

RESEARCH PAPER

# Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes

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## Abstract

The involvement of ethylene response factor (ERF) transcription factor (TF) in the transcriptional regulation of ethylene biosynthesis genes during fruit ripening remains largely unclear. In this study, 15 ERF genes, designated as *MaERF1–MaERF15*, were isolated and characterized from banana fruit. These MaERFs were classified into seven of the 12 known ERF families. Subcellular localization showed that MaERF proteins of five different subfamilies preferentially localized to the nucleus. The 15 *MaERF* genes displayed differential expression patterns and levels in peel and pulp of banana fruit, in association with four different ripening treatments caused by natural, ethylene-induced, 1-methylcyclopropene (1-MCP)-delayed, and combined 1-MCP and ethylene treatments. *MaERF9* was upregulated while *MaERF11* was downregulated in peel and pulp of banana fruit during ripening or after treatment with ethylene. Furthermore, yeast-one hybrid (Y1H) and transient expression assays showed that the potential repressor *MaERF11* bound to *MaACS1* and *MaACO1* promoters to suppress their activities and that *MaERF9* activated *MaACO1* promoter activity. Interestingly, protein–protein interaction analysis revealed that MaERF9 and -11 physically interacted with MaACO1. Taken together, these results suggest that MaERFs are involved in banana fruit ripening via transcriptional regulation of or interaction with ethylene biosynthesis genes.

**Key words:** Banana, ERF, ethylene biosynthesis, fruit ripening, interaction, transcriptional regulation.

## Introduction

Ethylene is a simple gaseous plant hormone involved in many plant physiological processes, including fruit ripening (Guo and Ecker, 2004). Fruit ripening is a complex and genetically programmed process that results in marked changes in colour, flavour, aroma, texture, and nutritional value of the flesh (Giovannoni, 2004). These changes are the result of the coordinated activation of multiple genetic and biochemical pathways, which are influenced by internal and external cues regulated by many critical transcription factors (TFs) (Martel *et al.*, 2011). Ethylene response factor (ERFs) are plant-specific and belong to the AP2/EREBP-type TFs which function as *trans*-acting factors at the last step of ethylene signalling (El-Sharkawy *et al.*,

2009; Lin *et al.*, 2009; Klee and Giovannoni, 2011). Many ERF proteins are important in plant responses to developmental cues and stresses by specifically interacting with the GCC box (a core sequence of AGCCGCC) and/or dehydration-responsive elements/C-repeat (DRE/CRT, a core sequence of CCGAC) presented in the promoters of many ethylene-responsive genes, demonstrating that a transcriptional cascade is involved in ethylene signalling or biosynthesis (El-Sharkawy *et al.*, 2009; Zhang *et al.*, 2009; Yin *et al.*, 2010). Although accumulating studies have shown that ERF proteins play an important role in fruit ripening (Lin *et al.*, 2009; Klee and Giovannoni, 2011) and multiple ERF

proteins have been characterized in many crops (Xu *et al.*, 2011) and some fruits, such as tomato (Li *et al.*, 2007; Zhang *et al.*, 2009), apple (Wang *et al.*, 2007), plum (El-Sharkawy *et al.*, 2009), kiwifruit (Yin *et al.*, 2010), and longan (Kuang *et al.*, 2012), little information is available about the ERF proteins in transcriptional regulation of ethylene biosynthesis in relation to fruit ripening.

Ethylene synthesis starts from methionine, which is first converted to S-adenosylmethionine by S-adenosylmethionine synthetase. S-Adenosylmethionine is subsequently metabolized to the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS), a rate-limiting step in ethylene biosynthesis. ACC is then oxidized by ACC oxidase (ACO) to generate ethylene in a reaction that also produces CO<sub>2</sub> and hydrogen cyanide (Yang and Hoffman, 1984). Previous studies have revealed that the expression of ACS and ACO is related to ethylene production in most cases, including fruit ripening (Alexander and Grierson, 2002). It has been well documented that ethylene biosynthesis is modulated by many factors or regulators at both transcriptional and post-transcriptional levels (Chae *et al.*, 2003; Liu and Zhang, 2004; Wang *et al.*, 2004; Joo *et al.*, 2008; Zhang *et al.*, 2009; Prasad *et al.*, 2010; Wan *et al.*, 2011). For instances, an E3 ubiquitin ligase ETO1, targets type-2 ACS enzymes for degradation, thereby inhibiting its activity (Chae *et al.*, 2003). A member of the RING E3 ligase XBAT32 negatively modulates the abundance of ACS proteins and ethylene biosynthesis (Prasad *et al.*, 2010). ACS protein stability is also regulated by protein phosphorylation and dephosphorylation during ethylene induction (Joo *et al.*, 2008; Hahn and Harter, 2009). These reports suggest that modulation of ethylene biosynthesis at the protein level is crucial for ethylene production. In addition, transcriptional regulation mediated by TFs also plays an important role in ethylene biosynthesis. Transcription factors such as tomato RIN, LeERF2, tobacco TERF1, and rice OsDERF1, modulate the expression of ethylene biosynthesis genes whereas downstream stress-responsive genes have been reported recently (Ito *et al.*, 2008; Zhang *et al.*, 2009; Wan *et al.*, 2011; Qin *et al.*, 2012). These investigations demonstrate that transcriptional regulation is a pivotal mechanism in controlling ethylene synthesis. However, it is unclear whether and how ethylene biosynthesis is transcriptionally modulated during economic fruit ripening.

Increasing evidence suggests that the control of climacteric fruit ripening relies largely on the modulation of ethylene production and/or action, and autoregulation of ethylene biosynthesis at the transcriptional level and regulation in ACS and ACO genes is only one consequence of ethylene response in ripening fruits (Inaba *et al.*, 2007; Zhang *et al.*, 2009; Yin *et al.*, 2010). Banana (*Musa acuminata*, AAA group), a typical climacteric fruit, is characterized by a peak in ethylene production that orchestrates ripening-associated processes (Clendennen and May, 1997). Some genes associated with ethylene biosynthesis and perception pathways have been isolated from banana fruit, including ACS, ACO, ethylene receptor, a CTR1 orthologue, and ethylene insensitive 3-like genes (Clendennen *et al.*, 1997; Liu *et al.*, 1999; Inaba *et al.*, 2007; Mbégué-A-Mbeguie *et al.*, 2008). Like many other

climacteric fruits, banana ethylene biosynthesis and perception genes are differentially regulated at the transcriptional level during fruit ripening (Do *et al.*, 2005; Inaba *et al.*, 2007; Mbégué-A-Mbeguie *et al.*, 2008; Choudhury *et al.*, 2008). Very recently, a banana fruit Mcm1-agamous-deficiens-S (MADS) 5 TF was identified to bind to CArG-box sequence in the promoters of major ripening genes, including *MaACS1* and *MaACO1*, to be involved in fruit ripening through directly regulating these ripening genes (Choudhury *et al.*, 2012a). In addition, banana *MaACS1* is reported to be phosphorylated by a Ser/Thr protein kinase during fruit ripening (Choudhury *et al.*, 2012b). These reports provide important novel evidence of transcriptional and post-translational regulations of *MaACS1* in relation to fruit ripening. However, the mechanism of transcriptional regulation of the key ethylene biosynthetic genes *MaACS1* and *MaACO1* during banana fruit ripening is still largely unknown. In the present study, 15 ERF TFs were isolated and characterized from banana fruit. Their expression patterns in association with four different ripening treatments were analysed. Moreover, the interactions of the ripening-related MaERFs with *MaACS1* and *MaACO1* were investigated.

