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RESEARCH PAPER

# Molecular characterization of banana NAC transcription factors and their interactions with ethylene signalling component EIL during fruit ripening

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## Abstract

**The plant-specific NAC (NAM, ATAF1/2, and CUC2) transcription factors (TFs) play important roles in plant growth, development, and stress responses. However, the precise role of NAC TFs in relation to fruit ripening is poorly understood. In this study, six *NAC* genes, designated *MaNAC1–MaNAC6*, were isolated and characterized from banana fruit. Subcellular localization showed that MaNAC1–MaNAC5 proteins localized preferentially to the nucleus, while MaNAC6 was distributed throughout the entire cell. A transactivation assay in yeast demonstrated that MaNAC4 and MaNAC6, as well as their C-terminal regions, possessed *trans*-activation activity. Gene expression profiles in fruit with four different ripening characteristics, including natural, ethylene-induced, 1-methylcyclopropene (1-MCP)-delayed, and a combination of 1-MCP with ethylene treatment, revealed that the *MaNAC* genes were differentially expressed in peel and pulp during post-harvest ripening. *MaNAC1* and *MaNAC2* were apparently upregulated by ethylene in peel and pulp, consistent with the increase in ethylene production. In contrast, *MaNAC3* in peel and pulp and *MaNAC5* in peel were constitutively expressed, and transcripts of *MaNAC4* in peel and pulp and *MaNAC6* in peel decreased, while *MaNAC5* or *MaNAC6* in pulp increased slightly during fruit ripening. Furthermore, the *MaNAC2* promoter was activated after ethylene application, further enhancing the involvement of *MaNAC2* in fruit ripening. More importantly, yeast two-hybrid and bimolecular fluorescence complementation analyses confirmed that MaNAC1/2 physically interacted with a downstream component of ethylene signalling, ethylene insensitive 3 (EIN3)-like protein, termed MaEIL5, which was downregulated during ripening. Taken together, these results suggest that MaNACs such as MaNAC1/MaNAC2, may be involved in banana fruit ripening via interaction with ethylene signalling components.**

**Key words:** banana, EIL, fruit ripening, interaction, NAC, regulation.

## Introduction

Fruit ripening is a complex and genetically programmed process that results in marked changes in colour, flavour, aroma, texture, and nutritional content 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, including regulation by many critical

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AD, activation domain; BiFC, bimolecular fluorescence complementation; CaMV, cauliflower mosaic virus; DBD, DNA-binding domain; EIN3, ethylene insensitive 3; EIL, ethylene insensitive 3-like; GFP, green fluorescent protein; 1-MCP, 1-methylcyclopropene; MADS, Mcm1-Agamous-Deficiens-S; NAC, NAM ATAF1/2 and CUC2; PEG, polyethylene glycol; NLS, nuclear localization signal; pI, isoelectric point; qPCR, quantitative real-time PCR; TF, transcription factor; Y2H, yeast two-hybrid; YFP, yellow fluorescent protein.

transcription factors (TFs) (Martel *et al.*, 2011). Numerous plant TFs such as apetalous (AP2), basic leucine zipper domain (bZIP), myeloblastosis oncogene (MYB), myelocytomatosis oncogene (MYC), WRKY, Mcm1-Agamous-Deficiens-Srf (MADS), heat-shock TF (HSF), and several classes of Cys2/His2-type zinc finger proteins, have been identified and characterized according to their DNA-binding motifs (Yamasaki *et al.*, 2008). The NAC, named after NAM (no apical meristem), ATAF1,2, and CUC2 (cup-shaped cotyledon), constitutes one of the largest families of plant-specific TFs, with 106 members in *Arabidopsis* (Gong *et al.*, 2004), 149 members in rice (Xiong *et al.*, 2005), and 101 members in the soybean genome (Pinheiro *et al.*, 2009). The distinguishing characteristic of NAC TFs is possession of a highly conserved N-terminal NAC domain, which is divided into five conserved subdomains (A–E), and a variable transcriptional regulation C terminus, which is able to activate or repress the transcription of multiple target genes (Duval *et al.*, 2002; Hao *et al.*, 2010).

NAC TFs have been implicated to contribute to diverse and vital physiological processes, including shoot apical meristem development (Hibara *et al.*, 2003), floral morphogenesis (Sablowski and Meyerowitz, 1998), leaf formation and senescence (Berger *et al.*, 2009; Balazadeh *et al.*, 2011; Zhang and Gan, 2012), embryo development (Larsson *et al.*, 2011), seed development (Verza *et al.*, 2011), flavonoid biosynthesis (Morishita *et al.*, 2009), lateral root development (He *et al.*, 2005), grain nutrient remobilization (Waters *et al.*, 2009), shoot branching determination (Mao *et al.*, 2007), and secondary cell-wall biosynthesis (Zhao *et al.*, 2010; Zhong *et al.*, 2010). Moreover, some NAC TFs have been reported to participate in hormone signalling mediated by auxin (Xie *et al.*, 2000), ethylene (He *et al.*, 2005), abscisic acid (Fujita *et al.*, 2004; Gao *et al.*, 2010), gibberellic acid (Aida *et al.*, 1997; Kim *et al.*, 2008), salicylic acid (Delessert *et al.*, 2005), methyl jasmonate (Bu *et al.*, 2008; Yoshii *et al.*, 2010), and cytokinin (Kim *et al.*, 2007), as well as in the response to biotic and abiotic stresses such as fungus infection, drought, cold, high salinity, and mechanical wounding (He *et al.*, 2005; Meng *et al.*, 2009; Xia *et al.*, 2010; Nakashima *et al.*, 2012). These stress inducible NAC TFs have been shown to bind to *cis*-regulatory elements, termed NACRSs (NAC recognition sequences), upstream of other stress responsive genes and activate their expression (Tran *et al.*, 2004). Furthermore, a few NAC TFs are differentially regulated by various pathways, including microRNA-mediated cleavage of mRNAs and ubiquitination-mediated proteolysis (Greve *et al.*, 2003; Berger *et al.*, 2009). Although the functions of NAC TFs in many plants have been studied extensively, the involvement of NAC TFs in relation to ripening of economic fruits has generally received less attention.

Banana (*Musa acuminata*, AAA group), a typical climacteric fruit, is one of the most important food crops after rice, maize, and wheat in tropical and subtropical countries. It comprises an important part in the diet of millions of people around the world. Commercially, it is a very prosperous crop in the world trade. The post-harvest physiology of banana fruit is characterized by a pre-climacteric phase, followed by a peak in ethylene production that orchestrates ripening-associated processes, including climacteric respiration, pulp softening, peel degreening, and the production of aroma compounds (Clendennen and May, 1997). However, the physiological climacteric attribute of banana fruit results in a short post-harvest life of 10–15 d at ambient temperature (Bapat *et al.*, 2010). In addition, unlike many other climacteric fruits, the peel and pulp of banana fruit exhibit different ethylene biosynthesis patterns (Dominguez and Vendrell, 1993; Elitzur *et al.*, 2010). For these reasons, it is imperative to gain a better understanding of the mechanism of fruit ripening, as this will aid in improving the quality and storage potential of this fruit. Some genes associated with ethylene biosynthesis and perception pathways have been identified in banana fruit, including 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, ACC oxidase, ethylene receptor, CTR1 orthologue, and ethylene insensitive 3-like (*EIL*) genes (Clendennen *et al.*, 1997; Liu *et al.*, 1999; Mbéguié-A-Mbeguié *et al.*, 2008). However, there is little information about how ripening-related TFs regulate the ripening of banana fruit in relation to ethylene signalling. Recently, several *MaMADS* TFs were reported to be induced by ethylene, suggesting that the *MADS* TF may participate in banana fruit ripening (Liu *et al.*, 2009; Elitzur *et al.*, 2010). Moreover, current research into fruit ripening is likely to focus on the identification of new candidate genes, as well as understanding the interactions among the already characterized components (Kumar *et al.*, 2012). *NOR* (non-ripening) is an NAC-domain TF whose mutation results in a non-ripening phenotype including ethylene synthesis, increased respiration, carotenoid accumulation, softening, and aroma volatile production (Martel *et al.*, 2011). Furthermore, *NAC* is also ethylene inducible (He *et al.*, 2005; Xia *et al.*, 2010) and may represent a TF downstream of the ethylene insensitive 2 gene (*EIN2*) in parallel with *EIN3* (Kim *et al.*, 2009; Al-Daoud and Cameron, 2011). Thus, it is interesting to study the transcriptional regulatory network concerning *NAC* in relation to the ethylene signal during fruit ripening. In the present study, six *NAC* TFs were isolated and characterized from banana fruit, and the expression patterns of six *MaNAC* genes in the fruit of four different ripening characteristics, including natural, ethylene-induced, 1-methylcyclopropene (1-MCP, a competitive inhibitor of ethylene action)-delayed, and a combination of 1-MCP with ethylene treatment (1-MCP+ethylene), were analysed by real-time quantitative PCR. In addition, the promoter of one ripening-related *NAC*, *MaNAC2*, was isolated and its response to ethylene was also investigated. More importantly, the direct interactions between MaNAC1/MaNAC2 and MaEIL5, a downstream component of ethylene signalling, were detected by yeast two-hybrid and the bimolecular fluorescence complementation (BiFC) assays. Our results suggest that *MaNAC* genes may be involved in banana fruit ripening via interaction with ethylene signalling components.

## Materials and methods

### *Plant materials and treatments*

Pre-climacteric banana (*Musa acuminata*, AAA group, cv. Carvendish) fruit at the 75–80% plump stage were obtained from a local commercial plantation near Guangzhou, south-eastern China. The hands were separated into individual fingers, and fruit of uniform weight, shape, and maturity, as well as being free of visual defects, were selected. The fruit were first surface sterilized by dipping in a 1% hypochloride solution for 1 min and then immersed in 0.05% Sporgon (with 46% Prochloraz–Mn; Aventis, Valencia, Spain) for 3 min to prevent fungal disease. They were then allowed to air dry at 25 °C for 2 h and treated as follows.

