

The Banana MaLBD (LATERAL ORGAN BOUNDARIES DOMAIN) Transcription Factors Regulate *EXPANSIN* Expression and Are Involved in Fruit Ripening

Liang-jie Ba · Wei Shan · Jian-fei Kuang · Bi-hong Feng · Yun-yi Xiao · Wang-jin Lu · Jian-ye Chen

Published online: 5 April 2014
© Springer Science+Business Media New York 2014

Abstract The LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD) proteins are plant-specific transcriptional factors (TFs) functioning in the growth and development of *Arabidopsis* and other plant species. However, the involvement of LBD TFs in regulating the ripening of economically important fruits is largely unknown. In the present study, four full-length *LBD* genes, designated as *MaLBD1–MaLBD4*, were isolated and characterized from banana fruit. Expressions of *MaLBD1–MaLBD4* in fruit with three ripening characteristics revealed that *MaLBD1/2/3* were ethylene-inducible, and their transcript levels obviously increased during fruit ripening, while *MaLBD4* changed slightly. Moreover, the activities of *MaLBD1/2/3* promoters were activated after ethylene treatment, further supporting their involvement in fruit ripening. Subcellular localization showed that *MaLBD1/2/3* were nuclear proteins, and a transactivation assay in protoplasts demonstrated that *MaLBD1/2/3* had transactivation activity. More importantly, a transient expression assay further indicated that *MaLBD1/2/3* were transcriptional activators that regulated ripening-related *MaEXP1/2* expressions by directly binding to *MaEXP1/2* promoters. These results suggest that MaLBDs are involved in regulating

fruit ripening, in part by transcriptional activation of the *EXPANSIN* expression related to cell wall modification. Taken together, our findings provide some new information about the functions of LBD TFs involved in the regulation of fruit ripening.

Keywords Banana fruit · LBD transcriptional factor · *EXPANSIN* · Transcriptional activation · Fruit ripening

Introduction

Fruit ripening represents a complex and highly coordination of physiological and biochemical processes, accompanying with changes of thousand genes associated with color, flavor, aroma, texture, and nutritional value of the flesh (Klee and Giovannoni 2011; Gapper et al. 2013), which are influenced by internal and external cues regulated by many critical transcription factors (TFs) (Martel et al. 2011). A major breakthrough in dissecting the transcriptional control of fruit ripening was the identification of several genes underlying rare mutations that completely abolish the normal ripening process of tomato, including *ripening inhibitor (RIN)*, *Colorless nonripening (CNR)*, and *nonripening (NOR)* (Seymour et al. 2013). These three mutants loci that all harbor TFs belong to MADS, NAC, and SBP TFs family, respectively (Seymour et al. 2013). Other ripening TFs, such as HD-zip protein LeHB1, TAGL1, SIAP2a, and SIERF6, also have been identified in tomato (Gapper et al. 2013). In addition, several TFs, like ERFs and EIN3-like, are suggested to be involved in the ripening of other fruits, such as apples, kiwifruit, plums, and longan fruit (Wang et al. 2007; El-Sharkawy et al. 2009; Yin et al. 2010; Kuang et al. 2012). Together, these studies highlight the dedicated transcriptional control of the fruit ripening process.

Electronic supplementary material The online version of this article (doi:10.1007/s11105-014-0720-6) contains supplementary material, which is available to authorized users.

L.-j. Ba · W. Shan · J.-f. Kuang · B.-h. Feng · Y.-y. Xiao · W.-j. Lu · J.-y. Chen (✉)

State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources/Guangdong Key Laboratory for Postharvest Science, College of Horticultural Science, South China Agricultural University, Guangzhou 510642, People's Republic of China
e-mail: chenjianye@scau.edu.cn

B.-h. Feng
College of Agriculture, GuangXi University, Nanning 530004, People's Republic of China

The LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD) (also known as ASL for ASYMMETRIC LEAVES2-LIKE) proteins are plant-specific TFs, characterized by a typical N-terminal LOB/AS2 domain with a CX₂CX₆CX₃C motif and a Leu zipper-like sequence (Husbands et al. 2007; Majer and Hochholdinger 2011). LBD proteins exhibit varied expression patterns ranging from temporal to tissue differences, indicating that they may function in diverse processes (Shuai et al. 2002). Functional characterization of several LBD members in *Arabidopsis*, rice, maize, and poplar reveals that LBD proteins play critical roles in defining lateral organ boundaries and in regulating many aspects of plant development, including root, leaf, inflorescence, and embryo development (Liu et al. 2005; Soyano et al. 2008; Yordanov et al. 2010; Majer and Hochholdinger 2011; Lee et al. 2013; Wang et al. 2013). In addition to developmental processes, LBD proteins also have been found to be directly involved in the metabolic regulation in response to nitrogen deficiency (Rubin et al. 2009), anthocyanin synthesis (Rubin et al. 2009), auxin signal cascades (Okushima et al. 2007; Lee et al. 2009; Rast and Simon 2012), and jasmonate signaling (Thatcher et al. 2012a). However, LBD TFs have not been previously implicated in the regulation of fruit ripening.

Bananas are the world's fourth most important food crop after rice, wheat, and maize, with a vast majority of the crop grown and consumed in the tropical and subtropical countries (Cruz-Hernández and Paredes-López 2012). Banana is a typical climacteric fruit, characterized by a peak in ethylene production that orchestrates ripening-associated processes, which results in a short postharvest life of 10–15 days at ambient temperature (Bapat et al. 2010; Shan et al. 2012; Xiao et al. 2013). Accordingly, it is important to gain a better understanding of the mechanism of banana fruit ripening, in order to explore effective technologies to maintain the quality and storage potential of this fruit. Very recently, several ripening TFs, including MADS-box, NAC, and ERF, have been identified in banana fruit, as well as their transcriptional control of ripening (Elitzur et al. 2010; Choudhury et al. 2012; Shan et al. 2012; Xiao et al. 2013). However, it is clear that multiple TFs influence the ripening process (Klee and Giovannoni 2011), indicating that the full complexity of transcriptional regulatory mechanism remains elusive. Moreover, recently, the whole-genome sequence for banana is reported (D'Hont et al. 2012), which has given researchers access to a new source of vast numbers of TFs. In this study, four LBD TFs were isolated and characterized from banana fruit. Their expression patterns in association with three different ripening treatments were analyzed. Moreover, the interactions of the ripening-related MaLBDs with cell wall-modifying genes *MaEXPs* encoding for EXPANSIN protein were investigated. Our findings suggest that MaLBDs may be involved in banana fruit ripening via in part by transcriptional activation of cell wall-loosening-related genes *MaEXPs*.

Materials and Methods

Plant materials and Treatments

Pre-climacteric banana (*Musa acuminata*, AAA group, cv. Cavendish) fruits at 75–80 % maturation were obtained from a local commercial plantation near Guangzhou, China. Three postharvest treatments, including a control (natural ripening), ethylene-induced ripening (100 $\mu\text{l l}^{-1}$ ethylene, 18 h), and 1-methylcyclopropene (1-MCP)-delayed ripening (0.5 $\mu\text{l l}^{-1}$ 1-MCP, 18 h) were performed to create three different ripening characteristics, and samples were taken as described previously (Shan et al. 2012). All assessments were conducted using three biological replicates.

RNA Extraction, Gene Isolation, and Sequence Analysis

Total RNA was extracted using the hot borate method of Wan and Wilkins (1994). Total RNA extract was treated with DNase I (Promega, Madison, WI, USA), and the resulting DNA-free total RNA was used as the template for reverse transcription PCR (RT-PCR). The first-strand cDNA of the product was subjected to PCR amplification.

According to gene annotation and bioinformatics analysis, four full-length *LBD* genes with complete start and stop codes, termed *MaLBD1-MaLBD4*, were selected randomly from banana whole-genome sequence (D'Hont et al. 2012), and the sequences were verified by further cloning from banana fruit pulp and sequencing. Alignments were carried out on ClustalX (version 1.83) and GeneDoc software, and a phylogenetic tree was constructed using the minimum evolution test method with the MEGA5 program.

