within MaDREB2 targets in unripe and ripe banana fruits. Red and green colors indicate up- and down-regulated genes, respectively.

Table 1 Top 50 up-regulated *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2) target genes during banana fruit ripening

Musa acuminata ID	Readcount_ ripe	Readcount_ unripe	log2.Fold_ change	*P*-value	Description
GSMUA_Achr11G22960_001	6063.232	0	13.566	0	Expansin-A8
GSMUA_AchrUn_ randomG23970_001	2467.52	0	12.269	9.3E-259	Cytochrome P450-1
GSMUA_Achr5G26280_001	38.479	0	8.172	1.05E-08	Probable ADP-ribosylation factor GTPase-activating protein AGD5
GSMUA_Achr2G11240_001	52.49737	0	6.7142	2.99E-12	LOB domain-containing protein 30
GSMUA_Achr3G03290_001	473.9068	4.802809	6.6246	8.16E-98	Soluble starch synthase 1
GSMUA_Achr8G34060_001	10.72835	0	6.3294	0.001554	Putative Uncharacterized protein
GSMUA_Achr7G16250_001	42.34121	0	5.7251	3.82E-10	UDP-glucuronate 4-epimerase 1
GSMUA_Achr9G15500_001	6.437008	0	5.5924	0.014886	Expressed protein
GSMUA_Achr9G00170_001	152.6286	4.669398	5.0306	2.63E-31	UDP-glucose 4-epimerase GEPI48
GSMUA_Achr6G28340_001	58.50525	2.134582	4.7765	1.15E-12	Expressed protein
GSMUA_Achr2G12470_001	298.3911	12.54067	4.5725	1.40E-56	DUF1336 domain containing protein
GSMUA_AchrUn_ randomG14270_001	9.440945	0	4.56	0.004851	Pto-interacting protein 1
GSMUA_Achr8G24090_001	86.39895	4.135752	4.3848	4.17E-17	Granule-bound starch synthase 2
GSMUA_Achr3G28010_001	10.29921	0	4.3645	0.003754	Tetraspanin family protein
GSMUA_Achr6G09870_001	66.80184	3.468695	4.2674	2.49E-13	MYB family transcription factor
GSMUA_Achr4G18220_001	125.5932	7.604448	4.0458	9.13E-23	Putative Extended synaptotagmin-2
GSMUA_Achr1G25940_001	14.16142	0	3.9226	0.001142	Putative Anaphase-promoting complex subunit cdc20
GSMUA_Achr6G36710_001	12.15879	0	3.7026	0.003365	Putative Uncharacterized protein
GSMUA_Achr5G03610_001	7.581365	0	3.5066	0.024424	Hypothetical protein
GSMUA_Achr2G15650_001	372.3451	34.95378	3.4131	1.95E-54	CK1_Casein kinase_1a.5
GSMUA_Achr7G15410_001	568.4593	57.36689	3.3088	2.60E-79	Hypothetical protein
GSMUA_Achr4G21280_001	301.252	31.35167	3.2644	3.20E-42	Autophagy-related protein 8C
GSMUA_Achr5G27680_001	15.30577	1.734348	3.1416	0.002721	Pyruvate kinase, cytosolic isozyme
GSMUA_Achr7G05520_001	201.836	24.54769	3.0395	1.61E-26	Pyruvate dehydrogenase E1 component subunit alpha-1
GSMUA_Achr4G32680_001	23.74541	3.068461	2.9521	0.000337	Hypothetical protein
GSMUA_Achr2G19710_001	32.04199	4.402575	2.8635	4.53E-05	Putative 60S ribosomal protein L18a-1
GSMUA_Achr5G28050_001	24.74672	3.468695	2.8348	0.000372	Hypothetical protein
GSMUA_Achr8G31130_001	14.16142	2.00117	2.823	0.007265	Expressed protein
GSMUA_Achr10G22620_001	41.0538	5.8701	2.8061	5.34E-06	Putative Telomere repeat-binding protein 5
GSMUA_Achr4G17750_001	17.16535	2.534816	2.7595	0.003641	Putative E3 ubiquitin protein ligase DRIP2
GSMUA_Achr8G31140_001	20.59842	3.068461	2.7469	0.0015	Putative Uncharacterized membrane protein
GSMUA_Achr5G05190_001	651.7113	102.9936	2.6617	3.57E-68	Putative Copper transporter 1
GSMUA_Achr6G14370_001	55.35827	9.071973	2.6093	5.48E-07	Putative Fatty acyl-CoA synthetase A
GSMUA_Achr2G18150_001	53.92782	9.605618	2.4891	1.89E-06	Probable ADP-ribosylation factor GTPase-activating protein AGD8
GSMUA_Achr6G05070_001	73.0958	13.60796	2.4253	5.57E-08	Vacuolar protein sorting-associated protein 2
GSMUA_Achr4G22810_001	239.1706	47.89468	2.3201	2.79E-21	Serine/threonine-protein phosphatase BSL3
GSMUA_Achr6G27000_001	39.33727	8.138093	2.2731	0.000161	Putative NAC domain-containing protein 8
GSMUA_Achr3G30860_001	41.76903	8.671739	2.268	0.000104	Putative BTB/POZ domain-containing protein KCTD9
GSMUA_Achr6G22300_001	82.67979	17.6103	2.2311	7.31E-08	Expressed protein
GSMUA_Achr10G05880_001	51.35302	11.07314	2.2134	2.52E-05	Expressed protein
GSMUA_Achr6G02670_001	61.08005	13.20772	2.2093	4.49E-06	Zinc finger, C3HC4 type
GSMUA_Achr4G22400_001	3500.874	765.7812	2.1927	3.4265E-261	Thioredoxin H1
GSMUA_Achr8G10700_001	67.08792	14.80866	2.1796	2.03E-06	Probable enoyl-CoA hydratase
GSMUA_Achr1G05110_001	661.1522	147.2861	2.1664	9.03E-50	Isocitrate dehydrogenase
GSMUA_Achr1G03260_001	93.55118	20.94558	2.1591	2.67E-08	Tyrosine protein kinase domain containing protein
GSMUA_Achr6G02930_001	58.93438	13.47455	2.1289	1.31E-05	F-box protein
GSMUA_Achr5G25500_001	85.82677	19.87829	2.1102	1.81E-07	Expressed protein
GSMUA_Achr2G12710_001	122.3031	29.88415	2.033	1.79E-09	Autophagy-related protein 8C
GSMUA_Achr11G18890_001	42.34121	10.40609	2.0246	0.0004219	mRNA-decapping enzyme-like protein
GSMUA_AchrUn_ randomG09810_001	95.41076	23.4804	2.0227	1.23E-07	BTBM1 – Bric-a-Brac