The selected banana fruit were randomly divided into four groups of 150 fingers each for the following treatments. Fifteen unsealed polyethylene plastic bags (0.01 mm in thickness) of ten fingers each were used for each treatment, and samples were taken based on the rate of ethylene production and fruit firmness change during ripening. Group 1 (control, non-conditioned for natural ripening) fruit were stored directly at 22 °C and 90% relative humidity for 25 days; samples were taken at 0, 1, 3, 5, 7, 15, 19, 21, and 25 days until the fruit ripened completely. Fruit of group 2 were treated with 100 µl $l^{-1}$ ethylene for 18 h in a closed chamber and then stored at 22 °C for 7 days; samples were taken at 0, 1, 3, 5, and 7 days of storage. Fruit of group 3 were treated with 0.5 µl $l^{-1}$ 1-MCP for 18 h and then stored at 22 °C for 35 days; samples were taken at 0, 1, 3, 5, 7, 21, 25, 30, and 35 days of storage. Fruit of group 4 were treated with 100 µl $l^{-1}$ ethylene followed by 0.5 µl $l^{-1}$ 1-MCP for 18 h, and then stored at 22 °C for 30 days and samples were taken at 0, 1, 3, 5, 7, 15, 17, 19, 21, 25, 28, and 30 days of storage.

All of the samples were frozen in liquid nitrogen immediately after sampling, and stored at –80 °C for further use. All assessments were conducted using three biological replicates.

### *Fruit ripening evaluations*

Fruit ripening was evaluated using the following two parameters: ethylene production and fruit firmness. Ethylene production was determined in three replicates each containing three fruit at the given sampling times. The three fruit were placed in a 2 l flask and capped with a rubber stopper for 2 h at 22 °C. A 1 ml sample of headspace gas was sampled and analysed for ethylene using gas chromatography (Model GC-17A; Shimadzu Co., Kyoto, Japan) fitted with a flame ionization detector and an activated alumina column (200 cm×0.3 cm), with an injector temperature of 120 °C, column temperature of 60 °C, and detector temperature of 60 °C, according to the method of Wang *et al.* (2006).

Fruit firmness was measured using a penetrometer (Model Instron 5542; Instron Co., USA) equipped with a cylindrical flat-surfaced plunger (6 mm diameter). A small slice of fruit skin was removed and firmness was then recorded from three different fruit, with three different points per fruit; means were expressed as newtons (N).

### *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 RT-PCR. The first-strand cDNA of the product was subjected to PCR amplification.

Six *NAC* genes, termed *MaNAC1–MaNAC6*, were isolated from our transcriptome database obtained using a high-throughput Solexa/Illumina sequencing platform (Beijing Genomics Institute, Shenzhen, China) and the sequences were first verified by reclonning and resequencing. Two of the six *NAC* genes, *MaNAC3* and *MaNAC4*, were full-length sequences in the database, with complete start and stop codes, while full-length sequences of the other four *NAC* genes, including *MaNAC1*, *MaNAC2*, *MaNAC5* and *MaNAC 6*, were obtained by 3'- or 5'-rapid amplification of cDNA ends (RACE) using a RACE kit (TaKaRa Biotechnology, Dalian, PR China) according to the manufacturer's instructions. The specific primers used for RACE are provided in Supplementary Table S1 at *JXB* online.

Alignments were carried out on CLUSTALW version 1.83 and GeneDoc software, and a phylogenetic tree was constructed using the neighbour-joining method in the MEGA5 program and visualized by TreeView software. The theoretical isoelectric points (pIs) and mass values for mature peptides were calculated using the PeptideMass program (http://web.expasy.org/peptide_mass/).

### *Subcellular localization of MaNAC proteins*

The coding sequences of *MaNAC1–MaNAC6* without the stop codon were amplified by PCR (primers are listed in Supplementary Table S2 at *JXB* online) and 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 (Zeiss Axioskop 2 Plus). All transient expression assays were repeated at least three times.

### *Transcriptional activation analysis in yeast cells*

The coding regions of *MaNAC1–MaNAC6* were cloned into the pGBKT7 vector (Clontech, USA) to create the pGBKT7-MaNAC1 to -6 constructs, respectively. Following the protocol of the manufacturer, pGBKT7-MaNAC1 to -6, the positive control pGBKT7-53+pGADT7-T, and the negative control pGBKT7 plasmids were used to transform the AH109 yeast strain using the lithium acetate method. The transformed strains were streaked onto minimal medium without Trp (SD/–Trp) or SD/–Trp–His–Ade plates, and the transactivation activity of each protein was evaluated according to their growth status and the activity of α-galactosidase. For mapping the activation domain, different truncated derivatives were constructed and transformed into the yeast strain for transcription activation activity as described above. The primers used for transcriptional activation analysis are listed in Supplementary Table S3 at *JXB* online.

### *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 analysis (qPCR). PCRs were performed in a total volume of 20 µl, containing 1 µl of each primer (10 µM; final concentration 200 nM) and 10 µl of SYBR® Green PCR Supermix (Bio-Rad Laboratories) on a Bio-Rad CFX96 Real-Time PCR System according to the manufacturer's instructions. The qPCR program included an initial denaturation step at 94 °C for 5 min, followed by 40 cycles of 10 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. 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 S4 at *JXB* online. *RPS2* (ribosomal protein 2) and *CAC* (clathrin adaptor complex) were selected as reference genes under different series samples according to our previous study on the selection of reliable reference genes for expression study by qPCR in banana fruit (L. Chen *et al.*, 2011). Each assay using the gene-specific primers amplified a single product of the correct size with high PCR 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 target gene were calculated using the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). The values represent the mean of three biological replicates.

### *Promoter isolation and its activity assay*

Genomic DNA was extracted from banana leaves using a DNeasy Plant Mini kit (Qiagen). Genomic DNA (2 µg for each) was digested separately with eight restriction enzymes (*Dra*I, *Ecl*136II, *Eco*RV, *Hpa*I, *Msc*I, *Stu*I, *Ssp*I, and *Sca*I). After enzyme digestion, all products were cleaned up using a DNA Clean and Concentration kit (Zymo Research) and then ligated to an adaptor (Clontech).

The 5'-upstream region of the *MaNAC2* gene was isolated using a Genome Walker kit (Clontech) with nested PCR. The nested PCR analysis was performed with two sets of primers, including two adaptor

primers that were obtained from the kit and two gene-specific primers that were designed by Primer 5.0, as listed in Supplementary Table S5 at *JXB* online. The amplification product was cloned into the pGEM-T Easy vector (Promega) and sequenced. Conserved *cis*-element motifs of the promoter were predicted using the PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and Plant-CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) databases.

For the promoter activity assay, the *MaNAC2* promoter region (~1.5 kb) was amplified by PCR using the specific primers listed in Supplementary Table S6 at *JXB* online. The PCR product was inserted into the vector pUC-GFP by replacing the CaMV 35S promoter to generate the construct $P_{MaNAC2}$::GFP containing the GFP-coding region under the control of the *MaNAC2* promoter. The construct $P_{MaNAC2}$::GFP and the positive control 35S::GFP were transfected into tobacco BY-2 protoplasts using a PEG method as described above. Transfected protoplasts were subjected to 0 (control) or 0.8 mmol $l^{-1}$ ethrel treatment and then incubated at 23 °C for 14 h. The GFP signal was detected before and after ethylene treatment using a fluorescence microscope (Zeiss Axioskop 2 Plus).

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

Y2H assays were performed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech). The coding regions of *MaNAC1–MaNAC6* and *MaEIL5* 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 S7 at *JXB* online). *MaEIL5* 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, and yeast cells were grown 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 $ml^{-1}$ X-α-Gal at 30 °C to test for possible interactions between *MaNAC1–MaNAC6* and *MaEIL5* according to their growth status and the activity of α-galactosidase.

### *BiFC assay*

To generate constructs for the BiFC assays, the full-length coding sequences of *MaNAC1*, *MaNAC2* and *MaEIL5* (without their stop codons) were subcloned into the pUC-pSPYNE or pUC-pSPYCE vector obtained from the laboratory of K. Harter and J. Kudla (Walter *et al.*, 2004). 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 previously (Abel and Theologis, 1994). The transformed protoplasts were then grown 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 (Zeiss Axioskop 2 Plus). The primers used in the BiFC assay are listed in Supplementary Table S8 in *JXB* online.

### *Statistical analysis*

The experiment was arranged in a completely randomized design. Each sample time point per treatment comprised three independent biological replicates. Data are plotted on figures as means ±standard error (SE).

## Results

### *Isolation and sequence analysis of* MaNAC *genes from banana fruit*

Six novel putative NAC full-length cDNAs were isolated from banana fruit and designated as *MaNAC1–MaNAC6*. *MaNAC1–MaNAC6* were predicted to encode proteins of 283, 293, 284, 257, 302, and 278 aa, with calculated molecular weights of 31.3, 33.2, 31.9, 29.1, 33.8, and 31.1 kDa and pI values of 8.71, 6.55, 9.20, 6.83, 7.60, and 5.43, respectively. Alignment of the full-length deduced proteins of the MaNACs clearly showed that the proteins contained the NAC conserved domain in their N-terminal regions, which was further divided into five subdomains (A–E) (Ooka *et al.*, 2003) (Fig. 1). However, the C-terminal regions showed no significant similarity to that of any other members of NAC family (Fig. 1). In addition, although all the MaNACs proteins had the conserved NAC domain, the similarities among the six MaNACs varied from 20% (MaNAC1 and MaNAC4) to 66% (MaNAC2 and MaNAC4) (Supplementary Table S9 at *JXB* online).