## Materials and methods

### Plant materials and treatments

Pre-climacteric banana (*M. acuminata* AAA group, cv. Cavendish) fruit at 75–80% maturation were obtained from a local commercial plantation near Guangzhou, China. Four postharvest treatments were performed, including a control (natural ripening), ethylene-induced ripening (100 µl ethylene l<sup>-1</sup>, 18 h), 1-methylcyclopropene (1-MCP)-delayed ripening (0.5 µl 1-MCP l<sup>-1</sup>, 18 h), and a combination of 1-MCP with ethylene treatment (1-MCP + ethylene), as described previously (Shan *et al.*, 2012). After each treatment, fruit were held at 22 °C and 90% relative humidity until production of climacteric ethylene and complete ripening. For each treatment, samples were taken based on the rate of ethylene production and fruit firmness changes during ripening as described by Shan *et al.* (2012). All of the samples were frozen in liquid nitrogen after sampling, and then stored at –80 °C for further use. All assessments were conducted using three biological replicates.

### RNA extraction, gene isolation, and sequence analysis

Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the hot borate method of Wan and Wilkins (1994). Potentially contaminating DNA was eliminated by treatment with DNase I digestion using an RNase-free kit (Promega Madison, WI, USA). The DNA-free total RNA was then used as template for reverse-transcription PCR. The first-strand cDNA of the product was subjected to PCR amplification.

According to gene annotation and bioinformatics analysis, 15 ERF genes, termed *MaERF1–MaERF15*, were isolated from a transcriptome database obtained using a high-throughput Solexa/Illumina sequencing platform (Beijing Genomics Institute, Shenzhen, China) and their sequences were verified by further cloning and sequencing. Four of the 15 ERF genes, *MaERF1*, -3, -9, and -12, were full-length sequences in the database, with complete start and stop codes, while the full-length sequences of the other 11 ERF genes were obtained by 3'- or 5'-rapid amplification of cDNA ends (RACE) using a RACE kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. The specific primers used for RACE are provided in Supplementary Table S1 (available at *JXB* online).

Alignments were carried out using CLUSTALX (version 1.83) and GeneDoc software, and a phylogenetic tree was constructed using the minimum-evolution method with MEGA5.

#### Quantitative real-time PCR analysis

Isolation of total RNA from the samples and synthesis of first-strand cDNA were performed as described above. The synthesized cDNA was diluted 1:40 with water, and 2 µl of the diluted cDNA was used as a template for quantitative real-time PCR (qPCR). Reactions were performed in a total volume of 20 µl, containing 1 µl each 10 µM primer and 10 µl SYBR Green PCR Supermix on CFX96 Real-Time PCR System (Bio-Rad Laboratories) according to the manufacturer's instructions. The cycling program included an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of 94 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. No-template controls for each primer pair were included in each run. The oligonucleotide primers for qPCR analysis were designed on the basis of the 3'-untranslated region using Primer 5.0 software. The sequences of all primers used for qPCR analysis are described in [Supplementary Table S2](#). Ribosomal protein 2 and clathrin adaptor complex were selected as reference genes under different experimental conditions according to this group's previous study on the selection of reliable reference genes for expression study by qPCR in banana fruit ([Chen et al., 2011](#)). Each assay using the gene-specific primers amplified a single product of the correct size with high efficiency (90–110%). All qPCRs were normalized using the cycle threshold ( $C_t$ ) value corresponding to the reference gene. The relative expression levels of the targeted genes were calculated using the formula  $2^{-\Delta\Delta C_t}$ . The values represent the mean of three biological replicates.

#### Subcellular localization of MaERF proteins

The coding sequences of *MaERFs* without the stop codon were amplified by PCR (primers are listed in [Supplementary Table S3](#)), and then subcloned into the pBI221-GFP vector, in frame with the green fluorescent protein (GFP) sequence, resulting in 35S::gene-GFP vectors under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The fusion constructs and the control GFP vector were used for transient assays using a modified polyethylene glycol (PEG) transfection method with tobacco BY-2 suspension culture cell protoplasts as described previously ([Abel and Theologis, 1994](#)). GFP fluorescence was observed with a fluorescence microscope (Axioskop 2 Plus, Zeiss). All transient expression assays were repeated at least three times.

#### Yeast one-hybrid (Y1H) assay

Yeast one-hybrid assay was carried out by using the Matchmaker Gold Yeast One-Hybrid System (Clontech). According to the reported sequences of the *MaACSI* and *MaACOI* promoters ([Wang and Peng, 2001a,b](#)), the *MaACSI* and *MaACOI* promoters were cloned into pAbAi (primers are listed in [Supplementary Table S4](#)). Plasmid was linearized and transformed into Y1H Gold strain. Positive yeast cells were then transformed with pGADT7-AD, which contained *MaERF9* or *MaERF11*. The DNA-protein interaction was determined based on the growth ability of the cotransformants on SD/-Leu medium with aureobasidin A, according to the manufacturer's protocol.

#### Transient expression assay and GUS activity analysis

The reporter construct was generated using full-length of *MaACSI* and *MaACOI* promoter sequences in the binary vector pBI101 cloned into the *SalI* and *BamHI* upstream sites of the GUS reporter gene. For the transient expression assay, tobacco (*Nicotiana benthamiana*) leaves were co-infiltrated with *Agrobacterium* GV3101 containing the reporter vector and the effector vector containing *MaERF9* or *-11* in the binary vector pCambia 1300 using the

*SalI* and *BamHI* sites as described by [Yu et al. \(2012\)](#). After 48 h of growth in the chamber, the infected leaves were used to analyse the GUS activity. The plant proteins were extracted and their fluorescence was measured as described by [Jefferson et al. \(1987\)](#), using a fluorometer (VersaFluor Fluorometer, Bio-Rad, Hercules, CA, USA). The aliquot at time zero was used as the control. The protein concentration was determined using a protein assay kit (Bio-Rad). The primers used in the construction of the effector and reporter vectors are listed in [Supplementary Table S5](#).

#### Yeast two-hybrid (Y2H) assay

Y2H assays were performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech). The coding regions of *MaERF9* and *-11*, *MaACSI* and *MaACOI* were cloned into pGADT7 and pGBKT7 to fuse with the activation domain (AD) and DNA-binding domain (DBD), respectively, to create different baits and preys (primers are shown in [Supplementary Table S6](#)). *MaACSI* and *MaACOI* did not show any transcriptional activation activity in yeast cells (data not shown). Different pairs of bait and prey constructs were then co-transformed into yeast strain Gold Y2H using the lithium acetate method. Yeast cells grew on a SD/-Leu/-Trp according to the manufacturer's protocol (Clontech) for 3 d. Transformed colonies were plated onto minimal medium quadruple dropout (SD medium with -Leu/-Trp/-His/-Ade) containing 125 µM aureobasidin A and 4 mg ml<sup>-1</sup> X-α-Gal at 30 °C to test for possible interactions between *MaERF9/MaERF11* and *MaACSI/MaACOI* by their growth status and α-galactosidase activities.