BTB/POZ, broad complex, tramtrack, and bric-à-brac/poxviruses and zinc finger; LOB, Lateral Organ Boundaries domain; MYB, myeloblastosis; NAC, NAM/ATAF1,2/CUC2.

patterns between ripe and unripe banana fruits (Lakhwani *et al.*, 2016). In the present study, we showed that the expression of *MaDREB1* to *MaDREB4* was up-regulated during ripening and was induced by ethylene (Fig. 2a). *MaDREB1* and *MaDREB2* promoters could be activated by ethylene treatment when transiently expressed in tomato fruit and tobacco BY-2 protoplasts

Table 2 Top 50 down-regulated *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2) target genes during banana fruit ripening

Musa acuminata ID	Readcount_ ripe	Readcount_ unripe	log2.Fold_ change	*P*-value	Description
GSMUA_Achr6G13090_001	0	57.76712	−8.6576	2.36E-14	Transcription elongation factor protein
GSMUA_Achr7G02290_001	0	153.2897	−8.2601	3.80E-37	LRR receptor-like serine/threonine-protein kinase ERECTA
GSMUA_Achr2G17630_001	0	37.22177	−7.0235	2.51E-11	Green ripe-like
GSMUA_Achr8G03130_001	0	17.47689	−5.9328	2.37E-06	Serine/threonine-protein kinase Nek2
GSMUA_Achr1G04490_001	1.001312	53.89819	−5.7503	9.61E-17	Putative uncharacterized protein
GSMUA_Achr8G23800_001	0	35.48742	−5.6328	1.47E-11	ZIM motif-containing protein
GSMUA_Achr5G24160_001	0	6.80398	−5.5718	0.0030999	DNA-binding protein
GSMUA_Achr4G06940_001	0	36.95495	−5.4282	5.26E-12	Zinc finger CCCH domain-containing protein 24
GSMUA_Achr3G06740_001	2.431758	80.18023	−5.0432	3.22E-24	14-3-3-like protein
GSMUA_Achr2G06270_001	3.71916	105.5284	−4.8265	3.36E-31	PDE225/PTAC7
GSMUA_Achr7G11220_001	1.430446	39.22294	−4.7772	1.48E-12	Alpha,alpha-trehalose-phosphate synthase
GSMUA_AchrUn_ randomG25040_001	1.001312	21.47923	−4.423	2.08E-07	Putative Predicted protein
GSMUA_Achr2G16760_001	17.59449	340.7326	−4.2754	4.54E-94	NAP domain containing protein
GSMUA_Achr10G03310_001	0	8.671739	−4.1163	0.0011109	DNA-damage-repair/toleration protein DRT100
GSMUA_Achr11G15520_001	0	4.802809	−4.0693	0.015458	Hypothetical protein
GSMUA_Achr8G20450_001	4.720472	79.24635	−4.0693	7.86E-23	Homocysteine S-methyltransferase 2
GSMUA_Achr9G05610_001	26.32021	427.5834	−4.022	4.96E-115	Ras-related protein Rab11D
GSMUA_Achr2G04570_001	3.71916	58.701	−3.9803	3.50E-17	Two-component response regulator-like PRR1
GSMUA_Achr9G17830_001	11.01444	170.0995	−3.9489	1.55E-46	Putative VHS and GAT domain containing protein
GSMUA_Achr5G25070_001	2.145669	32.8192	−3.935	3.24E-10	Conserved hypothetical protein
GSMUA_Achr4G21380_001	94.12336	1417.896	−3.9131	0	YT521-B-like family domain containing protein
GSMUA_Achr2G19940_001	1.85958	27.88297	−3.9063	7.21E-09	Putative chaperon P13.9
GSMUA_AchrUn_ randomG14070_001	0	7.471036	−3.9013	0.002753	Putative Peptide transporter PTR3-A
GSMUA_Achr4G16310_001	0	8.271505	−3.8536	0.001679	Hypothetical protein
GSMUA_Achr9G09860_001	19.74016	264.8216	−3.7458	8.34E-70	Expressed protein
GSMUA_AchrUn_ randomG21450_001	1.001312	13.34114	−3.7359	7.45E-05	Putative membrane protein ycf1
GSMUA_Achr3G31260_001	6.150918	81.51434	−3.7282	1.28E-22	30S ribosomal protein S19
GSMUA_Achr8G06870_001	0	5.603277	−3.7068	0.010396	Putative Ethylene-responsive transcription factor 4
GSMUA_Achr5G17390_001	0	6.403745	−3.6789	0.006254	Helix-loop-helix DNA-binding protein
GSMUA_Achr10G08800_001	0	6.270334	−3.6485	0.006937	Putative Salt stress-induced protein
GSMUA_Achr5G09890_001	0	5.8701	−3.5534	0.009479	Putative Protein UNUSUAL FLORAL ORGANS
GSMUA_Achr2G10060_001	6.437008	72.04214	−3.4844	1.57E-19	Putative Uncharacterized protein
GSMUA_Achr3G01940_001	4.005249	44.82622	−3.4844	9.99E-13	Probable Peroxisomal (S)-2-hydroxy-acid oxidase 2
GSMUA_Achr2G05390_001	8.296588	86.98421	−3.3902	6.47E-23	Tryptophan synthase beta chain 2
GSMUA_Achr4G25960_001	1.144357	11.33997	−3.3088	0.000411	Auxin response factor 12
GSMUA_AchrUn_ randomG03340_001	5.006562	47.36103	−3.2418	7.25E-13	NADH-ubiquinone oxidoreductase chain 2
GSMUA_Achr7G22620_001	1.144357	10.80632	−3.2393	0.000612	TBC domain containing protein
GSMUA_Achr11G14980_001	4.863517	45.49327	−3.2256	2.21E-12	Calreticulin-3
GSMUA_Achr7G23670_001	0	7.87127	−3.1971	0.00359	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
GSMUA_AchrUn_ randomG06460_001	0	6.270334	−3.1321	0.009826	Hypothetical protein
GSMUA_Achr2G19830_001	14.16142	116.3347	−3.0382	3.47E-28	Tetratricopeptide repeat domain containing protein
GSMUA_Achr7G02590_001	10.29921	81.78117	−2.9892	4.37E-20	Hypothetical protein
GSMUA_Achr1G04640_001	4.863517	37.22177	−2.9361	7.59E-10	Expressed protein
GSMUA_Achr10G29360_001	1.716535	12.27385	−2.838	0.000482	Expressed protein
GSMUA_Achr11G23980_001	10.72835	74.31013	−2.7921	1.38E-17	Ras-related protein ARA-3
GSMUA_Achr10G07260_001	2.860892	19.74488	−2.7869	1.10E-05	WUSCHEL-related homeobox 8
GSMUA_AchrUn_ randomG21770_001	15.16273	104.4611	−2.7844	4.94E-24	Predicted protein
GSMUA_Achr10G13190_001	2.717848	18.41077	−2.76	2.33E-05	Hypothetical protein
GSMUA_Achr6G24640_001	1.001312	6.670568	−2.7359	0.011146	Putative LRR receptor-like serine/threonine-protein kinase
GSMUA_AchrUn_ randomG23990_001	2.574803	16.67642	−2.6953	6.60E-05	Hypothetical protein

LRR, leucine-rich repeat; NAP, NAC-like, activated by APETALA3/PISTILLATA; TBC, Tre-2/Bub2/Cdc16.

