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MaSPL16 positively regulates fruit ripening in bananas *via* the direct transcriptional induction of *MaNAC029*

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Abstract

Fruit ripening (FR) is attributed to the selective expression of several genes precisely governed by various specific transcription factors (TFs). The NAC (NAM, ATAF, and CUC) TF, MaNAC029, positively regulated banana ripening by directly inducing ethylene biosynthesis and transcription of fruit quality-related genes. However, its upstream regulatory mechanism still needs to be clarified. Herein, yeast one-hybrid screening revealed that a SQUAMOSA promoter binding protein-like (SPL) TF, MaSPL16, was a potentially upstream regulator of *Musa acuminata NAC* (*NAM, ATAF, CUC*) *029* (*MaNAC029*). Furthermore, gel mobility shift assay revealed that MaSPL16 can directly bound with the "GTAC" element of the *MaNAC029* promoter. The gene expression and promoter activity assays demonstrated that *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16* (*MaSPL16*) expression was inducible by ethylene and ripening. MaSPL16 was localized to the nucleus, displayed a potenial capacity for transcriptional activation of *MaNAC029*. More critically, the transient expression of *MaSPL16* in bananas accelerated FR *via* the upregulation of *MaNAC029* and its downstream genes. Collectively, the mechanistic basis of a regulatory cascade involving MaSPL16-MaNAC029 that governed ethylene biosynthesis and fruit quality throughout the entire process of banana fruit ripening was unveiled. These outcomes increase the understanding of the gene-transcriptional regulatory mechanisms in FR. They are envisaged to help devise molecular techniques to regulate maturation and improve future fruit quality.

Keywords Banana fruit, Ripening, SPL, NAC, Regulatory cascade, Ethylene biosynthesis

Introduction

Fruit ripening (FR) is a precisely orchestrated and irreversible process involving significant modifications in color, aroma, flavor, and texture, producing a palatable fruit that exhibits desirable quality-related characteristics (Wang et al. 2022). FR is modulated by the differential expression of several functionally essential genes, a mechanism tightly controlled by specific transcription factors (TFs) (Chen et al. 2020). In tomato, the non-ripening mutants, namely *non-ripening* (*nor*), *ripening inhibitor* (*rin*), and *colorless non-ripening* (*cnr*), have been studied extensively. Hence, the function of TFs in crucially regulating FR has increasingly garnered interest. The *rin*, *nor,* and *cnr* encode TFs that belong to MCM1, AG, DEF and SRF (MADS); SQUAMOSA promoter binding protein-like (SPL); and NAM, ATAF1/2, and CUC2 (NAC) families of TFs, respectively (Giovannoni 2007; Wang et al. 2020). Among these, RIN has been studied most thoroughly in various species. It has been well established that the targets of RIN are involved in multiple ripening-associated metabolic pathways, which include ethylene synthesis and perception, aromatic biosynthesis, metabolism of sucrose, and energy production (Qin et al. 2016; Ito et al. 2017; Li et al. 2020). The TFs,

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FUL2 and SlHY5, which belong to the MADS-box and bZIP families, respectively, favorably regulated ethylene production and improvement in fruit quality throughout the ripening process of tomatoes (Wang et al. 2014, 2021). Nevertheless, SlMADS1 and AP2a negatively influenced the maturation of fruits (Karlova et al. 2011; Dong et al. 2013). The other types of TFs, including bHLH, EIL and MYB, contributed to FR in apples, strawberries, kiwis, longans, and papayas (Yin et al. 2010; Tisza et al. 2010; Zhou et al. 2015; Kuang et al. 2012; Fu et al. 2019).

SPL is a highly influential family of TFs exclusive to the plant kingdom and regulates numerous biological processes, including development, senescence, and stress responses (Agarwal and Lahiri 2020; Jerome Jeyakumar et al. 2020). SBP is a conserved DNA-binding domain of SPLs with double zinc-finger structures (Lai et al. 2020; You et al. 2021). The contribution of SPLs to the modulation of FR was initially observed in the *cnr* mutant of tomato, characterized by a lack of color and ripening, exhibited a significant decrease in ethylene production, and lowered pigment accumulation due to the *nor* mutation. Consequently, the fruit remained colorless and failed to ripen (Thompson et al. 1999). The *cnr* mutation was linked to a transient epimutation that triggered DNA hypermethylation in the promoter of *SQUAMOSA promoter binding protein-like-colorless non-ripening* (*SPL-CNR*) (Zhong et al. 2013). Genome-encoded analyses revealed that ethylene insensitive 3 (EIN3) acts as a regulatory factor upstream of *SPL-CNR*, and EIN3-SPL-CNR constituted a positive feedback loop that governed ethylene biosynthesis (Lv et al. 2018; Gao et al. 2019). Additionally, the function of SPL-CNR in ripening is not only dependent on its nuclear localization signal (NLS) and zinc-finger motifs (ZFMs) but also regulated by phosphorylation catalyzed by the sucrose nonfermenting1-related protein kinase1 (SlSnRK1) (Lai et al. 2020). Ripening induced the differential expression of 15 other *SPL*s in tomatoes (Salinas et al. 2012). Several studies have demonstrated the evidence supporting the functional role of SPLs throughout the ripening of various fruits. For example, 14 *Carica papaya* L. *SQUAMOSA promoter binding protein-like* (*CpSPL*) and 12 *Prunus avium* L. *SQUAMOSA promoter binding protein-like* (*PavSPLs*) were systematically identified from the genomes of papaya and sweet cherry, respectively, and their expression patterns in several vegetative tissues and throughout FR were clearly described (Xu et al. 2020; Sun et al. 2023). Nevertheless, the specific regulatory mechanisms, especially the regulatory pathways, are still unclear.