#### Bimolecular fluorescence complementation assay

To generate constructs for the bimolecular fluorescence complementation (BiFC) assays, the full-length coding sequences of *MaERF9* and *-11*, *MaACSI* and *MaACOI* without their stop codons were subcloned into the pUC-pSPYNE or pUC-pSPYCE vectors. The expression of target genes alone was used as negative controls. The resulting constructs were used for transient assays using a modified PEG transfection of tobacco BY-2 suspension culture cell protoplasts as described above. The transformed protoplasts then grew in liquid MS medium containing 0.4 M sucrose for 24–48 h, and transfected cells were imaged using a fluorescence microscope with a yellow fluorescent protein (YFP) filter (Axioskop 2 Plus). The primers used in the BiFC assay are listed in [Supplementary Table S7](#).

#### Statistical analysis

The experiment was arranged in a completely randomized design. Each sample time point for each treatment was comprised of three independent biological replicates. Data were plotted as means ± standard errors (SE) in figures. Least significant difference (LSD) at the 5% level was analysed by DPS software (version 3.01; Zhejiang University, Hangzhou, China). A complete linkage hierarchical clustering of 15 banana fruit *MaERF* genes was generated using the Pearson clustering algorithm according to their expression profiles.

## Results

### Sequence analysis of 15 *MaERF* genes

This study isolated 15 novel *ERF* full-length cDNAs from banana fruit, designated *MaERF1* to *MaERF15*. The sizes of the deduced amino acid sequences differed substantially (e.g. *MaERF3* codes 432 amino acids, while *MaERF11* codes 183 amino acids) ([Supplementary Fig. S1](#)). The sequence similarities of the *MaERFs* varied from 18.0% (*MaERF3* and *MaERF6*) to 76.7% (*MaERF12* and *MaERF13*)



(Supplementary Table S8). Alignments of the full-length deduced proteins of MaERFs and AterFs showed conserved motifs, including a DNA-binding domain designated the ERF domain (ranging from 58 to 59 amino acids, Supplementary Figs. S1A, B and S2), which is a defining character of the ERF transcriptional factor gene family (Fujimoto *et al.*, 2000; Nakano *et al.*, 2006; Yin *et al.*, 2010). In addition, MaERF10–MaERF15 also exhibited a repressor domain, the ERF-associated amphiphilic repression domain (EAR) located at the 3'-end of the sequence (Supplementary Fig. S1A, C).

Phylogenetic analysis of MaERFs, AterFs, and other fruit ERFs, including tomato, apple, and longan, revealed that MaERF sequences were clustered into seven out of 12 groups of ERF proteins (Nakano *et al.*, 2006) (Supplementary Fig. S3). MaERF1 and MaERF2 are classified as members of subfamily IXc, MaERF5 and -6 belong to subfamily X, and MaERF7 and -8 belong to subfamily VII. MaERF3, -4, and -9 are assigned to subfamilies I, IXb, and II ERFs respectively. MaERF10–MaERF15 belong to subfamily VIII. Overall, these data suggest that MaERF1–MaERF15 may exhibit diverse functions.

#### Nuclear localization of five MaERF proteins

Potential nuclear localization signals (NLS) sequences were predicted for all banana ERFs based on the sequence analysis. To examine the subcellular localization of MaERFs *in vivo*, five *MaERF* genes from different subfamilies, including *MaERF1* (subfamily IXc), *MaERF4* (subfamily IXb), *MaERF5* (subfamily X), *MaERF9* (subfamily II), and *MaERF11* (subfamily VIII) were selected, and then the full-length coding sequences of these five *MaERF* genes were fused in frame with the GFP gene. Transient expression of these constructs in tobacco BY-2 protoplasts showed that the fluorescence of all these MaERF proteins was localized exclusively in the nucleus (Fig. 1). By contrast, the GFP control showed ubiquitous distribution of GFP signal in the whole cells. Similar results were also obtained in tobacco leaves (Supplementary Fig. S4).

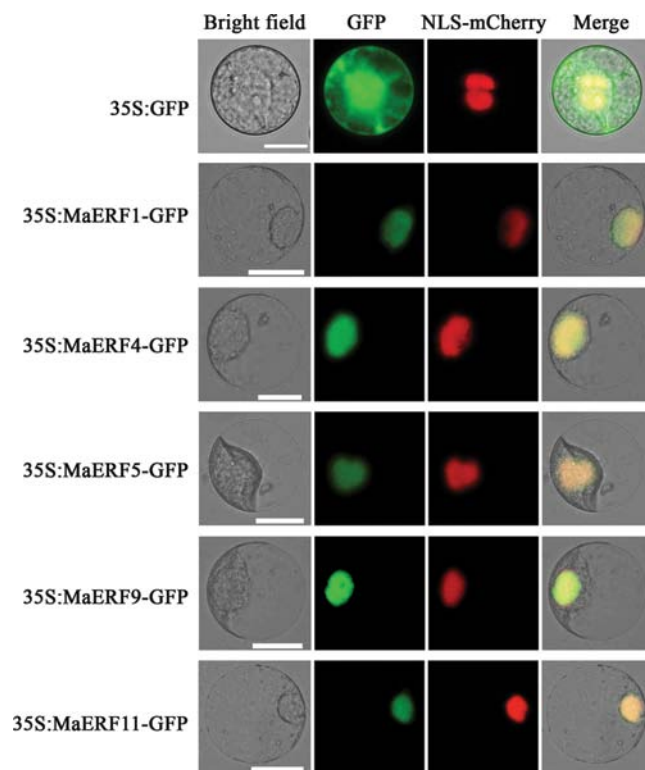
#### Differential expression of *MaERF* genes in peel and pulp during fruit ripening

The data related with fruit ripening and softening, including changes in fruit firmness and ethylene production in banana fruit in four different ripening treatments caused by natural, ethylene-induced, 1-MCP-delayed, and combined 1-MCP and ethylene treatments, have been described in Shan *et al.* (2012). Ethylene treatment accelerated fruit ripening and promoted a climacteric rise, with a peak appearance at 3 d. Control natural fruit started the ethylene climacteric at 15 d and reached a peak at 21 d. In contrast, 1-MCP treatment delayed ethylene production, with a peak appearance at day 30. In addition, application of ethylene to the 1-MCP-treated fruit promoted ethylene evolution, with the ethylene production starting to increase at day 25 and then reaching a peak at day 28 (Shan *et al.*, 2012).