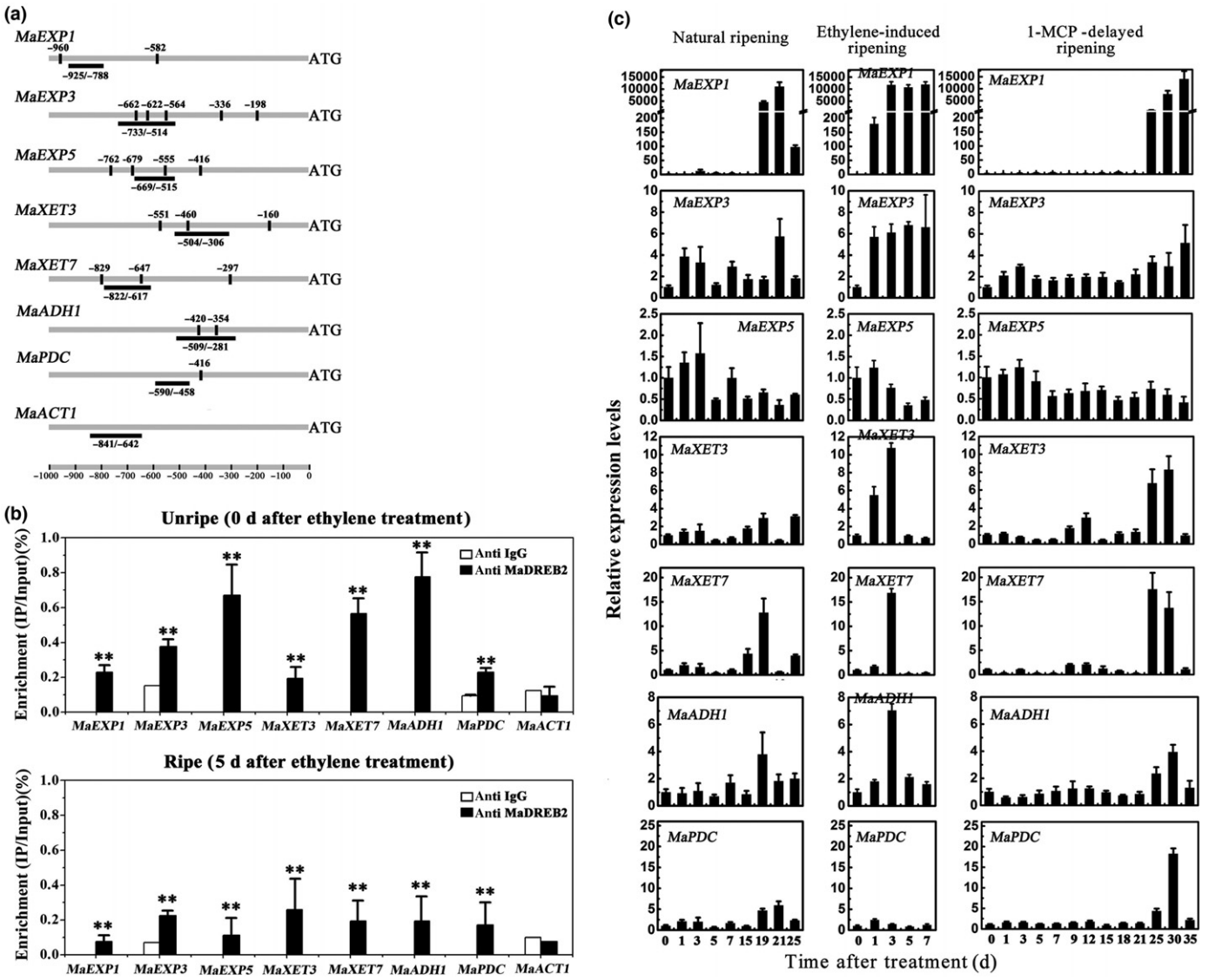

Fig. 7 Chromatin immunoprecipitation (ChIP)–quantitative PCR (qPCR) analysis of *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2) binding to genes in relation to cell wall modification and aroma production. (a) Schematic promoter structures of selected MaDREB2 target genes. Bold vertical bars indicate the MaDREB2-binding motif 1 in the promoter region and the numbers indicate the position of these motifs relative to the translational start site. The PCR products corresponding to each gene promoter are shown as bold horizontal bars. (b) ChIP-qPCR analysis of MaDREB2 binding to genes involved in cell wall modification and aroma production. Immunoprecipitated DNAs were recovered in unripe and ripe banana fruits with antibodies against MaDREB2 and immunoglobulin G (IgG) and the enrichment was evaluated by qPCR. *Musa acuminata Actin* (*MaACT*) was used as a reference gene. Notably, because of the higher sensitivity of ChIP-qPCR compared with ChIP-Seq, some MaDREB2-binding genes that were not detected in ChIP-Seq were identified in ChIP-qPCR assays. Fold of enrichment for antibodies against MaDREB2 relative to control IgG is presented as mean value ± SD of three replicates. Significant difference (according to Student's *t*-test): **, $P < 0.01$. (c) Expression patterns of *Expansin1* (*MaEXP1*), *MaEXP3*, *MaEXP5*, *xyloglucan endotransglycosylase3* (*MaXET3*), *MaXET7*, *alcohol dehydrogenase1* (*MaADH1*) and *pyruvate decarboxylase* (*MaPDC*) in pulp during banana fruit ripening. The expression level of each gene was expressed as a ratio relative to that at harvest time (0 d of natural ripening bananas), which was set at 1. Each value represents the mean ± SE of three biological replicates.

(Fig. 2b–d). Furthermore, *MaDREB2* directly binds to a number of ripening-related genes in banana. All these results indicate that MaDREB proteins are likely to be involved in regulating banana fruit ripening.