Quantitative Real-time PCR Analysis

Synthesis of first-strand cDNA and all RT-qPCR analysis were performed as described previously (Chen et al. 2011; Shan et al. 2012). The sequences of all primers used for RT-qPCR analysis are listed in Supplementary Table S1. *MaRPS4* (ribosomal protein 2) was selected as a reference gene according to our previous study on the selection of reliable reference genes under different experimental conditions (Chen et al. 2011). All RT-qPCR reactions were normalized using Ct value corresponding to the reference gene. The relative expression levels of target gene were calculated with the formula $2^{-\Delta\Delta\text{CT}}$. Three independent biological replicates were used in the analysis.

Promoter Activity Assay

Genomic DNA was extracted from banana leaves using the DNeasy Plant Mini Kit (Qiagen). The promoter of *MaLBDs* gene was isolated using the GenomeWalker Kit (Clontech)

with nest PCR according to the manufacturer's instructions. Two gene-specific primers for the nest PCR are listed in Supplementary Table S1. The amplification product was cloned into pGEM-Teasy vector (Promega, Madison, WI, USA) and sequenced. Conserved *cis*-element motifs of promoter were predicted using PLACE (<http://www.dna.affrc.go.jp/PLACE/signalscan.html>) and Plant-CARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) databases.

For promoter activity assay, the *MaLBD* promoter region was amplified by PCR using the specific primers listed in Supplementary Table S1. The PCR product was inserted into the pGreenII 0800-LUC double reporter vector (Hellens et al. 2005) at the *Hind*III and *Bam*HI sites to fuse it with the Firefly luciferase (LUC) reporter gene (*MaLBDs pro-LUC*); a Renilla (REN) luciferase under the control of the 35S promoter at the same vector was used as a positive control. The construct CaMV35S-REN/*MaLBDs pro-LUC* was transformed into tobacco BY-2 protoplasts by polyethylene glycol (PEG) methods as described previously (Abel and Theologis 1994). The promoter activity is indicated by the ratio of LUC to REN. The transformed protoplasts were subjected to 0 mM (control) or 0.8 mM of ethylene releaser (ethrel) treatment and then incubated at 23 °C for 14 h, and LUC and REN luciferase activities were assayed using the dual luciferase assay kits (Promega). The analysis was carried out using the Luminoskan Ascent Microplate Luminometer (Thermo) according to the manufacturer's instructions, with a 5-s delay and 15-s integrated measurements. At least six assay measurements were included for each.

Subcellular Localization of MaLBDs

The coding sequence of MaLBDs without the stop codon was amplified by PCR (primers are listed in Supplementary Table S1) and subcloned into the pUC-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 in tobacco BY-2 suspension culture cell protoplasts as described above. GFP fluorescence was observed with a fluorescence microscope (Zeiss Axioskop 2 Plus). All transient expression assays were repeated at least three times.

Transient Assay in Protoplast

A dual luciferase assay was performed in the transient assay. For transactivation analysis of MaLBDs, the coding sequence of MaLBDs without the stop codon was cloned into the reconstructed GAL4DBD vector as effector. The double reporter vector includes a GAL4-LUC and a internal control REN driven by the 35S promoter. GAL4-LUC contains five

copies of GAL4-binding element and minimal TATA region of the 35S promoter of CaMV, and these sequences are located upstream of the LUC.

For the assay of the binding activity of MaLBDs to the *MaEXPs* promoter, two pGreenII vectors, pGreenII 0800-LUC reporter vector and pGreenII 0029 62-SK effector vector, were used (Hellens et al. 2005). The *MaEXPs* promoter was cloned into pGreen 0800 at the *Hind*III and *Bam*HI sites to fuse it with the LUC reporter gene (*MaEXPs pro-LUC*). MaLBDs were cloned into the pGreenII 62-SK vector. The pGreenII 0800-LUC vector carried a REN under the control of the 35S promoter as a positive control.

The constructed effector and reporter plasmids were co-transfected into BY-2 protoplasts as described above. The resulted protoplasts were incubated at 23 °C for 16 h in the dark and then were harvested to determine the LUC and REN luciferase activities as described above. The transactivation ability of MaLBDs and the binding activity of MaLBDs to the *MaEXPs* promoter are indicated by the ratio of LUC to REN. At least six transient assay measurements were included for each pair. The primers used in the construction of the effector and reporter vectors are listed in Supplementary Table S1.

Statistical Analysis

The experiment was performed using a completely randomized design. Data were presented as mean±standard error (S.E.). Least significant difference (LSD) at the 5 % level was analyzed by DPS software (version 3.01; Zhejiang University, Hangzhou, China).

Results

Sequence Analysis of Banana Fruit *MaLBDs* Genes

Based on the banana whole-genome sequence (D'Hont et al. 2012), four *LBD* full-length cDNAs, designated as *MaLBD1* to *MaLBD4*, were cloned from banana fruit pulp. *MaLBD1*–*MaLBD4* were predicted to encode proteins of 236, 211, 287, and 250 aa, with calculated molecular weights of 24.4, 23.0, 30.7, and 26.7 kDa, and pI values of 7.46, 9.22, 6.20, and 6.14, respectively. Alignment of the deduced amino acid sequences of MaLBDs clearly showed that the conserved DNA-binding domain, designated as the LOB domain, existed in the N-terminal regions of the MaLBDs proteins (Fig. 1), which is a defining character of the LBD TF family (Majer and Hochholding 2011). The LOB domain comprises a C-domain presumably required for DNA binding which contains four perfectly conserved cysteine (C) residues in a CX2CX6CX3C zinc finger-like motif (Fig. 1). Moreover, an invariant glycine residue and a C-terminal leucine-zipper-like motif responsible for probable protein-protein

Fig. 1 Alignment of the deduced amino acid sequences of four banana MaLBDs and their respective homologs from grape (VvLBD1, XP_002279521), cacao (TcLBD1, EOY20040), and soybean (GmLBD1, XP_003541285). Identical and similar amino acids were presented by *black and gray shading*, respectively. Gaps were introduced to optimize alignment. The LOB domain was *underlined*. The C-motif (CX2CX6CX3C) was included in the *rectangle*, and the invariant glycine residue was indicated by the *asterisk*