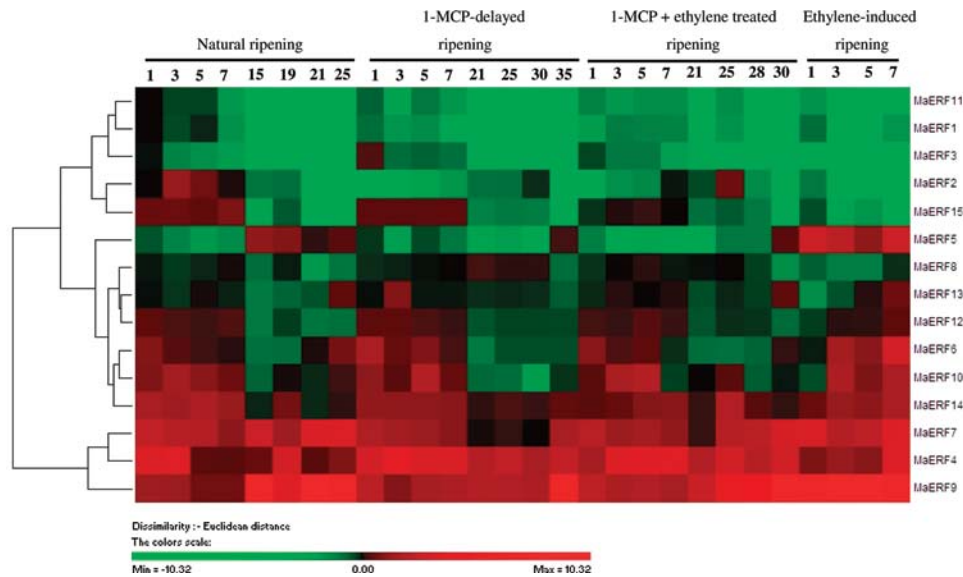
The expression patterns of *MaERF1*–*MaERF15* in peel and pulp of banana fruit with the four different ripening

treatments were investigated by qPCR. Figs. 2 and 3 showed complicated expression patterns of *MaERF* genes. In the peel, among the 15 *MaERF* genes, *MaERF4*, -7, and -9 showed increased transcript levels, followed by the increase in ethylene production at about 15, 1, 25, and 25 d of storage in natural, ethylene-induced, 1-MCP-delayed ripening, or combined 1-MCP and ethylene-treated fruit, respectively. A more marked increase was observed for *MaERF9* transcript level (Fig. 2 and Supplementary Fig. S5). In contrast, *MaERF1*, -2, -3, -8, -11, and -15 transcript levels decreased after ethylene treatment, following the increase in ethylene production, with a more obvious decrease for *MaERF1* and -11 transcript levels (Fig. 2 and Supplementary Fig. S5). In addition, *MaERF5*, -6, -10, -12, -13, and -14 were induced in ethylene-treated fruit, while their transcript levels in natural, 1-MCP-delayed ripening, or combined 1-MCP and ethylene-treated fruit decreased or changed only slightly following the increase in ethylene production (Fig. 2 and Supplementary Fig. S5).

In the pulp, transcript levels of *MaERF3*, -7, -9, and -14 were higher in ethylene-treated fruit, and their transcript levels



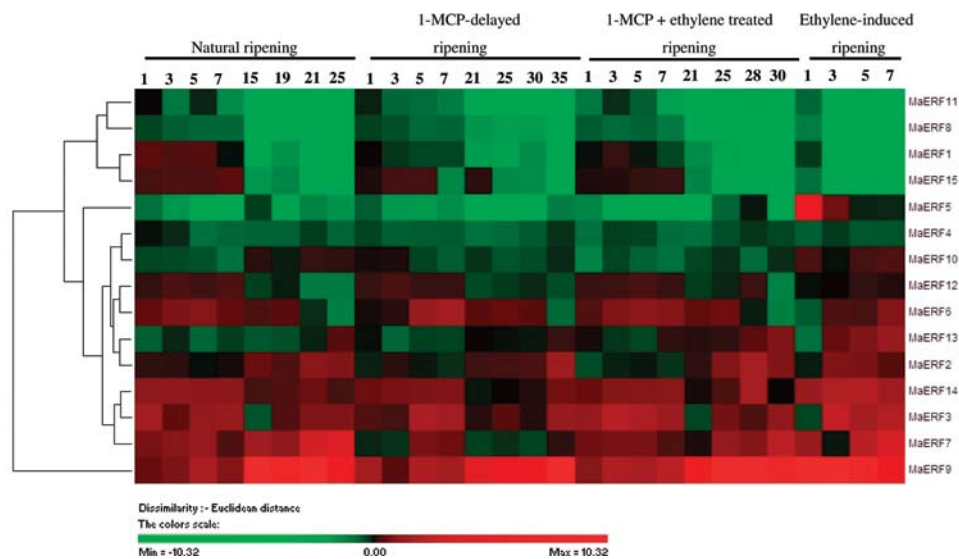
**Fig. 1.** Subcellular localization of MaERFs in tobacco BY-2 protoplasts. Protoplasts were transiently transformed with MaERF–GFP constructs or GFP vector using a modified PEG method. GFP fluorescence was observed with a fluorescence microscope. VirD2NLS-mCherry was included in each transfection to 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 outline of the cell and the merged were photographed in a bright field. Bars, 25  $\mu$ m (this figure is available in colour at JXB online).



**Fig. 2.** Agglomerative hierarchical cluster analysis of transcript levels from 15 *MaERF* genes in peel of banana fruit with four different ripening characteristics caused by natural (control), ethylene-induced, 1-MCP-delayed, and combined 1-MCP and ethylene treatments. The cluster was generated using the Pearson clustering algorithm according to gene expression profile analysis by qPCR. Data are log<sub>2</sub>-transformed (or  $-\Delta\Delta Ct$ ) value of gene expression of each time point compared to day 0 of control fruit. The scale indicates log<sub>2</sub> variations: red, increase; green, decrease. Means  $\pm$  SE from three repeats are provided in [Supplementary Fig. S5](#). The physiology data related with fruit ripening and softening, including changes in fruit firmness and ethylene production in banana fruit with these four different ripening characteristics has been described in [Shan et al. \(2012\)](#) (this figure is available in colour at [JXB](#) online).

in natural, ethylene-induced, 1-MCP-delayed ripening, or combined 1-MCP and ethylene-treated fruit clearly increased with ethylene evolution, with a more obvious accumulation for *MaERF9* (Fig. 3 and [Supplementary Fig. S6](#)). *MaERF2*,

*-6*, *-10*, *-12*, and *-13* transcript levels slightly increased in ethylene-treated fruit, and their transcript levels in natural, 1-MCP-delayed ripening, or combined 1-MCP and ethylene-treated fruit decreased or changed only slightly during