To examine the phylogenetic relationship among the NAC domain proteins in banana, *Arabidopsis* and rice, a phylogenetic tree was constructed based on their translated amino acid sequences. As shown in Fig. 2, the whole NAC proteins of *Arabidopsis* and rice could be classified into two major groups, I and II, which comprised 14 and four subgroups, respectively (Ooka *et al.*, 2003). Based on the tree, the banana six *NAC* genes were clustered into four subfamilies. MaNAC2 and MaNAC4 belonged to ONAC022, and MaNAC5 and MaNAC6 belonged to NAC1, whereas MaNAC1 and MaNAC3 belonged to ONAC003 and NAM, respectively (Fig. 2). Collectively, these data suggested that MaNAC1–MaNAC6 may exhibit diverse functions.

### *Subcellular localization of the MaNAC proteins*

NAC proteins are TFs that are usually located in the nucleus; however, some NAC proteins are also membrane associated (Seo *et al.*, 2010; Hao *et al.*, 2011). To validate the subcellular localizations of the MaNAC1–MaNAC6 proteins, the full-length coding sequences of *MaNAC1–MaNAC6* were fused in frame with the GFP gene. Transient expression of these constructs in tobacco BY-2 protoplasts showed that the fluorescence of MaNAC1–MaNAC5 was localized exclusively in the nucleus (Fig. 3). By contrast, the fluorescence from the GFP control was distributed rather evenly within the cells. Interestingly, the fluorescence of MaNAC6 was not targeted exclusively to the nucleus, and the GFP signal was also found in the cytoplasm and cell membrane (Fig. 3), although it contained a nuclear localization sequence (NLS).

### *Transcriptional activation ability of MaNAC proteins in yeast*

It has been reported that the N-terminal regions of NAC proteins show DNA-binding ability while the C-terminal regions function as a transcriptional activation domain (Olsen *et al.*, 2005). To investigate the abilities of MaNAC1–MaNAC6 to activate transcription, a transient expression assay using a GAL4-responsive reporter system in yeast cells was performed. First, the full-length coding regions of *MaNAC1–MaNAC6* were fused to the GAL4 DBD to generate pGBKT7-MaNAC1 to -6 fusion plasmids (DBD–MaNAC1 to -6) (Fig. 4A). The plasmids were then transformed into yeast strain AH109 and the transformants were assayed for their ability to activate

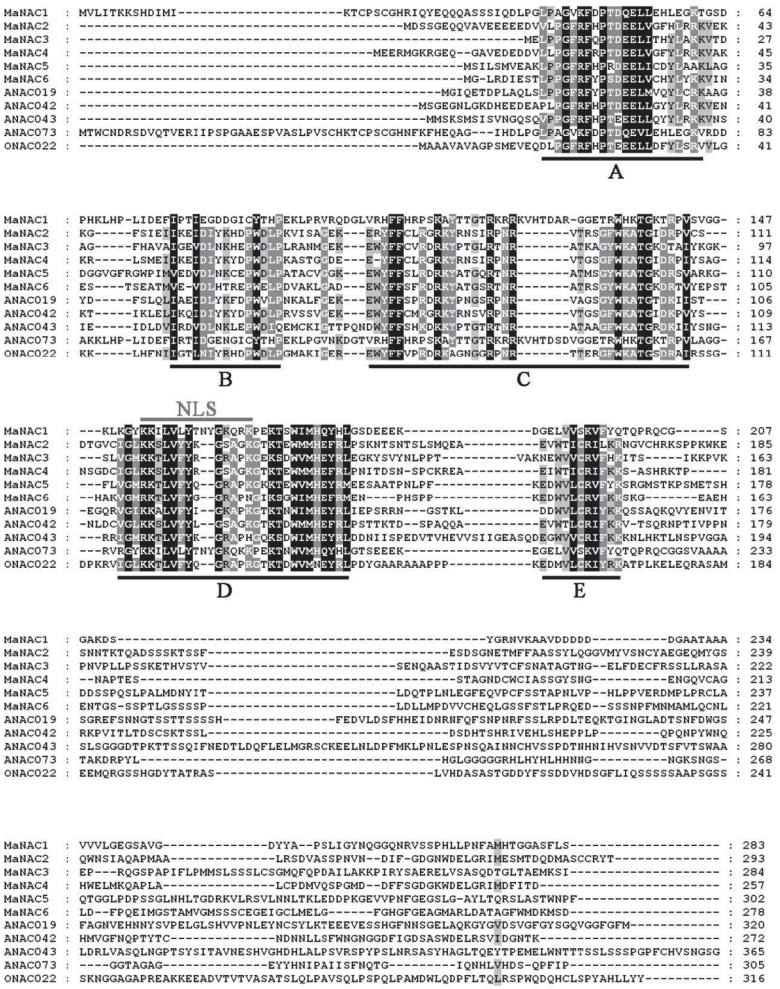


**Fig. 1.** Amino acid sequence alignment of the MaNACs proteins with other plant NAC proteins. MaNACs were aligned with *Arabidopsis* ANAC019 (At1g52890.1), ANAC042 (At2g43000.1), ANAC043 (At2g46770.1), and ANAC073 (At4g28500.1), and rice ONAC022 (AK107090). Identical and similar amino acids are indicated by black and grey shading, respectively. Gaps were introduced to optimize alignment. The five highly conserved amino acid motifs (A–E) and the nuclear localization signal (NLS) are indicated by black and grey lines, respectively.

transcription from the GAL4 upstream activation sequence and to promote yeast growth in medium lacking histidine. The transformants containing pGBKT7-53+pGADT7-T (DBD–P53+T-antigen) and pGBKT7 vectors (empty) were used as positive and negative controls, respectively. Fig. 4A showed that the transformed yeast cells harbouring DBD–MaNAC4, DBD–MaNAC6 and DBD–P53+T-antigen (positive control) grew well in SD medium lacking tryptophan, histidine, and adenine, and showed α-galactosidase activity, whereas cells containing DBD–MaNAC1, DBD–MaNAC2, DBD–MaNAC3, DBD–MaNAC5, or pGBKT7 (negative control) showed no α-galactosidase activity. To identify further the transcriptional activation domains of MaNAC4 and MaNAC6, the N- and C-terminal regions of MaNAC4 and MaNAC6 were fused to the GAL4 DBD (Fig. 4B) and transformed into yeast strain AH109. As shown in Fig. 4B, yeast cells harbouring DBD–MaNAC4-C and DBD–MaNAC6-C showed α-galactosidase activity. These data confirmed that MaNAC4 and MaNAC6 exhibited transcriptional activation ability in their C-terminal regions.

### *Changes in fruit firmness and ethylene production in fruit with four different ripening characteristics*

Changes in banana fruit firmness and ethylene production in fruit with four different ripening characteristics, comprising natural, ethylene-induced, 1-MCP-delayed, and a combination of 1-MCP with ethylene treatment, were shown in Fig. 5. The firmness of fruit ripened naturally began to decline at day 19, reaching a firmness of ~4 N at day 25, with ethylene production obviously increased at day 15, reaching a maximum at day 21 and then decreasing at day 25. The decline in fruit firmness and increase in ethylene production were substantially faster in ethylene-treated fruit, reaching the same ripening stage at day 7 as natural ripening at day 35, and ethylene production in ethylene-treated fruit began to be detected at 1 d of treatment, peaking at day 3. In contrast, treatment with 1-MCP delayed fruit softening and the increase in ethylene production, its firmness began to decline at day 28 reaching ~5 N at day 35, and ethylene production was detected at day 25 and reached a peak at day 30. However, application of ethylene to the 1-MCP-treated fruit promoted a decline

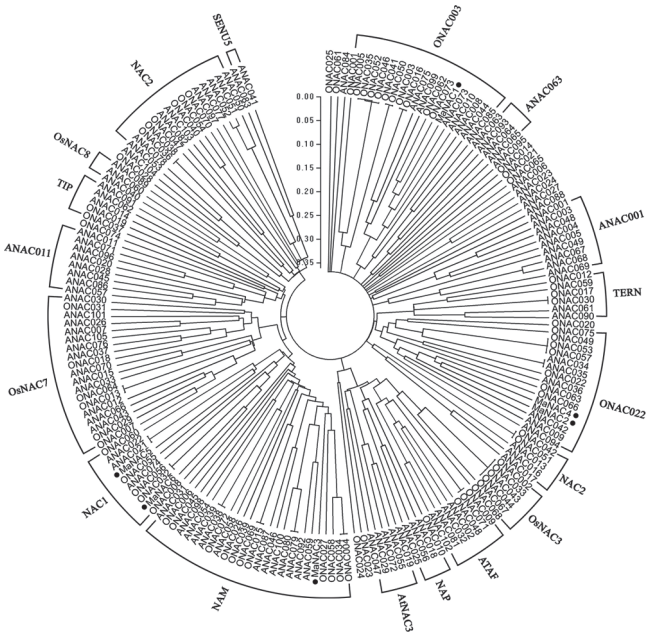


**Fig. 2.** Phylogenetic tree of NACs. Six banana MaNACs (black circles) were aligned with those of the *Arabidopsis* and rice NAC families as designated by Ooka *et al.* (2003). Multiple alignment was carried using CLUSTALW and the phylogenetic tree was constructed with MEGA5.0 using a bootstrap test of phylogeny with minimum evolution test and default parameters. The GenBank accession numbers of the *Arabidopsis* and rice NAC proteins are listed as Supplementary Data at *JXB* online.