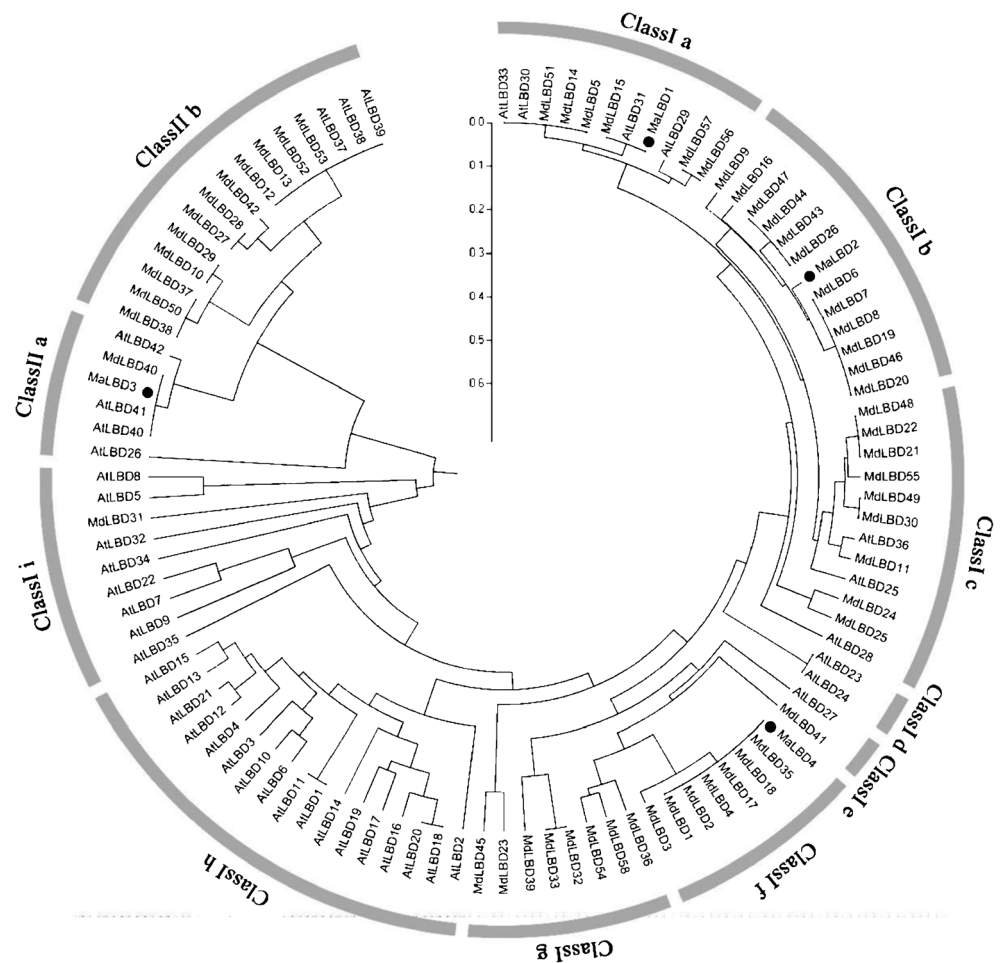
MaLBD1	-----MSGSIGPNNGGGGGGGGG--SGGGGGG-----	26
MaLBD2	-----MSTPRERIEEVGKKIKREADVVTDRTRR--LSLVGTIN-----	37
MaLBD3	-----	-
MaLBD4	MASCSSALALYMYSPGLEASHHLDSPFPSSKAMKSSDPTTTTSSFQHSSESSSSSSSPTSSPSSS	70
VvLBD1	-----MSANTSSGGGGGGGGGGSTSGGGGGG-----	28
TcLBD1	-----MSRERERFDEIGKKIKREADVSSHMMGRRHMLGPPPTLN-----	39
GmLBD1	-----	-
C-motif		
MaLBD1	-----FCGACKFLRRKCVVGCIFAPYFN---SEQGAHFAAVHKMFGASNASKLLLHI	76
MaLBD2	-----TITFCAAKLLRRRCACQDCPPAFYFA---EHE-PQRFASVHKMFGASNISRMLLEV	89
MaLBD3	-----MRMFCNGCRVLRKCCSENCISIRECLOWIKSESOANATVFLAKFYGRAGUMSLINAG	57
MaLBD4	PPSLPPTTPPPFVIHSPFCGACKFLRRRCAGKCLAPYFP---ETE-PLKFTTAHRVFGASNTIKLLQDL	135
VvLBD1	-----FCGACKFLRRKCVVGCIFAPYFD---EQGAHFAAVHKMFGASNISRMLLEV	78
TcLBD1	-----TITFCAAKLLRRRCACQDCPPAFYFS---EHE-PQRFASVHKMFGASNISRMLMEV	91
GmLBD1	-----MRMFCNGCRVLRKCCSEDCISIRECLOWIKNEESOANATVFLAKFYGRAGUMSLINAG	57
LOB domain		
MaLBD1	<u>BAHKRLDAVVVTCYEAQARLRDPVYGCVAHIFALQQQVNLHAEELSCLOHHLATLELPTPPS</u>	139
MaLBD2	<u>PESQRADAANSLVYEANRLRDPPIYGCMAISALQQQVQALELELQAVRAEILKHKFRQASAGVIEPTSHA</u>	159
MaLBD3	<u>BDHLRPAIFRSLLYEACCRIVNPIYGSVGLLWSGSSWQLCQAAVBAVLKGEIVQIPEYKAASPTAE</u>	125
MaLBD4	<u>PESQRADAVSSMVEANARIRDPVYGCAGATEQLKQVNELOAQEAQAQRLNLQAQHN</u>	198
VvLBD1	<u>BVHKRLDAVVVTCYEAQARLRDPVYGCVAHIFALQQQVNLQAEBSYLOAHLATLELPSPPPPPPPPV</u>	148
TcLBD1	<u>PESQRADAANSLVYEANVRLRDPVYGCMAISALQQQVQSLQAEELNAVRAEILKYKCREAN</u>	159
GmLBD1	<u>BNELRPAIFRSLLYEACCRIVNPIYGSVGLLWSGSSWQLCQAAVBAVLKGEIITPTTSEAAANGRAE</u>	125
MaLBD1	LASPTPFSMSDLSSSTLPESTADLSTLDFPFLP-SWPLQ--QRPTELFMQQRSTSTRSPSESSGCGGG	206
MaLBD2	AFLAPSEAVSMAAPPFRSVTSPPP-----ALGSSSSFMYSILDSSA---ITMNHNTLYFG-----	211
MaLBD3	KAYDIRHVSKESSAARAAAEHLHKVKNRPRFRKRSKATTRPDTPL-PEFQARSHES--AASHATEPGPS	192
MaLBD4	ALICVEVAQNQODRAPRSTDALAAGN---YLLQNDAYFLDEINQEG---SVWDEPLWI-----	250
VvLBD1	MVTPPSLISLPLSASSMPPITYDLSSLFDFPMVQSSWPMQHRQIDPRQFGGRGP-TDLPSTAGGSGGG	217
TcLBD1	ALLS-SCAVSVAALPSAPTHPPPPPPPLPPTSSSSSMYTQPTTAADYSTISNENVSYFGMSRERERFD	228
GmLBD1	KAYDIRHVSKE---QNSANETPKT-KTRSRFKRTSGTLIKPKASKGTGFVVEPEMANRTASHESGLSHL	191
MaLBD1	DLQELARELLGRH--CLGDVT--AEPGVQESKSI-----	236
MaLBD2	-----	-
MaLBD3	AAAASGEEAECHRENESSASADTAEEASHVSGQEPQRAEEAQEEEEISLELTLCLEPASRSARPAEHAA	262
MaLBD4	-----	-
VvLBD1	DLQALARELLHRHSSALGGHVPCTESSMPPSISK-----	251
TcLBD1	EIGKKIKREADVSSHMMGRRHMLGPPPTLNTITPCAACKLLRRRCACQDCPPSFPYFSPHEPQKFASVHKVF	298
GmLBD1	SEATAMVEGESKESESVVSMDTSNLTHEEPEWAKTSDGTGESGNEGLGLETLGFEPVSRHLHVVMKRR	261
MaLBD1	-----	-
MaLBD2	-----	-
MaLBD3	PQCDVSSWDAVAR--TSGLGGLALPPPS-----	287
MaLBD4	-----	-
VvLBD1	-----	-
TcLBD1	GASNVSKMLMEVPESQRADAANSLVYEANVRLRDPVYGCMAISALQQQVQSLQAEELNAVRAEILKYKCR	368
GmLBD1	KIIEKSCGDSBAEKDSCMELGLEYP-----	288
MaLBD1	-----	-
MaLBD2	-----	-
MaLBD3	-----	-
MaLBD4	-----	-
VvLBD1	-----	-
TcLBD1	EANLIPSSHVALSSGAVSVAALPSAPTHPPPPPPPLPPTSSSSSMYTQPTTAADYSTISNENVSYFG	438
GmLBD1	-----	-

interactions, which includes five hydrophobic amino acids (valine, isoleucine, leucine) separated by six variable amino acids, are observed within the LOB domain (Fig. 1) (Shuai et al. 2002; Matsumura et al. 2009; Majer and Hochholdinger 2011).