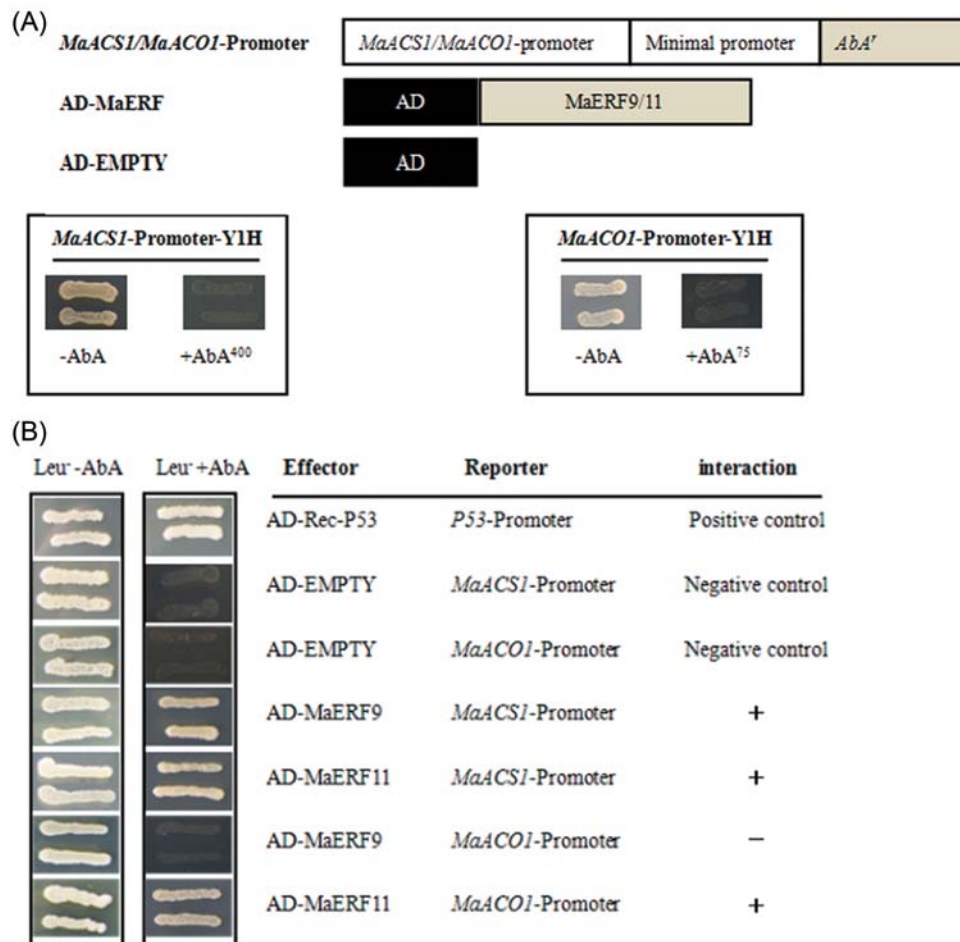
**Fig. 3.** Agglomerative hierarchical cluster analysis of transcript levels from 15 *MaERF* genes in banana fruit pulp with four different ripening characteristics caused by natural (control), ethylene-induced, 1-MCP-delayed, and combined 1-MCP and ethylene treatments. The cluster was generated using the Pearson clustering algorithm according to gene expression profile analysis by qPCR. Data are log<sub>2</sub>-transformed (or  $-\Delta\Delta Ct$ ) value of gene expression of each time point compared to day 0 of control fruit. The scale indicates log<sub>2</sub> variations: red, increase; green, decrease. Means  $\pm$  SE from three repeats are provided in [Supplementary Fig. S6](#). The physiology data related with fruit ripening and softening, including changes in fruit firmness and ethylene production in banana fruit with these four different ripening characteristics has been described in [Shan et al. \(2012\)](#) (this figure is available in colour at [JXB](#) online).

ripening (Fig. 3 and Supplementary Fig. S6). *MaERF1*, -4, -5, -8, -11, and -15 transcript levels decreased after ethylene treatment, following the increase in ethylene production, with more remarkable decrease for *MaERF1*, -11, and -15 (Fig. 3 and Supplementary Fig. S6). Based on these 15 *MaERFs* expression patterns during fruit ripening, two most ripening-related banana *ERF* genes, including ripening-induced *MaERF9* and ripening-downregulated *MaERF11*, were selected and focused upon for the following analysis.

#### Interaction of *MaERF9* and -11 with promoters of *MaACS1* and *MaACO1*

In previous studies, banana fruit *MaACS1* and *MaACO1* have been reported as key genes involved in ethylene biosynthesis. In peel and pulp tissues, *MaACS1* and *MaACO1* were ripening- and ethylene-induced genes during fruit ripening (Inaba et al., 2007). Similar to the previous report, this study also showed that *MaACS1* and *MaACO1* in peel and pulp tissues were ethylene-induced, and that their transcript levels increased with ethylene evolution during fruit ripening (data not shown).

Recently, several TFs, such as tomato RIN, LeERF2, tobacco TERF1, and rice OsDERF1, have been reported to modulate the expression of ethylene biosynthesis genes (Ito et al., 2008; Zhang et al., 2009; Wan et al., 2011; Qin et al., 2012). Therefore, the current study decided to investigate whether ripening-induced *MaERF9* and ripening-downregulated *MaERF11* could regulate *MaACS1* and *MaACO1* in banana fruit ripening. A yeast one-hybrid assay based on the Matchmaker Gold Yeast One-Hybrid System was first performed for this purpose. The promoters of *MaACS1* and *MaACO1* were cloned in front of the reporter gene *AURI-C*, an antibiotic resistance gene that confers aureobasidin A resistance in yeast. No basal activities of the *MaACS1* and *MaACO1* promoters were detected in yeast (Fig. 4A). After the Y1H reporter strains were transformed with plasmids carrying cassettes constitutively expressing *MaERF9* and -11 effectors, yeast cells harbouring *MaACS1* promoter grew well in the presence of aureobasidin A (Fig. 4B). While the yeast cells harbouring the *MaACO1* promoter grew well when co-transformed with *MaERF11* effector, but could not grow when co-transformed with *MaERF9* (Fig. 4B). These observations indicate that



**Fig. 4.** Yeast one-hybrid (Y1H) analysis of *MaERF9* and -11 binding to *MaACS1* and *MaACO1* promoters. (A) No basal activities of *MaACS1* and *MaACO1* promoters were detected in yeast grown on SD medium lacking Leu in the presence of 400 and 75 ng ml<sup>-1</sup> aureobasidin A, respectively. (B) Yeast growth assays after the Y1H reporter strains were transformed with plasmids carrying cassettes constitutively expressing *MaERF9* and -11 effectors. Interaction was determined based on the ability of transformed yeast to grow on SD medium lacking Leu in the presence of aureobasidin A.