In this study, 697 high-confidence MaDREB2-binding sites were identified in the banana genome using ChIP-Seq technology (Fig. 4a; Notes S1). Similar to the binding sites of *A. thaliana* phytochrome A (phyA) (Chen *et al.*, 2014) and *Populus* (*Populus trichocarpa*) ARBORKNOX1 (Liu *et al.*, 2015a), the binding regions of MaDREB2 are preferentially distributed in proximal promoters near TSSs of their target genes (Fig. 4b,c). In addition, a motif analysis using the MEME program identified three MaDREB2-binding motifs (Fig. 5; Notes S3). In particular, the most abundant motif ((A/G)CC(G/C)AC) is similar to the DRE/CRT-binding motif ((A/G)CCGAC), a typical binding site for DREB TFs. This motif is enriched in 1240 MaDREB2-binding peaks, although there are some sequence variations in comparison with the canonical DRE/CRT-binding motif

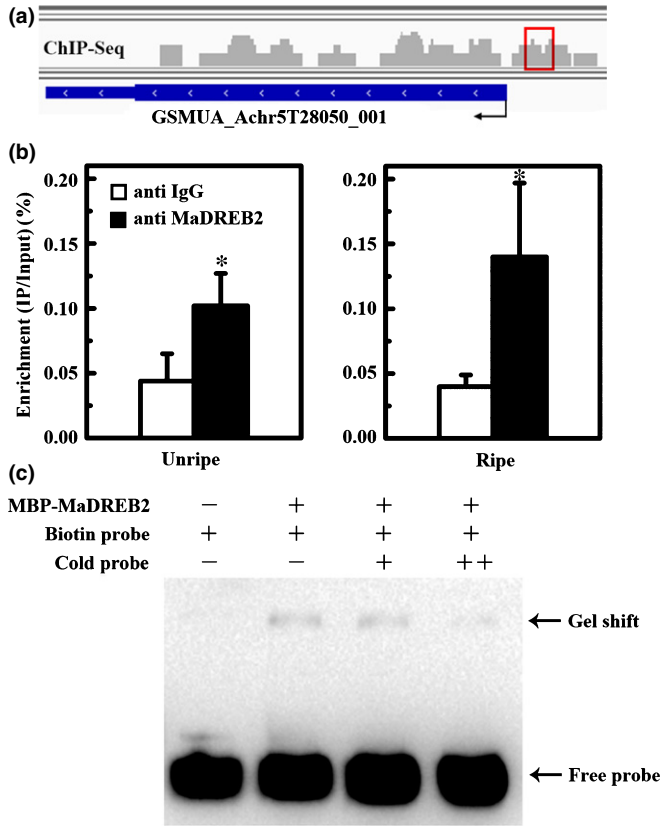

Fig. 8 *Musa acuminata* dehydration-responsive element binding 2 (MaDREB2) binds to its own promoter. (a) INTEGRATIVE GENOMICS VIEWER (IGV) image of the *MaDREB2* gene in chromatin immunoprecipitation and high-throughput sequencing (ChIP-Seq) reads. The red box indicates the amplicon located in the MaDREB2 promoter used for ChIP-qPCR and electrophoretic mobility shift assays. (b) ChIP-qPCR analysis of MaDREB2 binding to its own promoter. The relative enrichment of amplicon immunoprecipitated with the MaDREB2 antibody normalized to the control, in which the IgG antibody was used, is shown. Error bars represent + SE from three different biological replicates. Significant difference (according to Student's *t*-test): *, $P < 0.05$. (c) EMSA of the binding of MaDREB2 protein to its own promoter. Nonlabeled amplicon was added as a competitor DNA.

(Fig. 5a). Moreover, we also found that nearly 23 504 genes encompass at least one ((A/G)CC(G/C)AC) motif within the 1-kb region in front of ATG (Notes S5). The second and third motifs are only enriched in a small subset of predicted target regions, resulting in relatively low binding rates (Fig. 5b,c). It was noted that not all the MaDREB2-binding genes contain these three binding motifs, suggesting that there may be some other binding motifs for MaDREB2 in the banana genome.

In combination with the RNA-Seq data, we identified a total of 196 MaDREB2 target genes regulated during fruit ripening, with 81 genes up-regulated and 115 down-regulated (Fig. 6a; Notes S4). This suggests that MaDREB2 may act as both a transcriptional activator and repressor. It has been reported that ERF7 can interact with the histone deacetylase HDA19 in repression of stress-responsive genes in *A. thaliana* (Song *et al.*, 2005). In the present work, MaDERB2 may associate with some MaHDAC members which are required for repression of ripening-related gene expression. In addition, we showed that quite a large number of potential MaDREB2 target genes did not show altered expression during fruit ripening, suggesting that these MaDREB2 targets may be involved in other developmental processes or only expressed under certain physiological conditions.

Interestingly, MaDREB2 was found to bind to the promoters of several genes with known functions in fruit ripening, including *expansin-A8* (*MaEXP2*), *cytochrome P450-1*, *ADP-ribosylation factor*, *14-3-3* and *green-ripe like* (Notes S1). *Expansin* is a cell wall modification gene and plays important roles in fruit softening (Hiwasa *et al.*, 2003). In previous studies, we have found that the TFs LATERAL ORGAN BOUNDARIES DOMAIN proteins (MaLOBs) and MaBSD1 are involved in fruit ripening, presumably through transcriptional regulation of *MaEXP1* and *MaEXP2* (Ba *et al.*, 2014a,b). Here, we found that MaDREB2 was able to bind to the *MaEXP2* promoter (Fig. 6b; Notes S1). Using ChIP-qPCR assays, several other target genes of MaDREB2 involved in cell wall modification and aroma production were identified, including *MaEXP1*, *MaEXP3*, *MaEXP5*, *MaXET3*, *MaXET7*, *MaADH1* and *MaPDC* (Fig. 7a,b). Expression of all these genes was increased in ripe bananas (Fig. 7c), suggesting that they are involved in the ripening processes. It should be pointed out that *MaEXP1*, *MaEXP5*, *MaXET3*, *MaXET7*, *MaADH1* and *MaPDC* were not detected in our ChIP-Seq database, implying discrepancies between ChIP-Seq and ChIP-qPCR. In general, there was good agreement between these two approaches, especially in identifying highly ranked enrichment regions (Fig. S7). ChIP-Seq requires high cell numbers (*c.* 10−50 ng ChIP-enriched DNA) to generate high-quality data sets, which may limit the application of ChIP-Seq to low numbers of cells (Kaufmann *et al.*, 2010). By contrast, ChIP-qPCR technology provides greater sensitivity and has the advantage of detecting low amounts of ChIP-enriched DNA (*c.* 20−100 pg) because the signal is augmented by PCR amplification, but this method relies on current knowledge of a protein's binding pattern (Strutt & Paro, 1999).