To determine the evolutionary relationship among MaLBDs and those of *Arabidopsis* and apple and then group them into established subfamilies, a phylogenetic tree was generated with full-length protein sequences (Fig. 2). The

whole LBD proteins of *Arabidopsis* and apple could be classified into two major groups, I and II. Class I and class II families were further divided into 8 and 2 groups, as reported by Wang et al. (2013). In class I, the subgroups were named from class I a to class I i, while class II a and class II b comprised the class II group. Based on the tree, the four banana MaLBD proteins were clustered into four subgroups. MaLBD1 to MaLBD4 belonged to class I a, class I b, class II a, and class I f, respectively (Fig. 2). Together, these data

Fig. 2 Phylogenetic tree of LBDs. Four banana *MaLBDs* (black circles) were aligned with the *Arabidopsis* and apple LBD family as designated by Wang et al. (2013). The multiple alignment was made using ClustalW, and the phylogenetic tree was constructed with MEGA 5.0 using a bootstrap test of phylogeny with minimum evolution test and default parameters



indicate that *MaLBD1* to *MaLBD4* may exhibit diverse functions.

Expression of *MaLBDs* Genes during Fruit Ripening

The data related with fruit ripening and softening, including changes in fruit firmness and ethylene production in banana fruit in three different ripening treatments caused by natural, ethylene-induced, and 1-MCP-delayed treatments, have been described in the previous publication (Shan et al. 2012). Control natural fruit showed the ethylene climacteric at 15 days and reached a peak at 21 days. Ethylene treatment accelerated fruit ripening, with a climacteric peak appearance at 3 days. In contrast, 1-MCP treatment delayed ethylene production, with a peak appearance at day 30 (Shan et al. 2012).

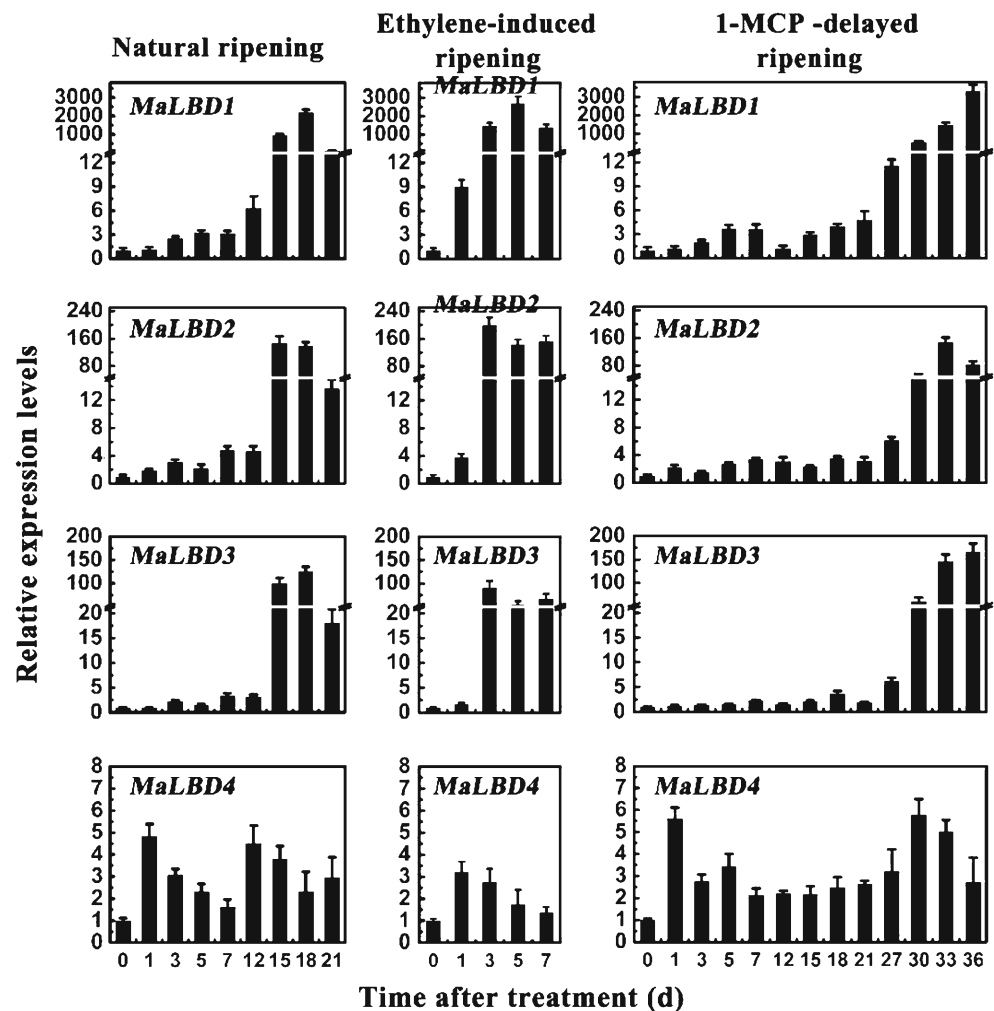
To investigate the possible role of *MaLBD* genes during banana fruit ripening, RT-qPCR was carried out to determine their mRNA accumulations in banana fruit pulp with those three different ripening characteristics. As shown in Fig. 3, among the four *MaLBD* genes, *MaLBD1*, *MaLBD2*, and *MaLBD3* were strongly induced after ethylene treatment, and their transcript levels in fruit pulp of natural, ethylene-

induced, or 1-MCP-delayed ripening clearly increased with ethylene evolution during ripening. However, *MaLBD4* was expressed constitutively and its transcripts changed slightly during ripening (Fig. 3). These results indicate that *MaLBD1*, *MaLBD2*, and *MaLBD3* may be more associated with fruit ripening. Thus, these three *MaLBD* genes were selected for the following analysis.

Promoter Activities of *MaLBD1/2/3* are Induced by Ethylene

To further understand the regulation of *MaLBD1/2/3* expression by ethylene, the upstream promoter region sequences from the transcription start site of *MaLBD1/2/3* were isolated from the genome of *M. acuminata* using genome walking PCR methods. Amplification using specific primers for *MaLBD1/2/3* upstream sequences resulted in 1,585-, 491-, and 1,067-bp amplicon, subsequently named *MaLBD1*, *MaLBD2*, and *MaLBD3* promoter, respectively. Analysis of these promoters using the PLACE and Plant-CARE databases was illustrated in Supplementary Tables S2, S3, and S4. In addition to the core *cis*-acting elements like the TATA box and CAAT box, several regulatory motifs that are potentially related to light response and phytohormones such as salicylic

Fig. 3 Expressions of *MaLBDs* in pulp during three ripening characteristics: natural (control), ethylene-induced, and 1-MCP-delayed ripening. The expression levels of each gene are expressed as a ratio relative to the harvest time (0 days of control), which was set at 1. Each value represents the mean±S.E. of three biological replicates. The physiology data related with fruit ripening and softening, including changes in fruit firmness and ethylene production in banana fruit with these three different ripening characteristics, has been described in the work of Shan et al. (2012)



acid (SA), methyl jasmonate (MeJA), auxin, abscisic acid (ABA), and gibberellin were also found. In particular, PERE motif, which has also been shown to function as an ethylene-responsive element (Solano et al. 1998; Yin et al. 2010), was observed in *MaLBD1/2/3* promoters (Supplementary Tables S2, S3, and S4). These results indicate that the activities of *MaLBD1/2/3* promoters may be modulated by ethylene.

To analyze *MaLBD1/2/3* promoter activities in response to ethylene, a transient protoplast assay was conducted using a dual luciferase reporter vector containing the Firefly LUC driven by the *MaLBD1/2/3* promoter and the REN luciferase driven by the CaMV35S promoter (CaMV35S-REN/*MaLBDs pro-LUC*, Fig. 4a). As shown in Fig. 4b–d, in BY-2 protoplast transfected with CaMV35S-REN/*MaLBD1/2/3 pro-LUC*, *MaLBD1/2/3 pro-LUC*/REN ratio apparently increased after ethylene treatment (Fig. 4b–d), suggesting that *MaLBD1/2/3* promoter activities were induced by ethylene. These observations, together with the accumulation of *MaLBD1/2/3* mRNA during ripening or following ethylene treatment, further strengthen the

notion that *MaLBD1/2/3* are likely to be involved in banana fruit ripening.