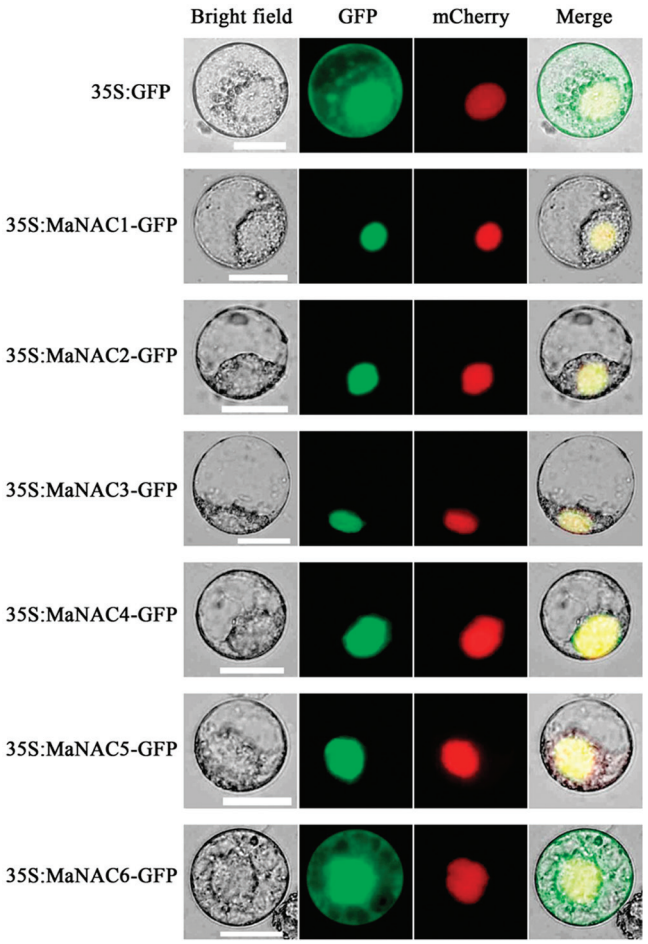


**Fig. 3.** Subcellular localization of MaNACs in tobacco BY-2 protoplasts. Protoplasts were transiently transformed with MaNAC–GFP constructs or GFP vector using a modified PEG method. GFP fluorescence was observed with a fluorescence microscope. VirD2NLS-mCherry was included in each transfection to 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 μm.

in the firmness and an increase in ethylene evolution, the firmness of the fruit began to decline at day 21, reaching to ~4N at day 30, and ethylene production started to increase at day 25 and reached a peak at day 28. It should be noted that the higher ethylene production of 1-MCP+ethylene-treated fruit at day 1 might be the residual ethylene of the treatment.

### *Expression of* MaNAC *and* MaEIL *genes in peel and pulp during fruit ripening*

To understand the possible role of *MaNAC1–MaNAC6* in banana fruit ripening, the expression patterns of *MaNAC1–MaNAC6* in peel and pulp of fruit with the four different ripening characteristics described above were investigated by qPCR. As shown in Fig. 6, in peel tissues, among the six *MaNAC* genes, *MaNAC1* and *MaNAC2* were strongly induced at 1 d after ethylene treatment, with ~30- and ~350-fold increases at day 3, respectively. Their transcript levels in peel were low during early storage, but clearly began to increase with ethylene evolution. In contrast, *MaNAC4* and *MaNAC6* transcript levels decreased following ethylene treatment and ethylene evolution, and their transcript levels in peel decreased following the increase in ethylene production, with a more remarkable decrease for *MaNAC6*. In addition, *MaNAC3* and *MaNAC5* were expressed constitutively and their transcripts changed only slightly during ripening.

In pulp tissues, as shown in Fig. 7, expression of *MaNAC1*, *MaNAC2*, *MaNAC3*, and *MaNAC4* showed a similar change in pattern to their expression in peel. *MaNAC1* and *MaNAC2* were ethylene induced and their transcript levels in natural, ethylene-induced, 1-MCP-delayed ripening, or a combination of 1-MCP with ethylene-treated fruit clearly increased with ethylene evolution. The *MaNAC3* transcript level changed slightly and *MaNAC4* transcript decreased following ethylene production increase during the ripening stage. Unlike the expression patterns in peel, expression of *MaNAC5* and *MaNAC6* was slightly enhanced after ethylene treatment, and their transcript levels in natural, 1-MCP-delayed ripening, or a combination of 1-MCP with ethylene-treated fruit also increased following the increase in ethylene production. These results indicated that *MaNAC* genes are differentially expressed in peel and pulp during post-harvest ripening, and that *MaNAC1* and *MaNAC2* may be more related to fruit ripening.

Five *EIN*-like genes, designated *MaEIL1–MaEIL4*, and AB266321 (termed *MaEIL5* in the present work) have been

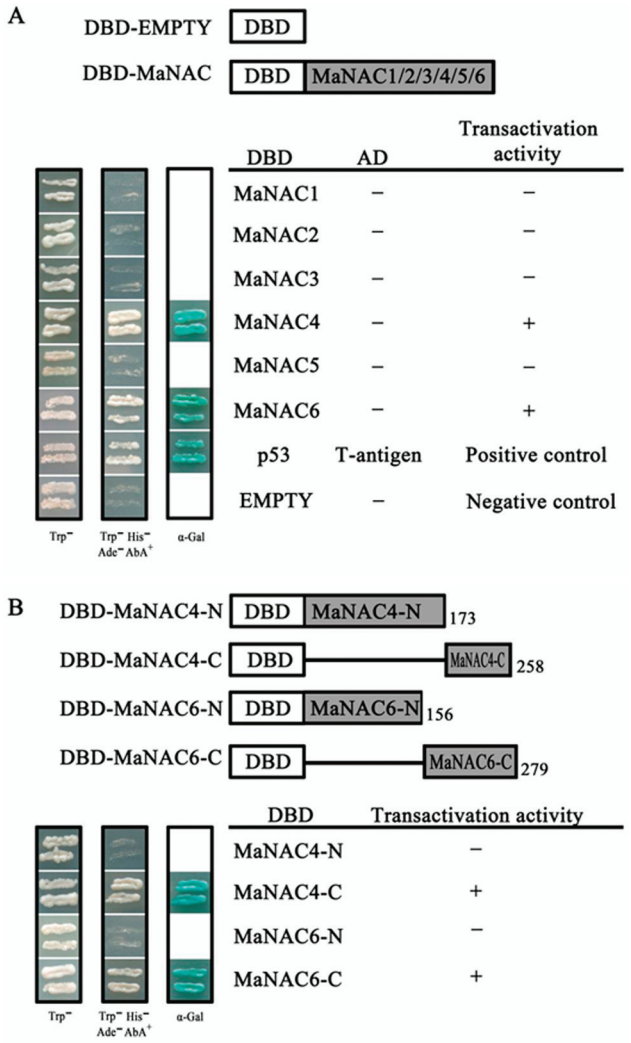


**Fig. 4.** Transcriptional activation of MaNACs in yeast. (A) The coding regions of *MaNAC1–MaNAC6* were cloned into the pGBKT7 (GAL4 DBD) vector to create the DBD–MaNAC1 to -6 constructs, respectively. (B) Truncation analysis of transcriptional activation of MaNAC4 and MaNAC6 for mapping activation domain. C- and N-terminal derivatives of MaNAC4 and MaNAC6 were fused with the pGBKT7 vector to create the DBD–MaNAC4 and -6 constructs, respectively. The numbers on the right indicate the last residues of the polypeptides. In (A) and (B), all of the constructs mentioned above, together with 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 or without tryptophan, histidine, and adenine but containing 125 μM Aureobasidin A for 3 d at 30 °C. Transcription activation was monitored by the detection of yeast growth and an α-galactosidase (α-Gal) assay.

isolated from banana fruit in a previous study, and it was shown that, in pulp tissue, *MaEIL2* was a unique ripening- and ethylene-induced gene, while the *MaEIL1*, *MaEIL4*, and AB266321 (*MaEIL5*) genes were downregulated during ripening (Mbéguié-A-Mbeguié *et al.*, 2008). As shown in Figs 6 and 7, similar to the previous report, *MaEIL2* in peel and pulp was ethylene induced and its transcript level increased, while that of *MaEIL5* in peel and pulp clearly decreased once ethylene evolution could be detected during ripening. *MaEIL1*and *MaEIL4* transcript levels in pulp also decreased during ripening but the changes were not as obvious as that of *MaEIL5* (data not shown).

### *The promoter activity of MaNAC2 is induced by ethylene*

Gene expression profiles of the *MaNAC* genes during post-harvest fruit ripening suggested that *MaNAC2* was ethylene inducible and might be involved in fruit ripening. To better establish the mechanisms by which *MaNAC2* expression is modulated, a 1529 bp upstream sequence from the start codon of *MaNAC2* was isolated from the genome of *Musa acuminata* using a genome-walking PCR method. Analysis of the promoter using the PLACE and Plant-CARE databases, as illustrated in Table 1, revealed that 15 distinct putative *cis*-acting elements were identified in the promoter region. Besides the core *cis*-acting elements like the TATA-box and CAAT-box, other potential *cis*-regulatory elements involved in the activation of abiotic stress (cold, water, and heat) and hormone-responsive (GA and SA) genes were also found. Interestingly, two sites for the ethylene-responsive element, ATTTCAAA, with one nucleotide change found in the promoter at positions –11 (ATTACAAA) and –680 (ATTTCACAA) upstream of ATG, could probably govern expression in response to ethylene.

To analyse *MaNAC2* promoter activity in response to ethylene, we conducted a transient protoplast assay using a GFP reporter construct containing the *MaNAC2* promoter (*MaNAC2pro*::GFP). As shown in Fig. 8, in tobacco BY-2 protoplast transfected with *MaNAC2pro*::GFP, GFP signals were not detected before ethylene treatment but were observed after ethylene treatment. As a positive control, a GFP signal was constitutively observed before or after ethylene treatment in protoplasts transfected with the CaMV 35S promoter-driven construct (35S::GFP), and no GFP signal was detected in protoplasts that were not transfected. Similar results were also obtained in *Arabidopsis* mesophyll protoplasts (Supplementary Fig. S1 at *JXB* online). The results that ethylene induced the *MaNAC2* promoter activity further support the suggestion that *MaNAC2* is ethylene inducible and might be involved in fruit ripening.