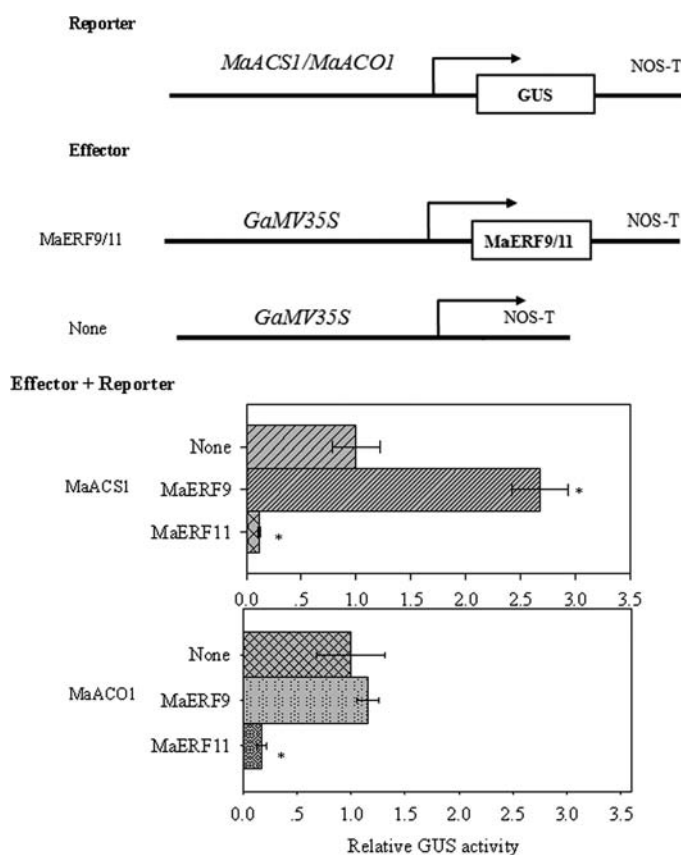
MaERF11 can bind to *MaACS1* and *MaACO1* promoters, while MaERF9 only binds to *MaACS1* promoter in yeast. Next, the tobacco transient cotransformational system was used to further confirm MaERF9 and -11 binding to the promoters of *MaACS1* and *MaACO1* *in vivo*. The full-length promoters of *MaACS1* and *MaACO1* were used to drive the GUS reporter gene, and the open reading frames of *MaERF9* and -11 were overexpressed under the control of the 35S promoter. Each promoter–GUS construct was then co-transferred with either 35S:MaERF9 and 35S:MaERF11, respectively, through *Agrobacterium tumefaciens* transfection. The interaction of MaERF9 with the *MaACS1* promoter resulted in a 3.27-fold enhancement of GUS activity, but no significant change was found with the *MaACO1* promoter (Fig. 5). However, a marked decrease in GUS activity was observed to the interaction of MaERF11 with the *MaACS1* or *MaACO1* promoter (Fig. 5). These results not only indicate that MaERF11 can bind to *MaACS1*

and *MaACO1* promoters, and MaERF9 binds to *MaACS1* promoter, but also suggest that MaERF9 might directly activate the transcription of *MaACS1* by interacting with the *MaACS1* promoter. In addition, MaERF11 might act as transcription repressors of *MaACS1* and *MaACO1* by suppressing their promoter activities.

#### Physical interactions between MaERF9 and -11 and *MaACS1* and *MaACO1*

The interactions between MaERF9 and -11 and *MaACS1*/*MaACO1* were investigated using the Matchmaker Gold Yeast Two-Hybrid System. *MaACS1*, *MaACO1* and MaERF9 and -11 coding sequences were subcloned into pGADT7 and pGBKT7 vectors for the Y2H assay, respectively. As shown in Fig. 6, yeast cells co-transformed with a positive control (pGBKT7-53 + pGADT7-T) and MaERF9 or MaERF11 with *MaACO1*, could grow on selective medium (synthetic medium lacking tryptophan, leucine, histidine, and adenine) supplied with the toxic drug aureobasidin A, and turned blue in the presence of the chromogenic substrate X- $\alpha$ -Gal, indicating that MaERF9 or MaERF11 physically interacted with *MaACO1*. However, yeast cells harbouring MaERF9 or MaERF11 with *MaACS1*, and the negative controls, could not grow on the selective medium and did not turn blue under the same conditions (Fig. 6).

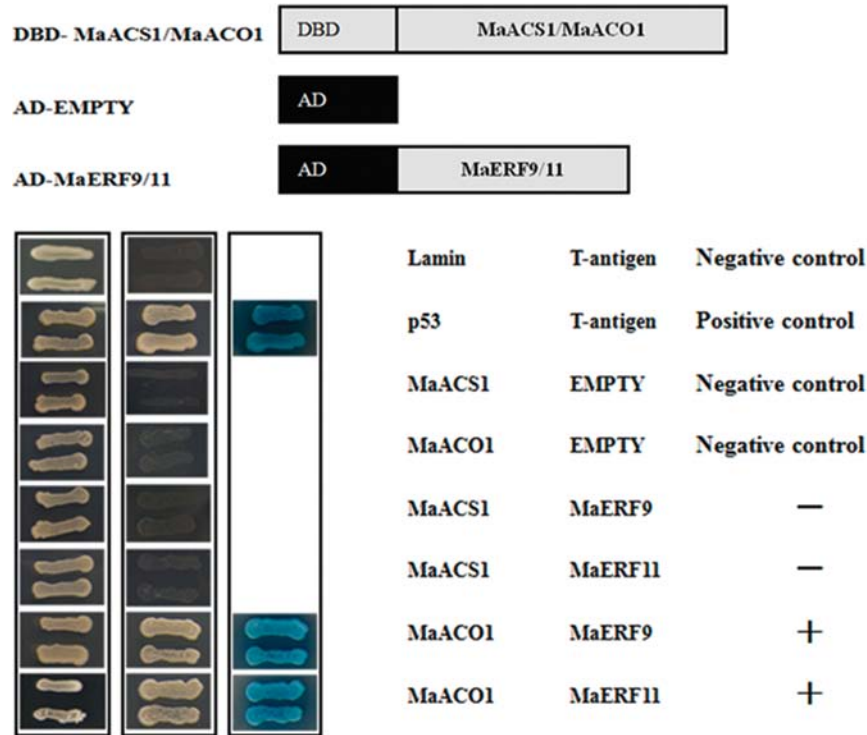
To further confirm the interactions between MaERF9 and -11 and *MaACO1* observed in the Y2H assay, this study performed a BiFC assay in tobacco BY-2 protoplasts. MaERF9 or MaERF11 tagged with pSPYNE (split YFP N-terminal fragment expression) and *MaACO1* tagged with pSPYCE (split YFP C-terminal fragment expression) were transiently co-expressed in tobacco BY-2 protoplasts following PEG transfection. A robust YFP fluorescent signal was detected in the nucleus of BY-2 cells which expressed MaERF9-pSPYNE or MaERF11-pSPYNE and *MaACO1*-pSPYCE (Fig. 7). No YFP fluorescent signal was observed either in the cells which expressed MaERF9 or -11-pSPYNE with only the pSPYCE or in those expressed *MaACO1*-pSPYCE with only the pSPYNE (Fig. 7). Similar results were also observed when MaERF9/MaERF11-pSPYCE were co-transfected with *MaACO1*-pSPYNE (Fig. 7). These results of Y2H and BiFC assays not only demonstrate *in vitro* and *in vivo* interactions between MaERF9 and -11 and *MaACO1*, but also the specific localization of the interacting proteins in the nucleus.