MaLBD1/2/3 are Nucleus-localized Proteins

LBD proteins are TFs that have previously been shown to localize to the nucleus (Husbands et al. 2007; Okushima et al. 2007; Rubin et al. 2009). To examine the subcellular localization of *MaLBD1/2/3* in vivo, the full-length coding sequences of *MaLBD1/2/3* were fused in-frame with the *GFP* gene and transiently expressed in tobacco BY-2 protoplasts. As shown in Fig. 5, the fluorescence of *MaLBD1/2/3* proteins was localized exclusively in the nucleus, whereas the fluorescence from the GFP control was distributed throughout the entire cell (Fig. 5). These results indicate nuclear localization of *MaLBD1/2/3*, consistent with their predicted role as TFs.

MaLBD1/2/3 Exhibit Transactivation Activity in Protoplasts

To investigate the abilities of *MaLBD1/2/3* to activate transcription, the transactivation activities of *MaLBD1/2/3* were

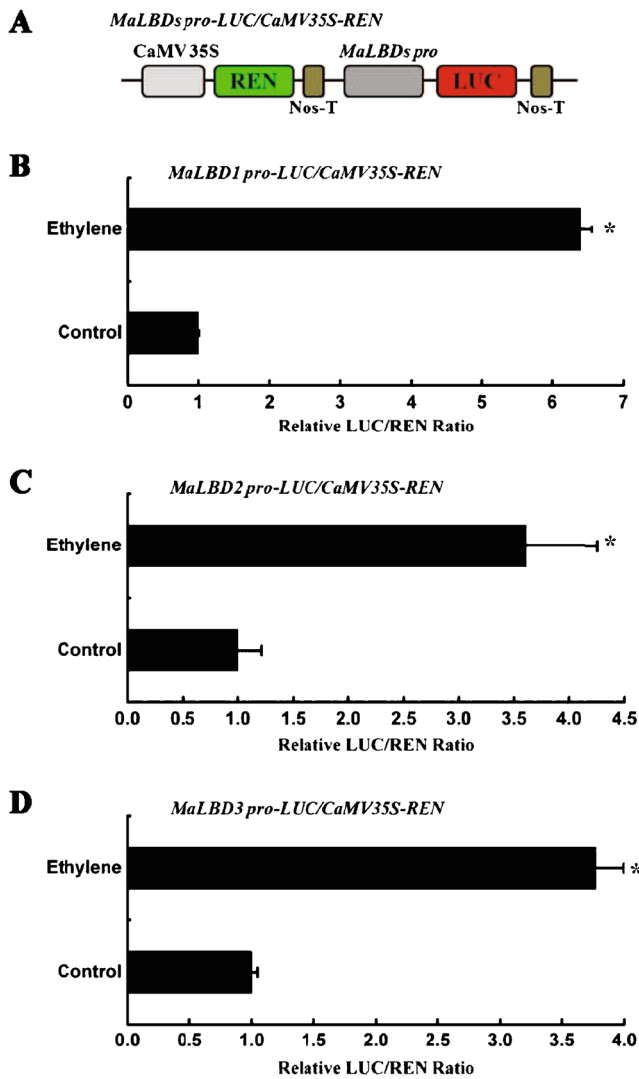


Fig. 4 *MaLBD* promoter activities in response to ethylene. The *top panel* (a) shows schematics of the dual luciferase reporter vectors containing *MaLBD* promoters (CaMV35S-REN/*MaLBDs pro*-LUC). The *bottom panel* (b–d) shows promoter activities of *MaLBD1/2/3* in response to ethylene. The reporter constructs containing *MaLBD* promoters were transiently transformed into tobacco BY-2 protoplasts using a modified PEG method and test for ethylene induction. After incubation for 14 h, LUC and REN luciferase activities were assayed, and the promoter activity is indicated by the ratio of LUC to REN. The *asterisk* indicates a significant difference at the 5 % level compared to the control. Each value represents the means of six biological replicates, and *vertical bars* represent the S.E

examined using transient expression assay in BY-2 protoplasts. We employed a dual luciferase reporter plasmid harboring five copies of the Gal4-DNA binding element and minimal TATA region of 35S promoter fused to the LUC reporter, and a REN reporter under the control of the 35S promoter at the same vector was used as an internal control for successful transfection, while an effector plasmid encoding full-length *MaLBD1/2/3* fused to the Gal4 DNA-binding domain (Gal4BD) (Fig. 6a). Compared with the GAL4-BD

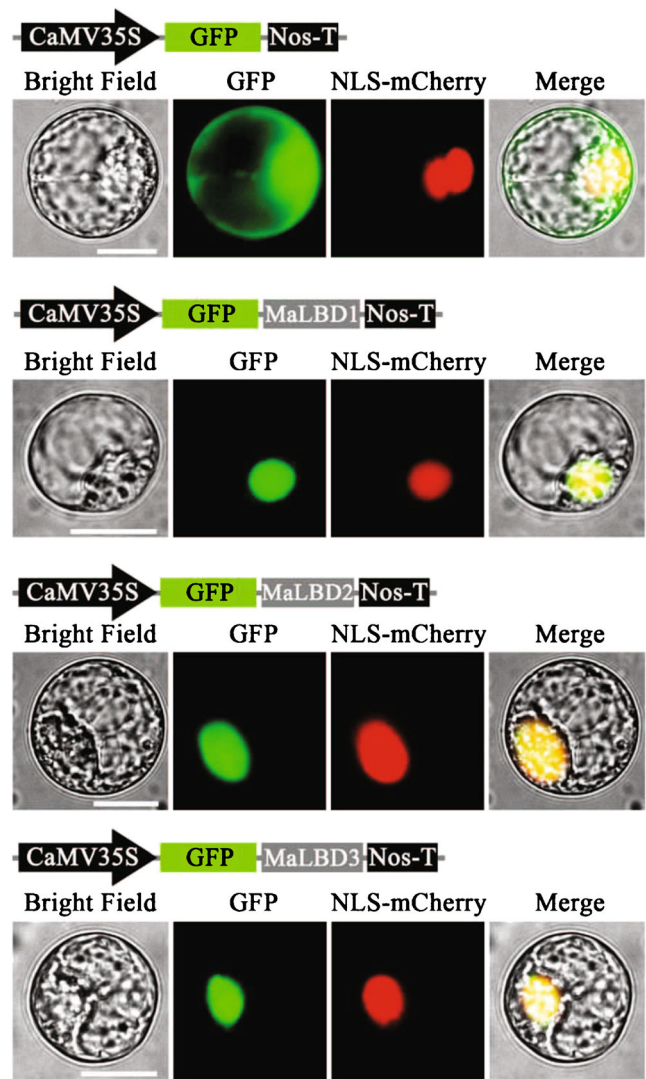


Fig. 5 Subcellular localization of *MaLBDs* in tobacco BY-2 protoplasts. Protoplasts were transiently transformed with 35S:*MaLBDs*-GFP constructs or 35S:GFP vector using a modified PEG method. GFP fluorescence was observed with a fluorescence microscope. VirD2NLS-mCherry was included in each transfection 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

(pBD) negative control, *MaLBD1/2/3* strongly activated the *LUC* reporter gene, and the LUC/REN ratio of *MaLBD1/2/3* was 4.5-, 5.8-, and 0.9-fold higher than that of the negative control, respectively (Fig. 6b). These results indicate that *MaLBD1/2/3* may function as transcriptional activators.