### *MaNAC1 and MaNAC2 physically interact with MaEIL5*

Several reports have suggested that NAC TFs are involved in ethylene signalling pathways through interaction with ethylene signalling components, such as EIN2 (He *et al.*, 2005; Kim *et al.*, 2009; Al-Daoud and Cameron, 2011). Based on the expression characteristics of the *MaNAC* and *MaEIL* genes during fruit ripening, it is thought that possible interactions between MaNAC and MaEIL proteins might exist.

In order to investigate whether MaNACs physically interact with MaEILs, the Matchmaker™ Gold Yeast Two-Hybrid System was used to investigate the interactions between MaNACs and MaEILs. *MaNAC1–MaNAC6* and *MaEIL5*

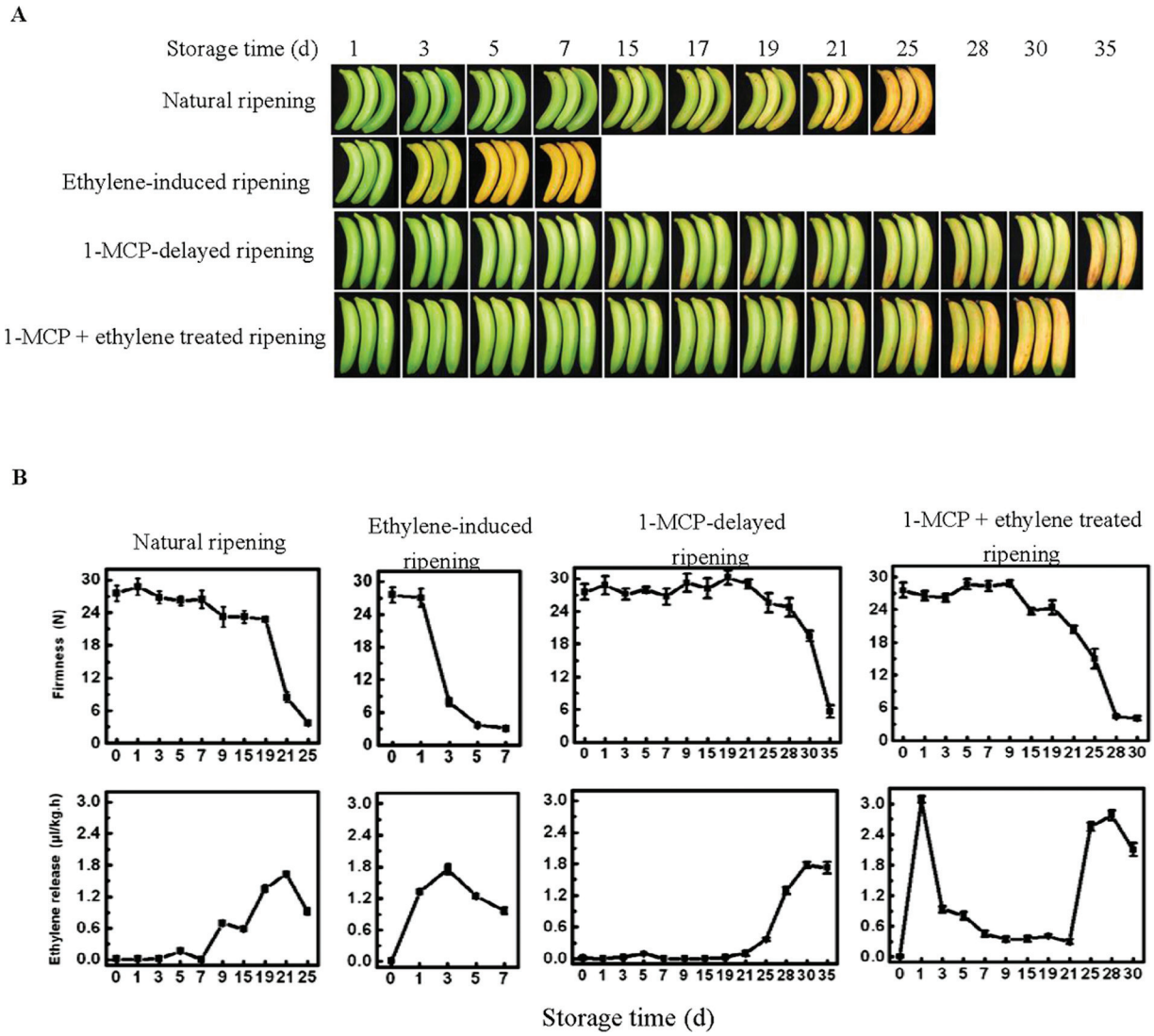


**Fig. 5.** Photograph of fruit with four different ripening characteristics, comprising natural (control), ethylene-induced, 1-MCP-delayed, and a combination of 1-MCP+ethylene treated ripening (A), and changes in fruit firmness and ethylene production (B) during ripening. Each value represents the mean ±SE of three replicates.

coding sequences were subcloned into pGADT7 and pGBKT7 vectors for a Y2H assay, as MaNAC4 and MaNAC6 showed transactivation activity in yeast when fused with the DBD (Fig. 4). As shown in Fig. 9A, yeast cells co-transformed the positive control (pGBKT7-53+pGADT7-T) and MaNAC1 or MaNAC2 with MaEIL5, could grow on selective medium (synthetic medium lacking tryptophan, leucine, histidine, and adenine) supplement with the toxic drug Aureobasidin A, and turned blue in the presence of the chromagenic substrate X-α-Gal. However, yeast cells harbouring MaNAC3, MaNAC4, MaNAC5, or MaNAC6 with MaEIL5, and the negative controls, could not grow on the selective medium and did not turn blue under the same conditions (Fig. 9A). Interactions between MaNAC1 or MaNAC2 and MaEIL5 were also observed, when MaNAC1 or MaNAC2 and MaEIL5 coding sequences were subcloned into pGBKT7 and pGADT7, respectively (Fig. 9B). Moreover, the C terminus of MaNAC1 or MaNAC2 was sufficient for the interaction with MaEIL5 (Fig. 9C). In addition, we also examined the interactions between *MaNAC1–MaNAC6* and MaEIL2 but detected no interactions among them (data not shown).

To further confirm the interactions between MaNAC1/MaNAC2 and MaEIL5 observed in the Y2H assays, we performed BiFC assays in tobacco BY-2 protoplasts. MaNAC1 or MaNAC2 tagged with pSPYNE (split YFP N-terminal fragment expression) and MaEIL5 tagged with pSPYCE (split YFP C-terminal fragment expression) were transiently co-expressed in tobacco BY-2 protoplasts following PEG transfection. As shown in Fig. 10A, robust YFP fluorescent signal was detected in the nucleus of BY-2 cells expressing MaNAC1-pSPYNE or MaNAC2-pSPYNE and MaEIL5-pSPYCE, while no YFP fluorescent signal was observed either in the cells expressing MaNAC1/MaNAC2-pSPYNE with only the pSPYCE or in those expressing MaEIL5-pSPYCE with only the pSPYNE (Fig. 10A). Similar results were also observed when MaNAC1/MaNAC2-pSPYCE was co-transfected with MaEIL5-pSPYNE

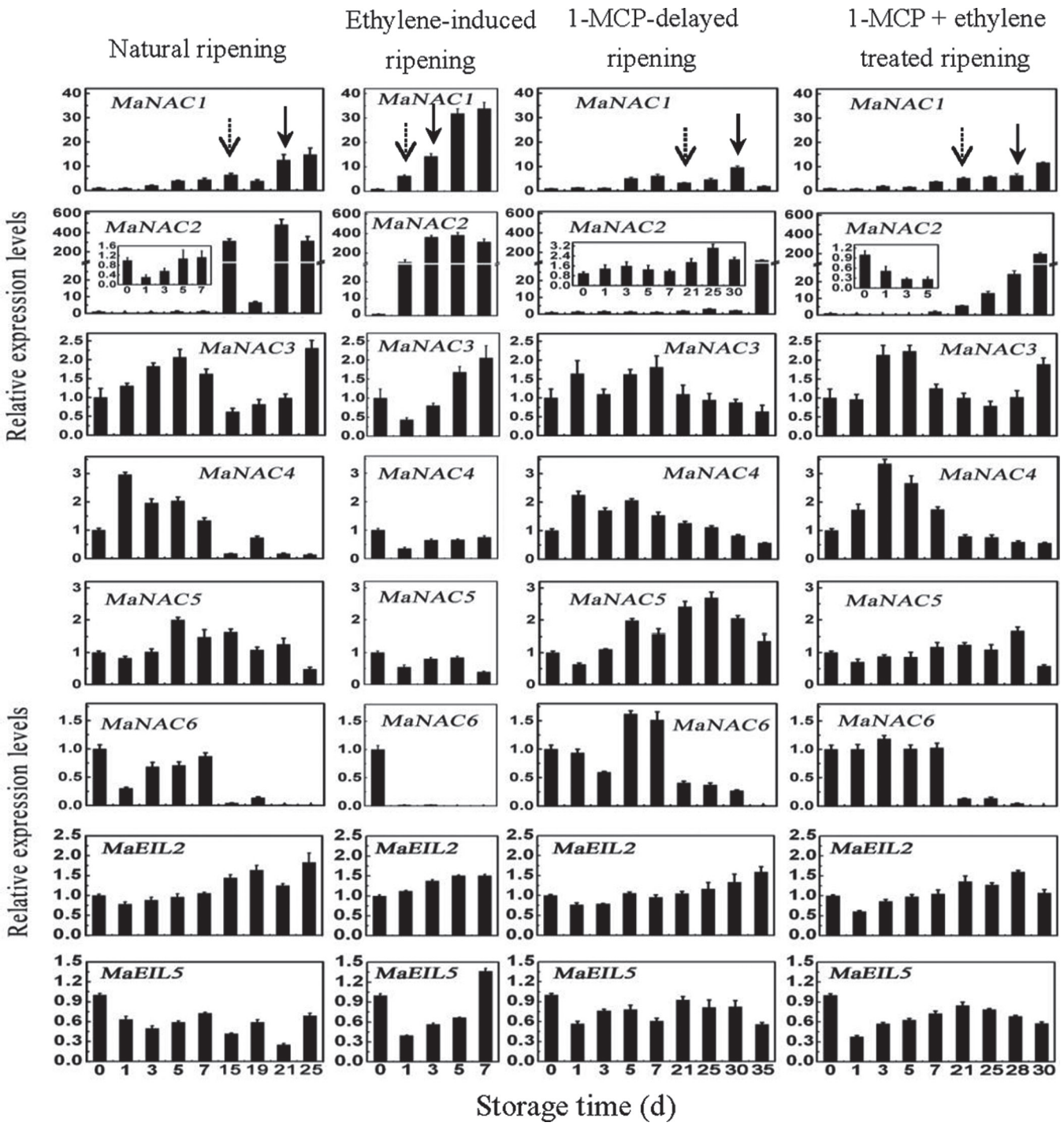


**Fig. 6.** Expression of *MaNAC* and *MaEIL* genes in banana fruit peel with four different ripening characteristics: natural (control), ethylene-induced, 1-MCP-delayed, and a combination of 1-MCP and ethylene-treated ripening. The expression levels of each gene are expressed as a ratio relative to the harvest time (0 d of control), which was set at 1. Each value represents the mean ±SE of three replicates. The broken arrow and full arrow represent the time point of ethylene production beginning to increase and its peak for each treatment, respectively.