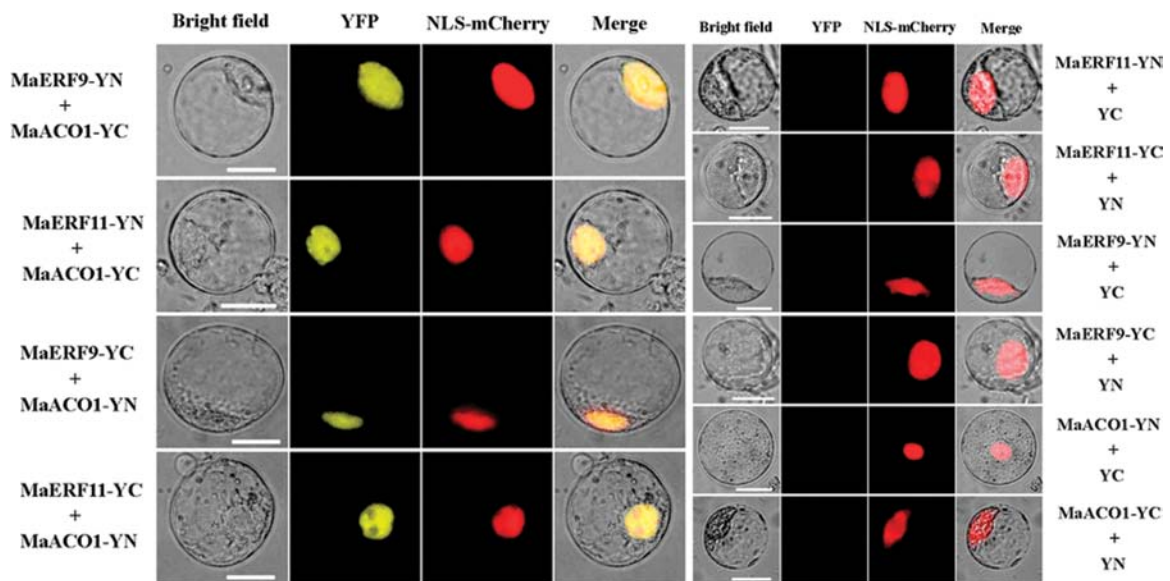
**Fig. 5.** Binding of *MaACS1* and *MaACO1* promoters to MaERF9 and -11 *in vivo* transient expression assay. The top panel shows the schematics of the GUS reporter vectors containing the *MaACS1* and *MaACO1* promoters and the MaERF9 and -11 effector vectors. The bottom panel shows the interactions of MaERF9 and -11 with *MaACS1* and *MaACO1* promoters through the activity of GUS. Asterisks indicate a significant difference at the 5% level compared to the leaves with no effector. Value are means  $\pm$  SE of three replicates.

## Discussion

ERFs are plant-specific TFs that bind to conserved motifs in promoter regions of target genes, thus regulating the expression of genes involved in the response to ethylene. ERFs comprise one of the largest TF families, with 122 members in *Arabidopsis* and 139 in rice (Nakano *et al.*, 2006). Although sequence identity can be as low as 13% among these different ERFs, all ERFs exhibited a highly conserved AP2/ERF DNA-binding domain of 57–66 amino acids (Nakano *et al.*, 2006; Yin *et al.*, 2010). In this study, based on a transcriptome



**Fig. 6.** Physical interactions between MaERF9 and -11 and MaACS1 and MaACO1 detected in an Y2H assay. The coding regions of *MaERF9* and -11 were cloned into the pGADT7 vector to create the AD-MaERF9 and -11 constructs, while the coding regions of *MaACS1* and *MaACO1* were cloned into the pGBKT7 vector to create the DBD-MaACS1/MaACO1 constructs. Gold Y2H yeast strains were co-transformed with DBD-MaACS1/MaACO1 and AD-MaERF9 and -11, respectively. The ability of yeast cells to grow on synthetic medium lacking tryptophan, leucine, histidine, and adenine but containing 125 μM aureobasidin A and to turn blue in the presence of the chromagenic substrate X-α-Gal was scored as a positive interaction. Yeast cells transformed with pGBKT7-53 + pGADT7-T, DBD-MaACS1 + pGADT7-T, DBD-MaACO1 + pGADT7-T, or pGBKT7-Lam + pGADT7-T were included as positive or negative controls, respectively (this figure is available in colour at JXB online).



**Fig. 7.** BiFC visualization of the MaERF9/MaERF11 and MaACO1 interactions in transiently co-expressed tobacco BY-2 protoplasts. MaERF9/MaERF11 and MaACO1 proteins were fused with the N (YN) and C (YC) termini of YFP, respectively. Expression of MaERF9/MaERF11 or MaACO1 alone was used as a negative control. VirD2NLS-mCherry was included in each transfection to serve as a control for successful transfection, as well as for nuclear localization. YFP fluorescence is yellow; the merged image is a digital merge of bright field and fluorescent images. Bars, 25 μm (this figure is available in colour at JXB online).



database, 15 banana fruit *ERF* genes, designated *MaERF1* to *MaERF15*, were cloned. Similar to the previous reports (Nakano *et al.*, 2006; Yin *et al.*, 2010; Liu *et al.*, 2011), alignment of the 15 MaERFs proteins showed that they shared a highly conserved ERF domain (Supplementary Fig. S1A, B), although they were highly diverse in size, ranging from 183 to 482 amino acids. The lowest similarity observed was 18% while the highest similarity observed was 76% (Supplementary Fig. S2 and Table S8). The 15 MaERFs fell into seven out of the 12 different groups of the previously characterized ERF proteins (Nakano *et al.*, 2006; Yin *et al.*, 2010) (Supplementary Fig. S3). In subfamily VIII, MaERF10 to MaERF15 also had an EAR repressor domain (Supplementary Fig. S1C), suggesting that these five members might act as transcription repressors (Ohta *et al.*, 2001). Consistent with their role as TFs, all the 15 MaERFs have basic amino acid regions that potentially serve as NLS to target the proteins to the nucleus. Nuclear localization was further confirmed by transient expression of five MaERFs from different subfamilies in tobacco BY-2 protoplasts or leaves (Fig. 1 and Supplementary Fig. S4).