MaLBD1/2/3 are Transcriptional Activators That Regulate *MaEXP1/2* Genes

Recently, *LBD18* is reported to function as a transcriptional activator that regulates the expression of *EXP14/17* by directly binding to *EXP14/17* promoters, facilitating the emergence of lateral root (Lee et al. 2013; Lee and Kim 2013). Five *EXP*

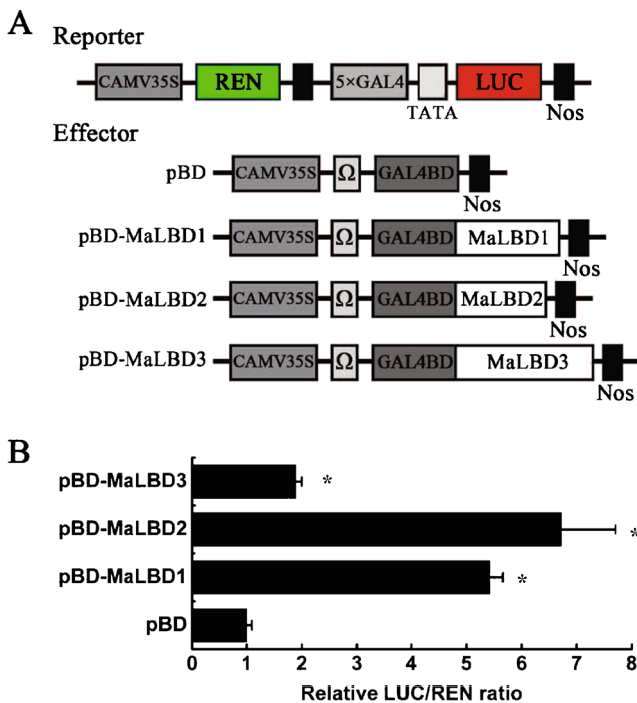


Fig. 6 Transcriptional activation of MaLBDs in tobacco BY-2 protoplasts. **a** Reporter and effector constructs. The dual luciferase reporter construct contained the LUC reporter gene driven by the mini-35S (TATA box) plus five GAL4-binding elements. Each of the effectors contained a GAL4 DNA-binding domain (GAL4-BD), and pBD was used as a negative control. MaLBDs were fused with the GAL4-BD and driven by the 35S promoter plus the translation enhancer Ω sequence. **b** Transactivation ability of MaLBDs. Plasmid combinations of dual REN/LUC reporter, and effectors were cotransformed into BY-2 protoplasts. The protoplasts were incubated for 16 h, and the transactivation ability of MaLBDs is indicated by the ratio of LUC to REN. Each value represents the means of six biological replicates, and vertical bars represent the S.E. The asterisk indicates a significant difference at the 5 % level compared to the negative control pBD

genes have been isolated from banana fruit, and cell wall loosening mediated mainly by *MaEXP1/2* is related to fruit ripening (Mbéguié-A-Mbéguié et al. 2009). We also investigated expressions of *MaEXP1/2* in banana fruit pulp during ripening. As shown in Supplementary Fig. S1, similar with the expression patterns of *MaLBD1/2/3*, *MaEXP1/2* were strongly induced after ethylene treatment, and their transcript levels in fruit pulp of natural, ethylene-induced, or 1-MCP-delayed ripening obviously increased with ethylene evolution during ripening (Supplementary Fig. S1), further confirming that *MaEXP1/2* are associated with fruit ripening. Thus, it is reasonable to speculate that ripening-related MaLBDs might function as transcriptional activators to regulate *MaEXP1/2* expression in banana fruit.

To confirm the speculation, transient expression assay in BY-2 protoplasts was performed with a dual luciferase reporter plasmid harboring the *MaEXP1/2* promoter fused to LUC, and the REN driven by the CaMV35S promoter (CaMV35S-REN/*MaLBDs* pro-LUC, Fig. 7a), while an effector plasmid

harboring *MaLBD1/2/3* was expressed under the control of the CaMV 35S promoter (Fig. 7a). The results showed that *MaLBD1/2/3* obviously activated the *MaEXP1* promoter activity, with 10.1-, 9.8-, and 4.9-fold enhancement of LUC/REN ratio than that of the negative empty control vector (Fig. 7b). Similarly, *MaLBD1/2/3* also activated the *MaEXP2* promoter activity (Fig. 7b). These results clearly demonstrate that *MaLBD1/2/3* are transcriptional activators that directly regulate *MaEXP1/2* gene expressions.

Discussion

LBD proteins are a plant-specific TFs family with 42 members in *Arabidopsis* (Shuai et al. 2002), 35 in rice (Yang et al. 2006), 57 in poplar (Zhu et al. 2007), 43 in maize (Schnable et al. 2009), and 58 in apple (Wang et al. 2013). LBD proteins are characterized by the presence of a highly conserved N-terminal DNA-binding domain termed the LOB domain, whereas little conservation is found in their C-termini (Majer and Hochholdinger 2011). In this study, based on the banana whole-genome sequence, four banana fruit *LBD* genes, designated as *MaLBD1* to *MaLBD4*, were cloned. Similar to the previous reports, alignment of the four MaLBD proteins showed that they shared a highly conserved LOB domain (Fig. 1). Phylogenetic analysis and evolutionary relationship of the LBD proteins have been intensively investigated in *Arabidopsis* and rice (Shuai et al. 2002; Yang et al. 2006; Matsumura et al. 2009), which divides the LBD gene family into class I and class II, characterizing by the presence or absence of functional leucine-zipper-like domains, respectively, and the majority of LBD proteins belong to class I (Majer and Hochholdinger 2011). Similarly, the four MaLBDs fell into class I and class II of the previously characterized LBD proteins, in which *MaLBD1/2/4* belong to class I, while *MaLBD3* belongs to class II (Fig. 2).

Most LBD proteins so far identified have been functionally characterized in *Arabidopsis* and crop plants. These LBD proteins are involved in regulating plant lateral organ such as embryo, root, leaf, inflorescence and flower development, anthocyanin or nitrogen metabolism, or responses to phytohormones such as cytokinin, auxin, gibberellins, and JA (Majer and Hochholdinger 2011). Recently, LBD proteins involved in biotic stress response are reported (Thatcher et al. 2012a, b). In the present work, among the four banana fruit *MaLBD* genes, *MaLBD1/2/3* were ripening-inducible; moreover, their expressions also apparently upregulated after ethylene treatment (Fig. 3). Further, when *MaLBD1/2/3* promoters transiently expressed in protoplasts, they were all activated by ethylene applications (Fig. 4). These data indicate that *MaLBD1/2/3* might be associated with banana fruit ripening. This is the first time, to our knowledge, that a possible role for LBD proteins has been demonstrated in the involvement of