(Fig. 10B). The BiFC results not only demonstrated the *in vivo* interaction among the three proteins tested but also showed the specific localization of the interacting proteins in the nucleus, which was consistent with the subcellular localization of MaNAC1, MaNAC2, and MaEIL5 in the nuclear compartment (Fig. 3 and Supplementary Fig. S2 at *JXB* online).

## Discussion

### *Identification of banana* MaNAC *genes*

NAC is a family of plant-specific TFs that have been identified in various species (Berger *et al.*, 2009; Meng *et al.*, 2009; Zhong *et al.*, 2010). There are approximately 100 NAC TFs in both the

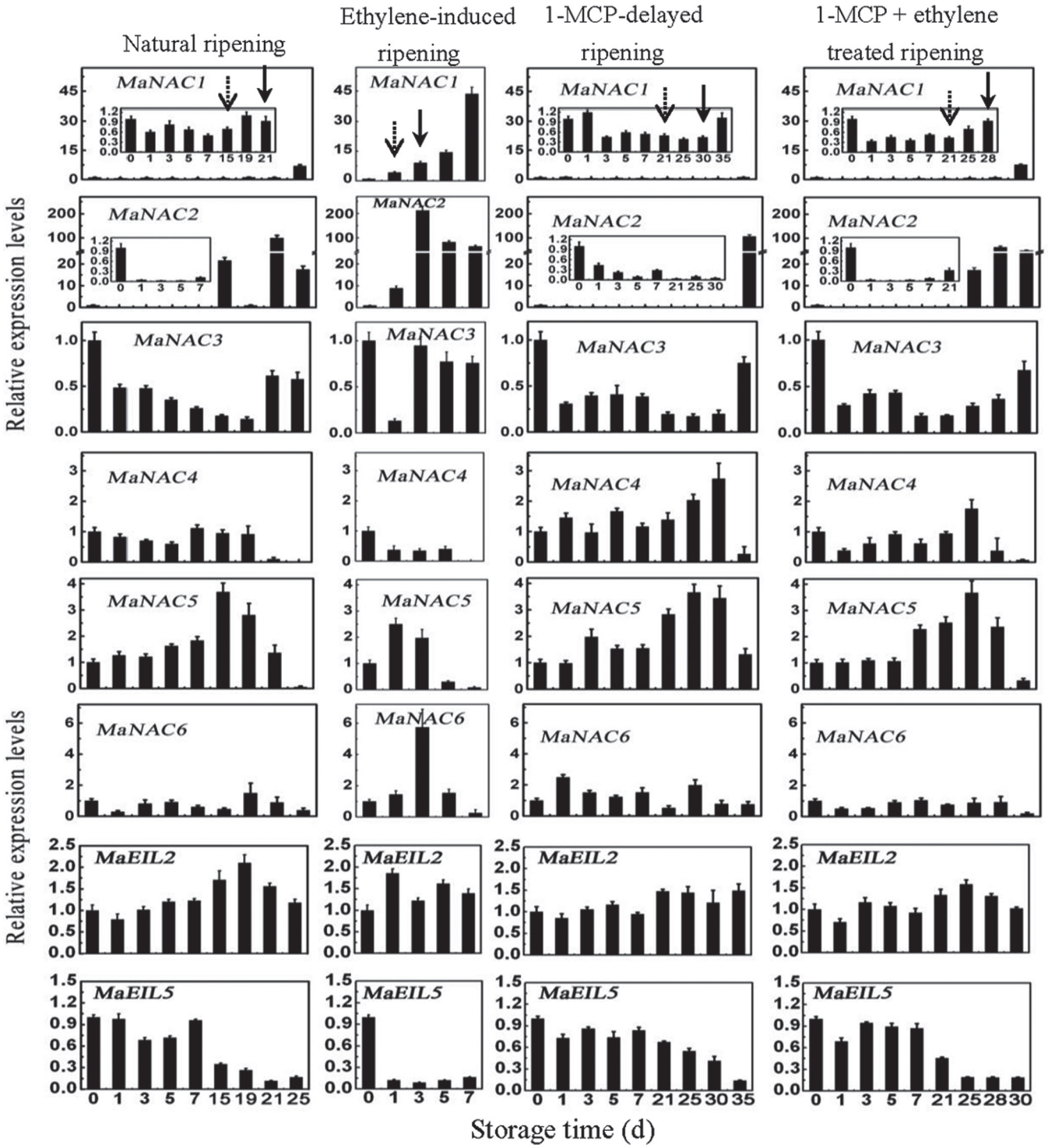


**Fig. 7.** Expression of *MaNAC* and *MaEIL* genes in banana fruit pulp with four different ripening characteristics: natural (control), ethylene-induced, 1-MCP-delayed, and a combination of 1-MCP and ethylene-treated ripening. The expression levels of each gene are expressed as a ratio relative to the harvest time (0 d of control), which was set at 1. Each value represents the mean ±SE of three replicates. The broken arrow and full arrow represent the time point of ethylene production beginning to increase and its peak for each treatment, respectively.

*Arabidopsis* and rice genomes (Gong *et al.*, 2004; Xiong *et al.*, 2005). From our transcriptome database, there are at least six putative proteins that exhibit a significant sequence identity with *Arabidopsis* AtNAC and rice OsNAC homologues, further confirming the notion that NAC proteins are encoded by a large multigene family (Ooka *et al.*, 2003). The predicted MaNAC paralogues were 20–66% identical to each other at the amino acid level (Supplementary Table S9). Alignment of the six MaNACs proteins showed that they shared a highly conserved N-terminal DBD, termed the NAC domain (Ooka *et al.*, 2003), which

**Table 1** Main regulatory motifs found within the promoter sequence of *MaNAC2*

| Factor or site name | Site | Signal sequence | Function |
|---|---|---|---|
| MYB1AT | 181(+); 147(+); 451(–); 900(–) | WAACA | MYB recognition site found in the promoters of the dehydration-responsive gene *rd22* and many other genes |
| MYB2AT | 1018(–) | TAACTG | Binding site for regulation of genes that are responsive to water stress |
| MYBCORE | 162(+); 394(+); 633(+); 1018(+); 159 (–) | CNGTTR | Binding site for regulation of genes that are responsive to water stress |
| MYCCONSENSUSAT | 375(+); 481(+); 499(+); 1239(+); 1523(+); 375(–); 481(–); 499(–); 1239(–); 1523(–) | CANNTG | Regulates the transcription of CBF/DREB1 genes in the cold |
| HSE | 646(–); 972(+); 886(+); 973(+) | AAAAAATTTC | *Cis*-acting element involved in heat stress responsiveness |
| GARE | 603(+) | TCTGTTG | Gibberellin-responsive element |
| LTRE1HVBLT49 | 1491(–) | CCGAAA | *Cis*-acting element involved in low-temperature responsiveness |
| LTRECOREATCOR15 | 1154(–) | CCGAC | Core of low temperature responsive element (LTRE) of *cor15a* gene |
| GAREAT | 866(+) | TAACAAR | Gibberellin-responsive element |
| P-box | 670(–) | GCCTTTTGAGT | Gibberellin-responsive element |
| MBS | 989(–) | TAACTG | MYB-binding site involved in drought inducibility |
| TC-rich repeats | 444(+); 970(–);850(–) | ATTTTCTCCA | *Cis*-acting element involved in defense and stress responsiveness |
| TCA element | 1223(+); 1226(–) | CAGAAAAGGA | *Cis*-acting element involved in salicylic acid responsiveness |
| WBOXNTERF3 | 425(–);1290(–);1275(–);1335(–) | TGACY | May be involved in activation of *ERF3* gene by wounding |
| WRKY71OS | 77(+); 359(+); 695(+); 104(–); 426(–); 1276(–); 1281(–); 1291(–); 1336(–) | TGAC | Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway |

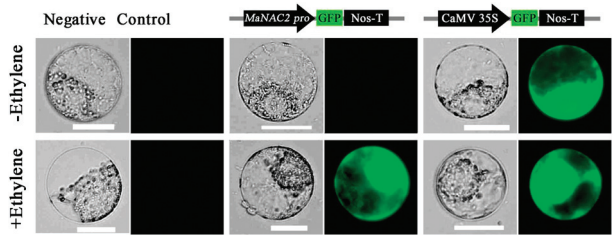


**Fig. 8.** *MaNAC2* promoter activity in response to ethylene. GFP reporter constructs containing the *MaNAC2* promoter (*MaNAC2pro*::GFP) and the CaMV 35S promoter (35S::GFP, positive control) were transiently transformed into tobacco BY-2 protoplasts using a modified PEG method and test for ethylene induction. Non-transformed protoplasts were used as a negative control. After incubation for 12 h, GFP fluorescence was observed by fluorescence microscopy. Bar, 25 μm. The experiment was repeated at least three times.