As one of the important TFs in the ethylene pathway, the involvement of the ERF gene family in the regulation of fruit ripening has been well studied. Subfamilies III and IV of ERFs have been reported to be associated with stress responses (Sakuma *et al.*, 2006; Qin *et al.*, 2008), while subfamilies VII, VIII, and IX are ethylene responsive (Tournier *et al.*, 2003; Yang *et al.*, 2005; Yin *et al.*, 2010). Moreover, subfamily VII genes have been particularly associated with fruit ripening and the genes of tomato *LeERF2* (Tournier *et al.*, 2003), apple *MdERF1* (Wang *et al.*, 2007), plum *PsERF2a/PsERF2b* (El-Sharkawy *et al.*, 2009), and kiwifruit *AdERF4/AdERF6* (Yin *et al.*, 2010) accumulate during this developmental process. However, no clear and consistent pattern in mRNA level changes were observed in the kiwifruit *ERFs* across the three subfamilies VII, VIII, and IX. The transcript levels of subfamily VII member *AdERF5*, subfamily VIII members *AdERF7/AdERF8*, and subfamily IX members *AdERF11–13* decreased during ripening (Yin *et al.*, 2010). In the present work, similar to the report of Yin *et al.* (2010), gene expression profiles in banana fruit with four different ripening treatments (natural ripening, ethylene-induced ripening, 1-MCP-delayed ripening, and 1-MCP and ethylene-treated ripening) revealed that banana *MaERF* genes were expressed in intricate patterns in peel and pulp during fruit ripening (Figs. 2 and 3 and Supplementary Figs. S5 and S6). Among the 15 banana *ERF* genes, subfamily VII member *MaERF7* and subfamily II member *MaERF9*, were apparently upregulated by ethylene in peel and pulp, showing strong correlation to the increase in ethylene production associated with the ripening climacteric. The transcript levels in peel and pulp of subfamily IX member *MaERF1*, subfamily VII member *MaERF8*, and subfamily VIII members *MaERF11* and *-15* decreased after ethylene treatment, following the increase in ethylene production, with a more obvious decrease for *MaERF11* than the other *MaERFs* (Figs. 2 and 3 and Supplementary Figs. S5 and S6). In addition, other banana *ERF* genes, changed slightly or showed different expression patterns in banana fruit peel and pulp

with four different ripening treatments (Figs. 2 and 3 and Supplementary Figs. S5 and S6). It has been shown that most reported fruit ERFs are induced in natural ripening or by ethylene treatment (Thara *et al.*, 1999; Tournier *et al.*, 2003; Huang *et al.*, 2004; Wang *et al.*, 2007; El-Sharkawy *et al.*, 2009). Recently, mRNA levels of some *ERF* genes, including seven kiwifruit *ERFs*, two petunia *ERFs*, and two longan *ERFs*, decreased during ripening or senescence (Yin *et al.*, 2010; Liu *et al.*, 2011; Kuang *et al.*, 2012). However, the mechanism of these ripening-downregulated *ERFs* involved in ripening was not studied. Therefore, this study chose to investigate what was considered to be the two most ripening-related banana *ERF* genes (*MaERF9* and *-11*) and their possible role in banana fruit ripening, including the mechanism of their action.

Recently, a few key transcriptional regulators involved in the transcriptional regulation of *ACS* and *ACO* genes were identified in tomato, such as RIN-MADS is a MADS-box transcription factor that binds to the promoter of *ACS2*, *ACS4*, and other ripening-associated genes (Qin *et al.*, 2012). In tomato, HB-1, a homeodomain-zip homeobox TF, directly interacts with the promoter of *ACO1*, which is responsible for the activation of *ACO1* expression during ripening (Lin *et al.*, 2008). Ethylene response factor 2 (ERF2), on the other hand has been shown to interact with the GCC box of the *ACO3* promoter (Zhang *et al.*, 2009). A negative regulator of ethylene biosynthesis *ERF* gene *AP2a* has also been identified, as repression of *AP2a* expression resulting in an overproduction of ethylene (Chung *et al.*, 2010; Karlova *et al.*, 2011). Downregulation of ethylene biosynthesis by ethylene-induced ERFs would mean that ethylene turns down its own production during fruit ripening, similar to observations made on system 1 and system 2 ethylene biosynthesis (Van de Poel *et al.*, 2012). Recently, banana fruit MADS5 TF is also identified to bind to CArG-box sequence in the promoters of major ripening genes, including *MaACS1* and *MaACO1* (Choudhury *et al.*, 2012a). However, it remains unclear whether banana fruit ERF TF directly regulate the expression of *MaACS1* and *MaACO1* during fruit ripening. In the present investigation, *in vitro* and *in vivo* assays showed that MaERF9 and -11 could bind to the *MaACS1* promoter and MaERF11 could bind to the *MaACO1* promoter (Figs. 4 and 5), providing insight into the role of the ERF TF in the transcriptional regulation of ethylene synthesis through regulating *MaACS1* and *MaACO1*. Importantly, it was observed that MaERF9 activated the promoter of *MaACS1*, while MaERF11 which includes an EAR repressor domain, repressed the promoters of *MaACS1* and *MaACO1* (Fig. 5). This suggests that MaERF9 and -11 may be, respectively, positive and negative regulators in the transcriptional regulation of ethylene production during fruit ripening. To date, no report has shown ERF protein interaction with *ACS* or *ACO*. The finding in this study that banana MaERF9 and -11 interacted with *MaACO1* *in vitro* and *in vivo* (Figs. 6 and 7) established such interaction. It will be interesting to investigate whether the interaction of MaERF9 and -11 with *MaACO1* is similar to that of DELLA with *SCARECROW-LIKE 3* (*SCL3*) in *Arabidopsis*. In *Arabidopsis* seedlings, *SCL3* is a

direct target gene of DELLA, whose expression is induced by DELLA and repressed by gibberellin (GA), moreover, *SCL3* autoregulates its own transcription by directly interacting with DELLA, indicating that *SCL3* seems to act as an attenuator of DELLA proteins (Zhang *et al.*, 2011). However, further studies are required to assess the biological significance of the interaction between MaERF and MaACO1.

In summary, 15 banana fruit *MaERF* genes were isolated and characterized. Gene expression profiles in banana fruit exposed to four different ripening treatments clearly revealed that *MaERF* genes are expressed differentially in peel and pulp tissues during fruit ripening. Yeast one-hybrid and transient expression assays showed that MaERFs bind *MaACS1* and *MaACO1* promoters and can activate or repress their activities during fruit ripening. MaERFs were shown to physically interact with MaACO1. Taken together, these results suggest that MaERFs may be involved in banana fruit ripening via transcriptional regulation of or interaction with ethylene biosynthesis genes. The study provides some new information in helping to understand the regulatory network of ERF TF in fruit ripening.

## Supplementary material

Supplementary data are available at *JXB* online.

[Supplementary Table S1](#). Primers used for RACE.

[Supplementary Table S2](#). Primers used for quantitative real-time PCR analysis.

[Supplementary Table S3](#). Primers used for fusing GFP.

[Supplementary Table S4](#). Primers used for Y1H assay.

[Supplementary Table S5](#). Primers used for transient expression assay.

[Supplementary Table S6](#). Primers used for Y2H analysis.

[Supplementary Table S7](#). Primers used for BiFC assays.

[Supplementary Table S8](#). Sequence similarities among the different MaERFs genes.

[Supplementary Fig. S1](#). Schematic analysis of MaERFs with ERF domains and EAR repressor domains.

[Supplementary Fig. S2](#). Amino acid sequence alignment of the MaERF proteins.

[Supplementary Fig. S3](#). Phylogenetic tree of ERFs.

[Supplementary Fig. S4](#). Subcellular localization of MaERFs in tobacco leaves.

[Supplementary Fig. S5](#). Expression of MaERFs genes in banana fruit peel with four different ripening treatments.

[Supplementary Fig. S6](#). Expression of MaERFs genes in banana fruit pulp with four different ripening treatments.

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