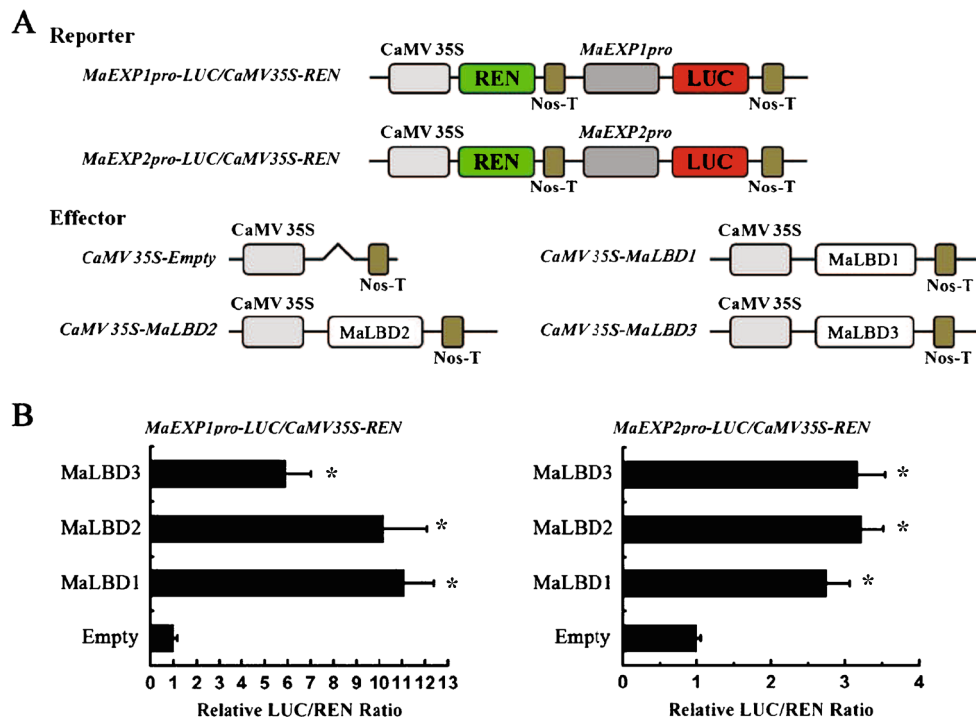


Fig. 7 MaLBDs activate the *MaEXP1/2* promoter in a dual luciferase assay. **a** Schematic representation of the double reporters and effector plasmids used in the assay. The double-reporter plasmid contained the *MaEXP1/2* promoter fused to LUC luciferase and REN luciferase drove by CaMV35S. The effector plasmid contained the MaLBDs drove by the CaMV35S. **b** MaLBDs activate the *MaEXP1/2* promoter. The reporter

and effector vectors, as indicated, were co-transformed into tobacco BY-2 protoplasts. The protoplasts were incubated for 16 h, and the activation of *MaEXP1/2* promoter by MaLBDs was indicated by the ratio of LUC to REN. Each value represents the means of six biological replicates, and vertical bars represent the S.E. The asterisk indicates a significant difference at the 5 % level compared to the empty effector

fruit ripening. LBD proteins are suggested to act as TFs based on their nuclear localization and DNA-binding activity (Shuai et al. 2002; Husbands et al. 2007; Naito et al. 2007; Rubin et al. 2009). It has been demonstrated that *Arabidopsis* ASL4 (AtLOB) specifically binds to a DNA motif 5'-GCGGCG-3' designated as the LBD motif (Husbands et al. 2007). Our present work also showed that MaLBD1/2/3 were localized in the nucleus (Fig. 5). Moreover, MaLBD1/2/3 possessed transcription-activating abilities in BY-2 protoplasts (Fig. 6). These results suggest that MaLBD1/2/3 may function as transcriptional activators. Accordingly, it is interesting to explore their downstream targets that related to banana fruit ripening.

Following the discovery of several important ripening-related TFs in tomato fruit, including MADS-RIN, NOR, and CNR, the identification of their downstream targets has been extensively investigated. For example, MADS-RIN directly controls the expression of a wide range of other ripening-related genes, targeting the promoters of genes involved in ethylene biosynthesis and perception, cell wall metabolism, carotenoid formation, aroma biosynthesis, and ATP generation (Seymour et al. 2013). As to LBD TFs, several downstream targets of LBD TFs have been identified (Majer and Hochholdinger 2011), and interestingly, *Arabidopsis* LBD18/ASL20 activates *EXPANSIN14/17* (*EXP14/17*) expression by directly binding to *EXP14/17*

promoters to enhance lateral root formation during the auxin response (Lee et al. 2013; Lee and Kim 2013). Expansins are primary wall-loosening factors that directly induce turgor-driven wall extension (Cosgrove 2000). Secondary wall-loosening factors such as xyloglucan endotransglycosylase/hydrolases (XET/XTH), glucanases, and polygalacturonase (PG), which enzymatically modify the structures of the cell wall, render it more responsive to wall-loosening events primarily mediated by expansins (Cosgrove 2000; Péret et al. 2009). Fruit softening is a typical textural change associated with ripening, and it has been suggested that cell wall modification enzymes and expansions contribute to fruit softening significantly (Cruz-Hernández and Paredes-lópez 2012). Previously, five *EXP* genes have been isolated from banana fruit, and *MaEXP1/2* expressions are related to fruit ripening (Mbéguié-A-Mbéguié et al. 2009). During fruit ripening, *MaEXP1/2* transcript levels obviously increased, as well as strongly induced after ethylene treatment (Supplementary Fig. S1). Using a transient expression assay in BY-2 protoplasts, we showed that ripening-related MaLBD1/2/3 are transcriptional activators that regulate *MaEXP1/2* gene expressions by directly binding to *MaEXP1/2* promoters (Fig. 7). In addition to *EXP*, other cell wall modification factors, such as XET/XTH, PG, and PME, are also reported to be associated with banana fruit ripening (Mbéguié-A-Mbéguié et al. 2009);

however, whether XET, PG, and PME are direct targets of MaLBD1/2/3 needs to be further investigated. In addition, it has been shown that several LBD proteins interact with MYB and bHLH TFs to co-regulate hormone-mediated organogenesis (Husbands et al. 2007; Guo et al. 2008; Wang et al. 2013); it is thus interesting to study whether MaLBD1/2/3 could interact with the reported ripening-related TFs of banana fruit, including MADSs (Choudhury et al. 2012), NACs (Shan et al. 2012), and ERFs (Xiao et al. 2013) to be involved in fruit ripening.

Overall, four banana fruit *MaLBD* genes were identified and characterized. Gene expression and promoter analysis demonstrated the possible involvement of *MaLBD1/2/3* in fruit ripening. MaLBD1/2/3 were nucleus-localized proteins and had transactivation activity in protoplasts. More importantly, MaLBD1/2/3 were transcriptional activators that regulated *MaEXP1/2* gene expressions by directly binding to *MaEXP1/2* promoters. Taken together, our results elucidate the molecular mechanism by which MaLBDs involved in regulating fruit ripening, in part by transcriptional activation of the *EXPANSIN* expression related to cell wall modification. Our findings provide some new information about the functions of LBD TFs involved in the regulation of fruit ripening.

Acknowledgments We thank Professor Seiichiro Hasezawa (Department of Integrated Biosciences, the University of Tokyo), Professor Shouyi Chen (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences), and Professor Junping Gao (Department of Ornamental Horticulture, China Agricultural University) for the generous gift of tobacco BY-2 suspension cells and the transient expression vectors, respectively. This work was supported in part by the National Natural Science Foundation of China (grant no. 31372111), the China Agriculture Research System (grant no. CARS-32-09) and Guangdong Modern Agricultural Industry Technology System (grant no. LNSG2011-12).