contains five consensus subdomains (Fig. 1). The NAC domain may function as a potential NLS (Duval *et al.*, 2002; Hegedus *et al.*, 2003; Ernst *et al.*, 2004) and most isolated NAC proteins are localized in the nucleus (Pinheiro *et al.*, 2009; Hao *et al.*, 2011). Similarly, the subcellular localizations of the MaNACs indicated that MaNAC1–MaNAC5 were all localized in the nucleus, while MaNAC6 was distributed throughout the whole cell, including nucleus, cytoplasm, and cell membrane (Fig. 3). Thirteen members of the membrane-associated type of NAC proteins have been found in *Arabidopsis* (Kim *et al.*, 2007). These NACs, which are larger in molecular weight than non-membrane-associated NAC TFs (~320 residues), have residue numbers ranging from 335 to 652, because of their C-terminal extensions, which contain the transmembrane motif (Morishita *et al.*, 2009). In eukaryotic cells, molecular mechanisms that regulate the localization of TFs have been investigated extensively, one of which is cytoplasmic sequestration caused by the presence of a membrane anchor (Lee and Hannink, 2003). Following specific cues, membrane-bound precursors are proteolytically cleaved, allowing nuclear localization of the TF (Morishita *et al.*, 2009). One *Arabidopsis* NAC protein, AtNAC78, has been located in the nucleus and cytoplasm in onion epidermal cells when fused with its full-length sequence (including the transmembrane motif), but was located in the nucleus when the putative transmembrane motif was deleted (Morishita *et al.*, 2009). However, the same is not true for MaNAC6, which includes a similar C-terminal extension to the other five MaNACs (Fig. 1). Thus, the mechanism and role of MaNAC6 localization within whole cells need to be elucidated further. In addition, the C-terminal regions of MaNAC1–MaNAC6 proteins are highly variable (Fig. 1). It has been reported that the non-conserved C-terminal regions of several NAC proteins, but not NAC domains, function as transcriptional activation domains (Hegedus *et al.*, 2003; Fujita *et al.*, 2004; Kim *et al.*, 2006), which may confer the regulation diversities of transcriptional activation activity. Our data also showed that, among the six MaNAC proteins, only MaNAC4 and MaNAC6 exhibited transcriptional activation ability in their C-terminal regions, and the NAC domain in their N-terminals did not activate reporter genes in yeast cells (Fig. 4). In addition, it should be pointed out that as the largest TF family in plants, other *NAC* genes may exist in banana fruit and their functions also need to be elucidated.

*Arabidopsis thaliana* and *Oryza sativa* NAC proteins have been classified previously into two groups based on similarities in the NAC domain structures (Ooka *et al.*, 2003). Group I is divided into 14 subgroups, namely TERN, ONAC022, SENU5, NAP, AtNAC3, ATAF, OsNAC3, NAC2, ANAC011, TIP, OsNAC8, OsNAC7, NAC1, and NAM, while the remaining

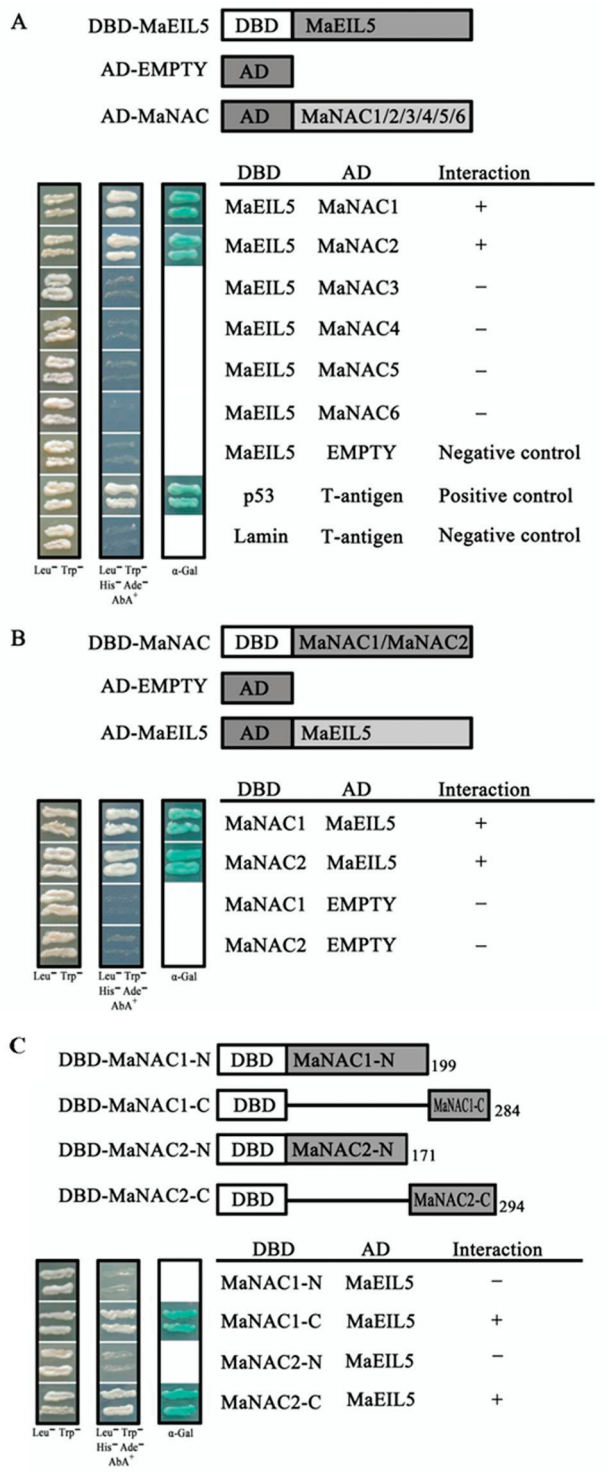


**Fig. 9.** Physical interactions between MaNAC proteins and MaEIL5 detected in Y2H assays. (A) The coding regions of *MaNAC1*–*MaNAC6* were cloned into the pGADT7 vector to create the AD–MaNAC1 to -6 constructs, while the coding region of *MaEIL5* was cloned into the pGBKT7 vector to create the DBD–MaEIL5 construct. Gold Y2H yeast strains were co-transformed with DBD–MaEIL5 and AD–MaNAC1 to -6, respectively. (B) The coding regions of MaNAC1/2 were cloned into the pGBKT7 vector to create the DBD–MaNAC1 and -2 constructs, while the coding region of MaEIL5 was cloned into the pGADT7 vector to create the AD–MaEIL5 construct. Gold Y2H yeast strains were co-transformed with DBD–MaNAC1 or -2 and AD–MaEIL5, respectively. (C) The N and C termini of MaNAC1 and MaNAC2 were tested for interaction with MaEIL5. Gold Y2H yeast strains were co-transformed with DBD–MaNAC1 or -2 derivatives and AD–MaEIL5, respectively. In (A), (B) and (C), 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–MaEIL5+pGADT7-T, or pGBKT7-Lam+pGADT7-T were included as positive or negative controls, respectively.

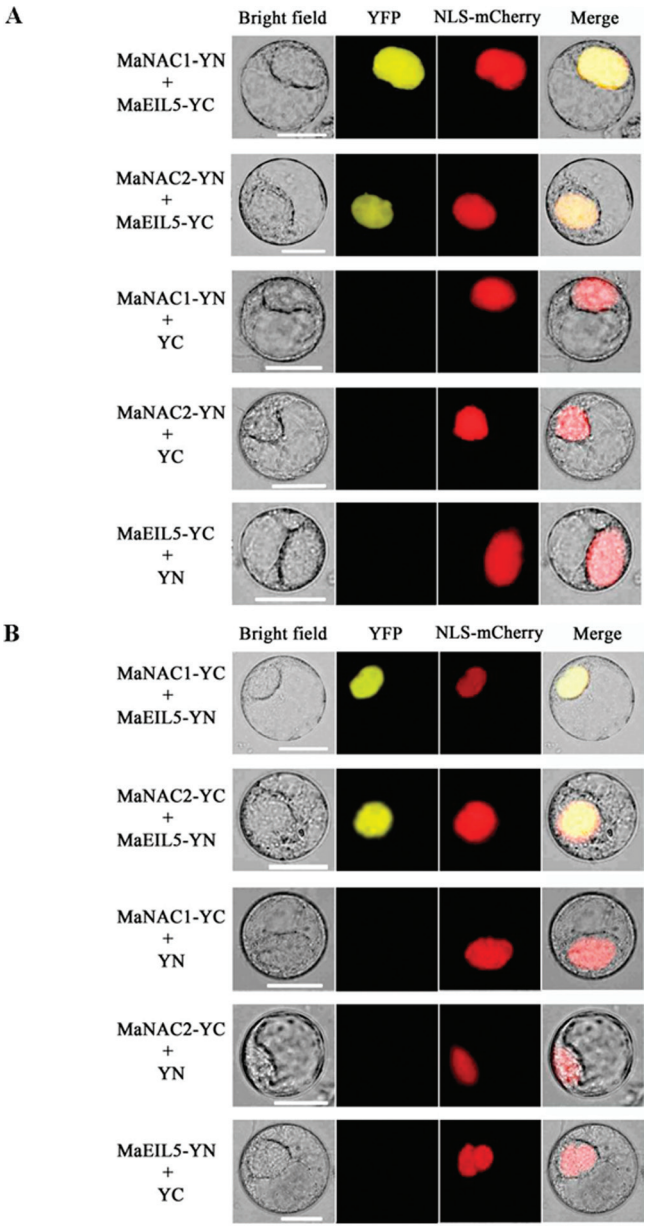


**Fig. 10.** BiFC visualization of the MaNAC1/MaNAC2 and MaEIL5 interaction in transiently co-expressed tobacco BY-2 protoplasts. (A) MaNAC1/ MaNAC2 and MaEIL5 proteins were fused with the N (YN) and C (YC) termini of YFP, respectively. (B) MaNAC1/ MaNAC2 and MaEIL5 proteins were fused with the C (YC) and N (YN) terminus of YFP, respectively. Expression of MaNAC1/ MaNAC2 or MaEIL5 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. Bar, 25 μm.