Other potential targets of MaDREB2, such as genes encoding cytochrome P450-1, ADP-ribosylation factor and 14-3-3 proteins, are induced by ethylene and differentially expressed in postharvest banana ripening (Pua & Lee, 2003; Wang *et al.*, 2010; Li *et al.*, 2012). In addition, a *green-ripe like* (*GRL*) gene, which showed down-regulation in banana fruit ripening, is also a target of MaDREB2. The tomato *GRL* orthologs *SlGR* and *SlGRL1* were previously reported to negatively regulate ethylene signaling (Barry & Giovannoni, 2006). All these results provide evidence that MaDREB2 is involved in banana fruit ripening by directly controlling the expression of ripening-associated genes.

In addition to the ripening-associated genes, MaDREB2 also bound the genes related to stress/defense responses (Fig. 6b). Transcripts of genes associated with plant defense responses, such as pathogenesis-related chitinase, endochitinase, β-1,3-glucanase, thaumatin, stress-related homologs of metallothioneins and ascorbate peroxidases, have been reported to accumulate in the pulp of ripening bananas (Clendennen & May, 1997). Moreover, a recent proteomic study in banana indicated high accumulation of chitinases during fruit ripening, suggesting the

importance of stress/defense-related genes in fruit ripening (Toledo *et al.*, 2012). In our study, several genes encoding autophagy and programmed cell death-related proteins were also found to be bound by MaDREB2 and up-regulated in the ripe fruits (Fig. 6b).

Another interesting finding of this study is that many TF genes were enriched in the MaDREB2 targets (Fig. 6b). For example, members of the *MYB*, *C3H zinc finger*, *NAC*, *homeodomain*, *APETALA2/ethylene-responsive element binding protein* (*AP2/EREBP*), *Gibberellic Acid Insensitive* (*GAI*)/*Repressor of GA1* (*RGA*)/*Scarecrow* (*SCR*) (*GRAS*), *ARF*, *basic region leucine-zipper* (*bZIP*) and *LOB* families were highly enriched in MaDREB2 targets. *NAC* (Shan *et al.*, 2012), *AP2/EREBP* (Xiao *et al.*, 2013), *bZIP* (He *et al.*, 2013) and *LOB* (Ba *et al.*, 2014b) have been previously identified as important regulators of fruit ripening. In this study, several TFs involved in hormone signaling, such as TIFY (characterized by a conserved TIFY motif (TIFF/YXG)), *ERF* and *Auxin Response Factor* (*ARF*), and other TFs including *homeoboxes*, *Pseudo Arabidopsis response regulator-B* (*ARR-B*), *basic helix-loop-helix* (*bHLH*) and *basic transcription factor 3* (*BTF3*) were enriched in MaDREB2 targets and were down-regulated during fruit ripening. By contrast, *LOB* and *MYB* genes were up-regulated in ripening. Differential expression patterns were also observed for TFs within the same family. These results suggest a hierarchal transcriptional network in banana fruit ripening and that MaDREB2 may function as an important regulator in this regulatory cascade.

The genes encoding proteins involved in posttranslational modification (PTM) such as phosphorylation and ubiquitination were also enriched in our ChIP-Seq data (Fig. 6b), indicating that direct activation of genes involved in PTM is another function of MaDREB2. During fruit ripening, proteins that are regulated posttranslationally have been reported. For example, Ma-ACS1 is phosphorylated in ripe banana fruit, possibly by the Ser/Thr family of protein kinases (Choudhury *et al.*, 2012b). In tomato, RIN functions to affect protein ubiquitination in nuclei and several ubiquitin conjugating enzymes (E2s) in the proteasome pathway were identified as direct targets of RIN, suggesting that ubiquitination may control fruit ripening (Wang *et al.*, 2014). In this study, the MaDREB2 target genes that are related to phosphorylation and ubiquitination included 11 kinases, two phosphatases, six E3 ligases, and one E2. RNA-Seq analysis revealed that six out of the 11 protein kinase genes and one E3 ligase gene were in the up-regulated gene subset, while the remaining genes were in the down-regulated gene subset during banana ripening (Fig. 6b). Further identification of specific substrates of these protein kinases/phosphatases and E2 conjugating enzyme/E3 ligases will be of great interest for understanding the functions of posttranslational modifications in fruit ripening.

Our data indicated that *MaDREB1* to *MaDREB4* are regulated by ethylene and hence probably act downstream of ethylene action. By comparison, RIN, one of the earliest-acting ripening regulators in tomato, seems to function upstream of ethylene as its mutant fruit could not induce ethylene production and hence failed to activate ethylene signaling and promote ripening (Vrebalov *et al.*, 2002). Similarly, two RIN homologs in banana, MaMADS1/2, also act upstream of ethylene and are probably involved in fruit ripening (Elitzur *et al.*, 2010). More recently, transgenic banana plants with repressed expression of either *MaMADS1* or *MaMADS2* were established and manifested delayed-ripening behaviors, supporting the conclusion that MADS-box TFs act as key players in fruit ripening (Elitzur *et al.*, 2016). Recently, ChIP-chip and transcriptome analysis identified 241 genes as direct RIN targets, which generally contain a RIN-binding site in their promoters and participate in many biological pathways or processes during ripening, especially in ethylene production, lycopene accumulation and stress responses (Fujisawa *et al.*, 2013). A comparison of our ChIP-Seq data and the reported RIN-binding sites revealed some overlapping genes in the targets of MaDREB2 and RIN (Table S3), suggesting conserved regulatory mechanisms of ripening between fleshy fruited monocot and dicot species. For example, *expansins*, *cytochrome P450*, and *stay-green protein* (*SGR*) are the targets of both MaDREB2 and RIN (Table S3). *Receptor-like protein kinases* were also found in MaDREB2- and RIN-associated targets, indicating that fruit ripening involves multiple signal transduction pathways via protein phosphorylation. In addition, *calmodulin-binding proteins*, *glycosyl transferases*, *thioredoxins* and *pyruvate decarboxylases* were found among the targets of MaDREB2 and RIN, although their roles in ripening are unclear. Strikingly, both MaDREB2 and RIN directly target a number of TFs. RIN positively regulates 14 TFs and negatively regulates 17 TFs (Fujisawa *et al.*, 2013), while MaDREB2 in banana showed positive or negative correlation with seven and 13 TFs, respectively (Fig. 6b). Additionally, both RIN and MaDREB2 can bind to their own promoters, supporting a MaDREB2- and RIN-mediated positive feedback loop in regulating fruit ripening (Fig. 8; Martel *et al.*, 2011; Fujisawa *et al.*, 2013). These data indicate that MaDREB2 modulates fruit ripening, to some extent, similarly to RIN.