References

- Abel S, Theologis A (1994) Transient transformation of *Arabidopsis* leaf protoplasts: a versatile experimental system to study gene expression. *Plant J* 5:421–427
- Bapat VA, Trivedi PK, Ghosh A, Sane VA (2010) Ripening of fleshy fruit: molecular insight and the role of ethylene. *Biotechnol Adv* 28:94–107
- Chen L, Zhong HY, Kuang JF, Li JG, Lu WJ, Chen JY (2011) Validation of reference genes for RT-qPCR studies of gene expression in banana fruit under different experimental conditions. *Planta* 234:377–390
- Choudhury SR, Roy S, Nag A, Singh SK, Sengupta DN (2012) Characterization of an AGAMOUS-like MADS box protein, a probable constituent of flowering and fruit ripening regulatory system in banana. *PLoS One* 7:e44361
- Cosgrove DJ (2000) Loosening of plant cell walls by expansins. *Nature* 407:321–326
- Cruz-Hernández A, Paredes-López O (2012) Fruit quality: new insights for biotechnology. *Crit Rev Food Sci Nutr* 52:272–289
- D'Hont A, Denoeud F, Aury JM, Baurens FC, Carreel F, Garsmeur O et al (2012) The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* 488:213–217
- Elitzur T, Vrebalov J, Giovannoni JJ, Goldschmidt EE, Friedman H (2010) The regulation of MADS-box gene expression during ripening of banana and their regulatory interaction with ethylene. *J Exp Bot* 61:1523–1535
- El-Sharkawy I, Sherif S, Mila I, Bouzayen M, Jayasankar S (2009) Molecular characterization of seven genes encoding ethylene responsive transcriptional factors during plum fruit development and ripening. *J Exp Bot* 60:907–922
- Gapper NE, McQuinn RP, Giovannoni JJ (2013) Molecular and genetic regulation of fruit ripening. *Plant Mol Biol* 82:575–591
- Guo M, Thomas J, Collins G, Timmermans MC (2008) Direct repression of KNOX loci by the ASYMMETRIC LEAVES1 complex of *Arabidopsis*. *Plant Cell* 20:48–58
- Hellens R, Allan A, Friel E, Bolitho K, Grafton K, Templeton M, Karunairatnam S, Gleave A, Laing W (2005) Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. *Plant Methods* 1:13
- Husbands A, Bell EM, Shuai B, Smith HMS, Springer PS (2007) LATERAL ORGAN BOUNDARIES defines a new family of DNA-binding transcription factors and can interact with specific bHLH proteins. *Nucleic Acids Res* 35:6663–6671
- Klee HJ, Giovannoni JJ (2011) Genetics and control of tomato fruit ripening and quality attributes. *Annu Rev Genet* 45:41–59
- Kuang JF, Chen JY, Luo M, Wu KQ, Sun W, Jiang YM, Lu WJ (2012) Histone deacetylase HD2 interacts with ERF1 and is involved in longan fruit senescence. *J Exp Bot* 63:441–454
- Lee HW, Kim J (2013) EXPANSIN17 up-regulated by LBD18/ASL20 promotes lateral root formation during the auxin response. *Plant Cell Physiol* 54:1600–1611
- Lee HW, Kim MJ, Kim NY, Lee SH, Kim J (2013) LBD18 acts as a transcriptional activator that directly binds to the EXPANSIN14 promoter in promoting lateral root emergence of *Arabidopsis*. *Plant J* 73:212–224
- Lee HW, Kim NY, Lee DJ, Kim J (2009) LBD18/ASL20 regulates lateral root formation in combination with LBD16/ASL18 downstream of ARF7 and ARF19 in *Arabidopsis*. *Plant Physiol* 151:1377–1389
- Liu H, Wang S, Yu X, Yu J, He X, Zhang S, Shou H, Wu P (2005) ARL1, a LOB-domain protein required for adventitious root formation in rice. *Plant J* 43:47–56
- Majer C, Hochholdinger F (2011) Defining the boundaries: structure and function of LOB domain proteins. *Trends Plant Sci* 16:47–52
- Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ (2011) The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. *Plant Physiol* 157:1568–1579
- Matsumura Y, Iwakawa H, Machida Y, Machida C (2009) Characterization of genes in the ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES (AS2/LOB) family in *Arabidopsis thaliana*, and functional and molecular comparisons between AS2 and other family members. *Plant J* 58:525–537
- Mbéguié-A-Mbéguié D, Hubert O, Baurens FC, Matsumoto T, Chillet M, Fils-Lycaon B, Sidibé-Bocs S (2009) Expression patterns of cell wall-modifying genes from banana during fruit ripening and in relationship with finger drop. *J Exp Bot* 60:2021–2034
- Naito T, Yamashino T, Kiba T, Koizumi N, Kojima M et al (2007) A link between cytokinin and ASL9 (ASYMMETRIC LEAVES 2 LIKE 9) that belongs to the AS2/LOB (LATERAL ORGAN BOUNDARIES) family genes in *Arabidopsis thaliana*. *Biosci Biotechnol Biochem* 71:1269–1278
- Okushima Y, Fukaki H, Onoda M, Theologis A, Tasaka M (2007) ARF7 and ARF19 regulate lateral root formation via direct activation of LBD/ASL genes in *Arabidopsis*. *Plant Cell* 19:118–130
- Péret B, De Rybel B, Casimiro I, Benková E, Swarup R, Laplace L, Beeckman T, Bennett MJ (2009) *Arabidopsis* lateral root development: an emerging story. *Trends Plant Sci* 14:399–408

- Rast MI, Simon R (2012) *Arabidopsis* JAGGED LATERAL ORGANS acts with ASYMMETRIC LEAVES2 to coordinate KNOX and PIN expression in shoot and root meristems. *Plant Cell* 24:2917–2933
- Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR (2009) Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in *Arabidopsis*. *Plant Cell* 21:3567–3584
- Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Pasternak S et al (2009) The B73 maize genome: complexity, diversity, and dynamics. *Science* 326:1112–1115
- Seymour GB, Østergaard L, Chapman NH, Knapp S, Martin C (2013) Fruit development and ripening. *Annu Rev Plant Biol* 64:219–241
- Shan W, Kuang JF, Chen L, Xie H, Peng HH, Xiao YY, Li XP, Chen WX, He QG, Chen JY, Lu WJ (2012) Molecular characterization of banana NAC transcription factors and their interactions with ethylene signaling component EIL during fruit ripening. *J Exp Bot* 63:5171–5187
- Shuai B, Reynaga-Peña CG, Springer PS (2002) The lateral organ boundaries gene defines a novel, plant-specific gene family. *Plant Physiol* 129:747–761
- Solano R, Stepanova A, Chao QM, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes Dev* 12:3703–3714
- Soyano T, Thitamadee S, Machida Y, Chua NH (2008) ASYMMETRIC LEAVES2-LIKE19/LATERAL ORGAN BOUNDARIES DOMAIN30 and ASL20/LBD18 regulate tracheary element differentiation in *Arabidopsis*. *Plant Cell* 20:3359–3373
- Thatcher LF, Powell JJ, Aitken EA, Kazan K, Manners JM (2012a) The lateral organ boundaries domain transcription factor LBD20 functions in *Fusarium* wilt susceptibility and jasmonate signaling in *Arabidopsis*. *Plant Physiol* 160:407–418
- Thatcher LT, Kazan K, Manners JM (2012b) Lateral organ boundaries domain transcription factors: new roles in plant defense. *Plant Signal Behav* 7:1702–1704
- Wan CY, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal Biochem* 223:7–12
- Wang A, Tan DM, Takahashi A, Li TZ, Harada T (2007) MdERFs, two ethylene-response factors involved in apple fruit ripening. *J Exp Bot* 58:3743–3748
- Wang XF, Zhang SZ, Su L, Liu X, Hao YJ (2013) A genome-wide analysis of the LBD (LATERAL ORGAN BOUNDARIES domain) gene family in *Malus domestica* with a functional characterization of MdLBD11. *PLoS One* 8:e57044
- Xiao YY, Chen JY, Kuang JF, Shan W, Xie H, Jiang YM, Lu WJ (2013) Banana ethylene response factors are involved in fruit ripening through their interactions with ethylene biosynthesis genes. *J Exp Bot* 64:2499–2510
- Yang Y, Yu X, Wu P (2006) Comparison and evolution analysis of two rice subspecies LATERAL ORGAN BOUNDARIES domain gene family and their evolutionary characterization from *Arabidopsis*. *Mol Phylogenet Evol* 39:248–262
- Yin XR, Allan AC, Chen KS, Ferguson IB (2010) Kiwifruit EIL and ERF genes involved in regulating fruit ripening. *Plant Physiol* 153:1280–1292
- Yordanov YS, Regan S, Busov V (2010) Members of the LATERAL ORGAN BOUNDARIES DOMAIN transcription factor family are involved in the regulation of secondary growth in *Populus*. *Plant Cell* 22:3662–3677
- Zhu QH, Guo AY, Gao G, Zhong YF, Xu M, Huang M, Luo J (2007) DPTF: a database of poplar transcription factors. *Bioinformatics* 23:1307–1308