four subgroups, comprising ANAC011, ONAC003, ONAC001, and ANAC063, constitute group II (Ooka *et al.*, 2003). Although many reported NAC TFs are known for their diverse functions, the functions of the majority of members in this family are still unclear. Generally, NAC genes with the same function show a tendency to fall into one subgroup. For example, *Arabidopsis ANAC072* (*RD26*), *ANAC019*, and *ANAC055*, which have been found to be involved in the response to various environmental stresses, are clustered into subgroup AtNAC3 (Tran *et al.*, 2004; Fujita *et al.*, 2004). Subgroup NAM encompasses *NAM* and *CUC2*, which have been shown to function in shoot apical meristem formation and development (Souer *et al.*, 1996; Aida *et al.*, 1997). *SND1* (*ANAC012*), together with *NST1* (*ANAC043*) and *VND6* (*ANAC101*), are grouped into subgroup OsNAC7, which has been proposed to regulate secondary wall synthesis (Zhong *et al.*, 2010). Additionally, the membrane-associated NAC proteins that harbour a transmembrane motif with a predicted α-helix in the far C-terminal region have been placed phylogenetically into subgroups ANAC001, TIP, and NAC2 (Kim *et al.*, 2006). The members of these subgroups are highly expressed in vegetative organs that are more susceptible to abiotic stress conditions (Kim *et al.*, 2006). In the present work, the six banana *MaNAC* genes fell into four subgroups, in which MaNAC1 and MaNAC3 belonged to ONAC003 and NAM, respectively, while MaNAC2 and MaNAC4 belonged to ONAC022, and MaNAC5 and MaNAC6 belonged to NAC1. These results suggest that *MaNAC* genes may have diverse functions in banana.

### *Possible roles of MaNAC TFs in relation to banana fruit ripening*

The regulation of fruit ripening has been well studied, and ripening is known to be influenced by hormones, light, temperature, and developmental gene regulation (Klee and Giovannoni, 2011). However, critical TFs involving in fruit ripening were identified only recently, even in tomato fruit (Martel *et al.*, 2011; Qin *et al.*, 2012). One of the tomato MADS-box TFs, *RIPENING INHIBITOR* (*RIN*), exhibited elevated expression at the onset of ripening (Vrebalov *et al.*, 2002). Moreover, recently, it has been confirmed that RIN interacts with the promoters of genes involved in the major pathways associated with observed and well-studied ripening phenotypes and phenomena, including the transcriptional control network involved in overall ripening regulation, ethylene biosynthesis, ethylene perception, the downstream ethylene response, cell-wall metabolism, carotenoid biosynthesis, and aroma formation (Martel *et al.*, 2011; Qin *et al.*, 2012). MADS-box TFs have also been suggested to be involved in the ripening of other fruit, such as strawberry (Seymour *et al.*, 2011), apple (Yao *et al.*, 1999), banana (Elitzur *et al.*, 2010), and avocado (Hershkovitz *et al.*, 2011). Interestingly, although banana *MaMADS2* showed a high sequence similarity to tomato *LeMADS-RIN*, complementation analysis demonstrated that it could not complement a ripening *rin* mutant in tomato (Elitzur *et al.*, 2010). In addition, other TFs, such as *FaSPT*, which encodes a basic helix–loop–helix TF, and *FaASR*, a TF involved in abscisic acid signal transduction, are related to strawberry ripening (Tisza *et al.*, 2010; J.Y. Chen *et al.*, 2011).

Little information about plant-specific NAC TFs associated with fruit ripening is available. In the present work, gene expression profiles in fruit with four different ripening characteristics – natural, ethylene-induced, 1-MCP-delayed, and 1-MCP and ethylene-treated ripening – revealed that banana *MaNAC* genes were differentially expressed in peel and pulp during ripening. *MaNAC1* and *MaNAC2* were apparently upregulated by ethylene in peel and pulp, consistent with the increase in

ethylene production (Figs 6 and 7). In contrast, *MaNAC3* in peel and pulp and *MaNAC5* in peel were expressed constitutively, and transcripts of *MaNAC4* in peel and pulp and *MaNAC6* in peel decreased, while *MaNAC5* or *MaNAC6* in pulp slightly increased during fruit ripening (Figs 6 and 7). These results suggest that the transcription complexes created during ripening are constantly changing and *MaNAC1*/*MaNAC2* expression may be more related to fruit ripening. Moreover, the *MaNAC2* promoter, when transiently expressed in protoplasts, was activated after ethylene application, further confirming that *MaNAC2* is ethylene inducible and may be involved in fruit ripening (Fig. 8 and Supplementary Fig. S1). Other plant NAC TFs, including *Arabidopsis AtNAC2* (He *et al.*, 2005), pepper *CaNAC1* (Oh *et al.*, 2005), rice *OsNAC19* (Lin *et al.*, 2007), and wheat *TaNAC4* (Xia *et al.*, 2010), are also ethylene inducible, supporting the notion that NAC TFs might be a common downstream component of the ethylene signalling pathway. In tomato, *NOR* (non-ripening) is a NAC-domain TF and fruit carrying its mutation, *nor*, cannot ripen even after exogenous ethylene application, suggesting an important role of *NOR* in regulating fruit ripening (Cara and Giovannoni, 2008). Thus, it will be interesting to investigate whether MaNAC1/MaNAC2 is able to complement ripening in a tomato *nor* mutant.

### *Interactions between MaNAC1/MaNAC2 and MaEIL5*

NAC TFs have been found not only to bind to DNA but also to interact with other proteins, including several NAC proteins interacting with a RING protein (Greve *et al.*, 2003), a TCP-domain TF (Weir *et al.*, 2004), and a SNF1-related kinase (Kleinow *et al.*, 2009). Moreover, some NAC proteins can interact with other NACs or with themselves to function by forming homodimers and/or heterodimers in plants, such as *Arabidopsis* NAC1 and ANA019 (Xie *et al.*, 2000; Ernst *et al.*, 2004), *Brassica* BnNAC14 with BnNAC3, BnNAC5-8, BnNAC5-11, and BnNAC485 (Hegedus *et al.*, 2003), and rice OsNAC5 with OsNAC6, OsNAC9, OsNAC10, and itself (Jeong *et al.*, 2009). Several groups have reported that NAC may represent a TF downstream of *EIN2* but in parallel with *EIN3*, which is also a TF downstream of *EIN2* (He *et al.*, 2005; Kim *et al.*, 2009; Al-Daoud and Cameron, 2011). In the present study, banana MaNAC1/MaNAC2 physically interacted with an EIN3-like protein, MaEIL5, in Y2H and BiFC assays (Figs 9 and 10). EIN3 and EIN3-like (EIL) proteins are key TFs in ethylene signalling, and play important roles in regulating fruit ripening (Mbéguié-A-Mbeguié *et al.*, 2008; Yin *et al.*, 2010). Our results, together with previous reports, clearly suggest that NAC TFs are involved in ethylene signalling pathways through interaction with ethylene signalling components. However, it should be pointed out that the MaEIL5 transcript level decreased following ethylene evolution during ripening (Figs 6 and 7). Generally, EILs act as positive regulators of ethylene response, but levels of *EIL* genes show no increase during fruit ripening, implying that changes in *EIL* mRNA levels may be not necessary to induce an ethylene response (Tieman *et al.*, 2001). At the same time, EILs may be regulated at the post-transcriptional or post-translational level, as demonstrated by Potuschak *et al.* (2003), who showed that EBF1/EBF2 physically interacted with EIN3/EIL1 and probably targeted these proteins for degradation. Accordingly, it is imperative to study the protein modifications of EILs during banana fruit ripening in the future. In addition, it will be interesting to find why only MaNAC1/MaNAC2, which showed no transcription activation ability in yeast cells, could interact with MaEIL5, while MaNAC4/MaNAC6 with transcription activation ability, could not interact with MaEIL5 (Figs 4 and 9), and why none of the six MaNACs interacted with MaEIL2 (data not shown), whose transcript level increased during fruit ripening (Figs 6 and 7).

In summary, six banana fruit *MaNAC* genes were isolated and characterized. Gene expression profiles in fruit with four different ripening characteristics revealed that *MaNAC* genes are expressed differentially in peel and pulp during ripening. More importantly, MaNAC1/MaNAC2 physically interacted with MaEIL5. Taken together, these results suggest that MaNACs such as MaNAC1 and MaNAC2 may be involved in banana fruit ripening via interaction with ethylene signalling components. To the best of our knowledge, this is the first report that NACs interact physically with EILs, and our findings reveal a novel mechanism of NAC TFs participating in ethylene signalling.

## Supplementary data

Supplementary data are available at *JXB* online.

## Supplementary data 1

Table S1. Primers used for RACE.
Table S2. Primers used for fusing GFP.
Table S3. Primers used for subcloning into pGBKT7.
Table S4. Primers used for quantitative real-time PCR analysis.
Table S5. Primers used for promoter isolation of MaNAC2.
Table S6. Primers used for MaNAC2 promoter fusing with GFP.
Table S7. Primers used for Yeast Two-Hybrid analysis.
Table S8. Primers used for BiFC assays.
Table S9. Sequence similarities among the different MaNACs genes.
Figure S1. MaNAC2 promoter activity in response to ethylene in Arabidopsis mesophyll protoplasts.
Figure S2. Subcellular localization of MaEIL5 in tobacco BY-2 protoplasts.

## Supplementary data 2

GenBank accession numbers of Arabidopsis and rice NAC proteins used for phylogenetic tree of NACs.

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