In conclusion, based on the results obtained in this study, a working model of the MaDREB2 regulatory network involved in banana fruit ripening is proposed (Fig. S9). *MaDREB2* is induced and up-regulated by exogenous or endogenous ethylene during fruit ripening, and the activation of MaDREB2 may initiate a transcriptional regulatory cascade or form a positive feedback loop to auto-amplify its own transcription. This would positively or negatively regulate the expression of its target genes involved in ripening, stress/defense responses, transcriptional regulation and posttranslational modifications, which may, in turn, directly or indirectly modulate the ripening of banana fruit.

Acknowledgements

We thank Professors Seiichiro Hasezawa, Shouyi Chen, and Junping Gao for the generous gifts of tobacco BY-2 suspension cells and the transient expression vectors, Dr Zhengkun Qiu and Dr Lin Chen for comments and suggestions during revisions, and Professor David G. Robinson (University of Heidelberg) for English language editing. This work was supported in part by grants from the National Natural Science Foundation of China (grant no. 31401922) and the China Agriculture Research

System (grant no. CARS-32-09) to South China Agricultural University (SCAU), the General Research Fund (codes 464412 and 14121915) and an AoE grant (AoE/M-05/12) from the Research Grants Council (RGC) of Hong Kong, and the Shenzhen Science & Technology Research & Development Funding – Peacock Scheme to the Chinese University of Hong Kong (CUHK).

Author contributions

W.L. and J.H. conceived and supervised the project; J.K., J.C. and X.L. planned and designed the research; J.K. performed most of the experiments and analyzed data; Y.H., Y.X., W.S. and Y.T. carried out some of the experiments; J.K., J.C., J.H. and W.L. wrote the manuscript; X.L. and K.W. gave advice and edited the manuscript.

References

Adams-Phillips L, Barry C, Giovannoni J. 2004. Signal transduction systems regulating fruit ripening. *Trends in Plant Science* **9**: 331–338.

Agarwal PK, Agarwal P, Reddy MK, Sopory SK. 2006. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. *Plant Cell Reports* **25**: 1263–1274.

Alba R, Payton P, Fei ZJ, McQuinn R, Debbie P, Martin GB, Tanksley SD, Giovannoni JJ. 2005. Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *Plant Cell* **17**: 2954–2965.

Anders S, Huber W. 2010. Differential expression analysis for sequence count data. *Genome Biology* **11**: R106.

Asif MH, Lakhwani D, Pathak S, Gupta P, Bag SK, Nath P, Trivedi PK. 2014. Transcriptome analysis of ripe and unripe fruit tissue of banana identifies major metabolic networks involved in fruit ripening process. *BMC Plant Biology* **14**: 316.

Ba LJ, Shan W, Kuang JF, Feng BH, Xiao YY, Lu WJ, Chen JY. 2014b. The banana MaLBD (LATERAL ORGAN BOUNDARIES DOMAIN) transcription factors regulate EXPANSIN expression and are involved in fruit ripening. *Plant Molecular Biology Reporter* **32**: 1103–1113.

Ba LJ, Shan W, Xiao YY, Chen JY, Lu WJ, Kuang JF. 2014a. A ripening-induced transcription factor MaBSD1 interacts with promoters of MaEXP1/2 from banana fruit. *Plant Cell Reports* **33**: 1913–1920.

Bapat VA, Trivedi PK, Ghosh A, Sane VA, Ganapathi TR, Nath P. 2010. Ripening of fleshy fruit: molecular insight and the role of ethylene. *Biotechnology Advances* **28**: 94–107.

Barry CS, Giovannoni JJ. 2006. Ripening in the tomato *Green-ripe* mutant is inhibited by ectopic expression of a protein that disrupts ethylene signaling. *Proceedings of the National Academy of Sciences, USA* **103**: 7923–7928.

Beekwilder J, Alavarez-Huerta M, Neef E, Verstappen FWA, Bouwmeester HJ, Aharoni A. 2004. Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiology* **135**: 1865–1878.

Chen F, Li B, Li G, Charron JB, Dai M, Shi X, Deng XW. 2014. *Arabidopsis* phytochrome A directly targets numerous promoters for individualized modulation of genes in a wide range of pathways. *Plant Cell* **26**: 1949–1966.

Chen JY, Liu DJ, Jiang YM, Zhao ML, Shan W, Kuang JF, Lu WJ. 2011a. Molecular characterization of a strawberry FaASR in relation to fruit ripening. *PLoS ONE* **6**: e24649.

Chen L, Zhong HY, Kuang JF, Li JG, Lu WJ, Chen JY. 2011b. Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. *Planta* **234**: 377–390.

Choudhury SR, Roy S, Nag A, Singh SK, Sengupta DN. 2012a. Characterization of an AGAMOUS-like MADS Box protein, a probable constituent of flowering and fruit ripening regulatory system in banana. *PLoS ONE* **7**: e44361.

Choudhury SR, Roy S, Sengupta DN. 2012b. A Ser/Thr protein kinase phosphorylates MA-ACS1 (*Musa acuminata* 1-aminocyclopropane-1-carboxylic acid synthase 1) during banana fruit ripening. *Planta* **236**: 491–511.

Clendennen SK, May GD. 1997. Differential gene expression in ripening banana fruit. *Plant Physiology* **115**: 463–469.

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**: 3674–3676.

D'Hont A, Denoeud F, Aury JM, Baurens FC, Carreel F, Garsmeur O, Noel B, Bocs S, Droc G, Rouard M *et al.* 2012. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* **488**: 213–217.

Ebrahimi M, Abdullah SNA, Aziz MA, Namasivayam P. 2015. A novel CBF that regulates abiotic stress response and the ripening process in oil palm (*Elaeis guineensis*) fruits. *Tree Genetics and Genomes* **11**: 56.

Elitzur T, Vrebalov J, Giovannoni JJ, Goldschmidt EE, Friedman H. 2010. The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene. *Journal of Experimental Botany* **61**: 1523–1535.

Elitzur T, Yakir E, Quansah L, Fei Z, Vrebalov J, Khayat E, Giovannoni JJ, Friedman H. 2016. Banana MaMADS transcription factors are necessary for fruit ripening and molecular tools to promote shelf-life and food security. *Plant Physiology* **71**: 380–391.

Fowler S, Thomashow MF. 2002. *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14**: 1675–1690.

Fujisawa M, Nakano T, Shima Y, Ito Y. 2013. A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. *Plant Cell* **25**: 371–386.

Fujisawa M, Shima Y, Nakagawa H, Kitagawa M, Kimbara J, Nakano T, Kasumi T, Ito Y. 2014. Transcriptional regulation of fruit ripening by tomato FRUITFULL homologs and associated MADS-Box proteins. *Plant Cell* **26**: 89–101.

Gilchrist DA, Fargo DC, Adelman K. 2009. Using ChIP-chip and ChIP-seq to study the regulation of gene expression: genome-wide localization studies reveal widespread regulation of transcription elongation. *Methods* **48**: 398–408.

Giovannoni JJ. 2004. Genetic regulation of fruit development and ripening. *Plant Cell* **16**: s170–s180.

Giovannoni JJ. 2007. Fruit ripening mutants yield insights into ripening control. *Current Opinion in Plant Biology* **10**: 283–289.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q *et al.* 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* **29**: 644–652.

Hao YJ, Song QX, Chen HW, Zou HF, Wei W, Kang XS, Ma B, Zhang WK, Zhang JS, Chen SY. 2010. Plant NAC-type transcription factor proteins contain a NARD domain for repression of transcriptional activation. *Planta* **232**: 1033–1043.

He S, Shan W, Kuang JF, Xie H, Xiao YY, Lu WJ, Chen JY. 2013. Molecular characterization of a stress-response bZIP transcription factor in banana. *Plant Cell, Tissue and Organ Culture* **113**: 173–187.

Heyndrickx KS, de Velde JV, Wang C, Weigel D, Vandepoele K. 2014. A functional and evolutionary perspective on transcription factor binding in *Arabidopsis thaliana*. *Plant Cell* **26**: 3894–3910.

Hiwasa K, Kinugasa Y, Amano S, Hashimoto A, Nakano R, Inaba A, Kubo Y. 2003. Ethylene is required for both the initiation and progression of softening in pear (*Pyrus communis* L.) fruit. *Journal of Experimental Botany* **54**: 771–779.

Huang FC, Do YY, Huang PL. 2006. Genomic organization of a diverse ACC synthase gene family in banana and expression characteristics of the gene member involved in ripening of banana fruit. *Journal of Agricultural and Food Chemistry* **54**: 3859–3868.

Inaba A, Liu X, Yokotani N, Yamane M, Lu WJ, Nakano R, Kubo Y. 2007. Differential feedback regulation of ethylene biosynthesis in pulp and peel tissues of banana fruit. *Journal of Experimental Botany* **258**: 1047–1057.

Ishida T, Hattori S, Sano R, Inoue K, Shirano Y, Hayashi H, Shibata D, Sato S, Kato T, Tabata S *et al.* 2007. *Arabidopsis* TRANSPARENT TESTA GLABRA2 is directly regulated by R2R3 MYB transcription factors and is

involved in regulation of GLABRA2 transcription in epidermal differentiation. *Plant Cell* **19**: 2531–2543.

Kaufmann K, Muino JM, Østeras M, Farinelli L, Krajewski P, Angenent GC. 2010. Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP). *Nature Protocols* **5**: 457–472.

Kuang JF, Chen JY, Luo M, Wu KQ, Sun W, Jiang YM, Lu WJ. 2012. Histone deacetylase HD2 interacts with ERF1 and is involved in longan fruit senescence. *Journal of Experimental Botany* **63**: 441–454.

Lakhwani D, Pandey A, Dhar YK, Bag SK, Trivedi PK, Asif MH. 2016. Genome-wide analysis of the AP2/ERF family in *Musa* species reveals divergence and neofunctionalisation during evolution. *Scientific Reports* **6**: 18878.

Lata C, Prasad M. 2011. Role of DREBs in regulation of abiotic stress responses in plants. *Journal of Experimental Botany* **62**: 4731–4748.

Li MY, Xu BY, Liu JH, Yang XL, Zhang JB, Jia CH, Ren LC, Jin ZQ. 2012. Identification and expression analysis of four 14-3-3 genes during fruit ripening in banana (Musa acuminata L. AAA group, cv. Brazilian). *Plant Cell Reports* **31**: 369–378.

Li R, Yu C, Lam TW, Yiu SM, Kristiansen K, Wang J. 2009. SOAP2: an improved ultrafast tool for short read alignment. *Bioinformatics* **25**: 1966–1967.

Lin ZF, Hong YG, Yin MG, Li CY, Zhang K, Grierson D. 2008. A tomato HD-Zip homeobox protein, LeHB-1, plays an important role in floral organogenesis and ripening. *Plant Journal* **55**: 301–310.

Liu M, Pirrello J, Chervin C, Roustan JP, Bouzayen M. 2015b. Ethylene control of fruit ripening: revisiting the complex network of transcriptional regulation. *Plant Physiology* **169**: 2380–2390.

Liu L, Zinkgraf M, Petzold HE, Beers EP, Filkov V, Groover A. 2015a. The *Populus* ARBORKNOX1 homeodomain transcription factor regulates woody growth through binding to evolutionarily conserved target genes of diverse function. *New Phytologist* **205**: 682–694.

Lu WJ, Nakano R, Kubo Y, Inaba A, Jiang YM. 2004. Cloning and expression analysis of an XET cDNA in the peel and pulp of banana fruit ripening and softening. *Acta Botanical Sinica* **46**: 355–362.

Maere S, Heymans K, Kuiper M. 2005. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* **21**: 3448–3449.

Manning K, Tor M, Poole M, Hong Y, Thompson AJ, King GJ, Giovannoni JJ, Seymour GB. 2006. A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nature Genetics* **38**: 948–952.

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ. 2011. The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. *Plant Physiology* **157**: 1568–1579.

Mbéguié-A-Mbéguié D, Hubert O, Baurens FC, Matsumoto T, Chillet M, Fils-Lycaon B, Sidibe-Bocs S. 2009. Expression patterns of cell wall-modifying genes from banana during fruit ripening and in relationship with finger drop. *Journal of Experimental Botany* **60**: 2021–2034.

Mizuno S, Hirasawa Y, Sonoda M, Nakagawa H, Sato T. 2006. Isolation and characterization of three DREB/ERF-type transcription factors from melon (*Cucumis melo*). *Plant Science* **170**: 1156–1163.

Mota RV, Cordenunsi BR, Nascimento JRO, Purgatto E, Rosseto MRM, Lajolo FM. 2002. Activity and expression of banana starch phosphorylases during fruit development and ripening. *Planta* **216**: 325–333.

Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006. Genome-wide analysis of the ERF gene family in *Arabidopsis* and rice. *Plant Physiology* **140**: 411–432.

Nakashima K, Ito Y, Yamaguchi-Shinozaki K. 2009. Transcriptional regulatory networks in response to abiotic stresses in *Arabidopsis* and grasses. *Plant Physiology* **149**: 88–95.

Pua EC, Lee YC. 2003. Expression of a ripening-related cytochrome P450 cDNA in Cavendish banana (*Musa acuminata* cv.Williams). *Gene* **305**: 133–140.

Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP. 2011. Integrative genomics viewer. *Nature Biotechnology* **29**: 24–26.

Seymour GB, Ostergaard L, Chapman NH, Knapp S, Martin C. 2013. Fruit development and ripening. *Annual Review of Plant Biology* **64**: 219–241.

Shan W, Kuang JF, Chen L, Xie H, Peng HH, Xiao YY, Li XP, Chen WX, He QG, Chen JY *et al.* 2012. Molecular characterization of banana NAC transcription factors and their interactions with ethylene signalling component EIL during fruit ripening. *Journal of Experimental Botany* **63**: 5171–5187.

Song CP, Agarwal M, Ohta M, Guo Y, Halfter U, Wang P, Zhu JK. 2005. Role of an *Arabidopsis* AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. *Plant Cell* **17**: 2384–2396.

Stockinger EJ, Gilmour SJ, Thomashow MF. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Sciences, USA* **94**: 1035–1040.

Strutt H, Paro R. 1999. Mapping DNA target sites of chromatin proteins *in vivo* by formaldehyde crosslinking. *Methods in Molecular Biology* **119**: 455–467.

Tadesse TN. 2014. Quality attributes and ripening period of banana (*Musa* spp.) fruit as affected by plant ethylene sources and packaging materials. *International Journal of Agricultural Research* **9**: 304–311.

Thomashow MF. 2010. Molecular basis of plant cold acclimation: insights gained from studying the CBF cold response pathway. *Plant Physiology* **154**: 571–577.

Toledo TT, Nogueira SB, Cordenunsi BR, Gozzo FC, Pilau EJ, Lajolo FM, do Nascimento JRO. 2012. Proteomic analysis of banana fruit reveals proteins that are differentially accumulated during ripening. *Postharvest Biology and Technology* **70**: 51–58.

Van Buskirk HA, Thomashow MF. 2006. Arabidopsis transcription factors regulating cold acclimation. *Physiologia Plantarum* **126**: 72–80.

Vrebalov J, Ruezinsky D, Padmanabhan V, White R, Medrano D, Drake R, Schuch W, Giovannoni J. 2002. A MADS-box gene necessary for fruit ripening at the tomato *ripening-inhibitor* (*Rin*) locus. *Science* **296**: 343–346.

Wan CY, Wilkins TA. 1994. A modified hot borate method significantly enhances the yield of high quality RNA from cotton (*Gossypium hirsutum* L.). *Analytical Biochemistry* **223**: 7–12.

Wang Y, Wang W, Cai J, Zhang Y, Qin G, Tian S. 2014. Tomato nuclear proteome reveals the involvement of specific E2 ubiquitin-conjugating enzymes in fruit ripening. *Genome Biology* **15**: 548.

Wang Y, Wu J, Xu BY, Liu JH, Zhang JB, Jia CH, Jin ZQ. 2010. Cloning of an ADP-ribosylation factor gene from banana (*Musa acuminata*) and its expression patterns in postharvest ripening fruit. *Journal of Plant Physiology* **167**: 989–995.

Xiao YY, Chen JY, Kuang JF, Shan W, Xie H, Jiang YM, Lu WJ. 2013. Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes. *Journal of Experimental Botany* **64**: 2499–2510.

Yamaguchi-Shinozaki K, Shinozaki K. 1994. A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell* **6**: 251–264.

Yang X, Song J, Fillmore S, Pang X, Zhang Z. 2011. Effect of high temperature on color, chlorophyll fluorescence and volatile biosynthesis in green-ripe banana fruit. *Postharvest Biology and Technology* **62**: 246–257.

Yin XR, Allan AC, Chen KS, Ferguson IB. 2010. Kiwifruit *EIL* and *ERF* genes involved in regulating fruit ripening. *Plant Physiology* **153**: 1280–1292.

Zhang Y, Liu T, Meyer CA, Eeckhoute J, Johnson DS, Bernstein BE, Nussbaum C, Myers RM, Brown M, Li W *et al.* 2008. Model-based analysis of ChIP-Seq (MACS). *Genome Biology* **9**: R137.

Zhao K, Shen X, Yuan H, Liu Y, Liao X, Wang Q, Liu L, Li F, Li T. 2013. Isolation and characterization of dehydration-responsive element-binding factor 2C (MsDREB2C) from *Malus sieversii* Roem. *Plant and Cell Physiology* **54**: 1415–1430.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Alignment of MaDREB amino acid sequences with DREBs from other plants.

Fig. S2 Phylogenetic alignment of MaDREB1–MaDREB9 with DREBs from other plants.

Fig. S3 qRT-PCR analysis of *MaDREB5–MaDREB9* in pulp during four ripening treatments: natural (control), ethylene-induced, 1-MCP-delayed, and a combination of 1-MCP and ethylene-treated ripening.

Fig. S4 Preparation of MaDREB2 antibody.

Fig. S5 Specificities of MaDREB2 antibody.

Fig. S6 Preparation of ChIP DNA samples recovered with MaDREB2 antibody.

Fig. S7 Validation of ChIP-Seq reads using ChIP-qPCR assays.

Fig. S8 EMSA validation of MaDREB2 binding to *MaEXP1*, *MaXET7*, *MaADH1* and *MaPDC* promoters.

Fig. S9 A postulated model of transcriptional regulatory network for MaDREB2-regulated fruit ripening in banana.

Table S1 Primers used in this study

Table S2 Overview of the ChIP-Seq results for MaDREB2 in three biological repeats

Table S3 Some common genes among the targets of MaDREB2 and RIN

Notes S1 List of MaDREB2-binding peaks from three biological replicates.

Notes S2 Detailed gene ontology (GO) analysis of MaDREB2-binding genes.

Notes S3 List of MaDREB2-binding motifs.

Notes S4 List of DEGs in unripe and ripe banana fruit and their combination with ChIP-Seq data.

Notes S5 List of banana genes that contain conserved binding motif 1 within the 1-kb region in front of ATG.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.