Various TFs act synergistically or antagonistically to regulate gene expression (Bemer et al. 2017). In citrus fruits, CitWRKY1 cooperates with CitNAC62 to transactivate *aconitase 3*, ultimately promoting the degradation of citric acid throughout FR (Li et al. 2017). Conversely, LcR1MYB1 interacts with LcNAC13 to weaken the LcNAC13-based transrepression of the anthocyanin biosynthesis genes in FR in litchi (Jiang et al. 2019). In addition to protein–protein interactions, TFs compose a series of transcriptional cascades that control FR (Wang et al. 2020). For example, in apple, an induction in the expression of *Malus domestica MYB* (*v-myb avian myeloblastosis viral*) *9* (*MdMYB9*) and *MdMYB11* was caused by the binding of MdNAC52 to their promoters, respectively, which in turn promoted the activating effect of MdMYB9 and MdMYB11 on proanthocyanidin and anthocyanidin biosynthesis pathways' genes in fruits (Sun et al. 2019). Similarly, PpNAC.A59, PpERF.A16, BLOOD, and PpMYB10.1 constitute a gene-transcriptional cascade that regulates fruit quality during ripening in peaches (Zhou et al. 2015; Guo et al. 2021). Thus, to construct a transcriptional regulatory network for FR, it is vital to determine the interrelationship between the ripening-related TFs.

Banana fruits (*Musa acuminata*, AAA group) are consumed globally and primarily cultivated in developing nations due to the specific climatic requirements for growth. Moreover, banana is a major commodity exported, generating billions of dollars in revenue, and is primarily consumed in developed countries (Aurore et al. 2009). Bananas are usually harvested at the mature green phase to enhance convenience during manufacturing and transportation. Subsequently, they are transported to the wholesale markets and ripened to achieve a desirable golden yellow color and an optimal edible quality. The ripening process was facilitated by applying ethylene, which contributed to determining the value of banana fruit as a commercial commodity (Pérez et al. 1997; Maduwanthi and Marapana, 2019). Therefore, much attention has been focused on understanding the regulatory mechanisms of postharvest FR in bananas. The involvement of various TFs, including bHLH (Xiao et al. 2018), EIL (Zhu et al. 2023) and MYB (Wei et al. 2023), in banana fruit ripening-associated transcriptional mechanisms has been elucidated. MaNAC029 directly impacted the expression of genes involved in ethylene biosynthesis and the degradation of cell walls, starch, and chlorophyll (Wei et al. 2022). Nevertheless, the upstream regulatory mechanisms governing the expression of *MaNAC029* remain unclear. This study identified a specific SPL-encoding gene, *MaSPL16*, induced by ripening and ethylene. MaSPL16 bound putatively to the promoter of *MaNAC029* and induced its transcriptional activation, which activated the genes associated with the biosynthesis of ethylene, determined fruit quality, promoted FR, and enhanced the quality of the bananas. These results

identified a new cascade involved in transcriptional regulation, MaSPL16-MaNAC029, which governed the biosynthesis of ethylene and an improvement in fruit quality throughout the ripening process in bananas. These findings broadened the comprehension of the transcriptional modulation of FR.

Materials and methods

Plant material and treatments

The fruits of banana (*Musa acuminata*, AAA group, cv. Cavendish) were obtained from a plantation near Guangzhou, China, upon reaching a maturation level of 75% – 80% at 90 days after flowering. The bananas were separated randomly into untreated control (natural ripening), ethylene (100 μL/L), and 1-methylcyclopropene (1-MCP) treatment (0.5 μL/L) groups. Following treatment, all fruits were incubated at 20°C until complete ripening, as delineated in an earlier study (Zhu et al. 2020). The samples were immediately frozen in liquid N_2 and cryopreserved at –80°C until subsequent utilization.

Gene expression analysis

The hot borate technique acquired the total RNA from the fruits. qRT-PCR was conducted using CFX96™ PCR (Bio-Rad, Hercules, CA, USA) and the Hieff® qPCR Kit (Yeasen, Shanghai, China). The primers were designed using the Primer software (ver 5.0) (Chen et al. 2011). *MaRPS4* was employed as the internal reference gene.

Yeast one-hybrid (Y1-H) assay

The Matchmaker™ yeast one-hybrid system (Clontech, Mountain View, CA, USA) was employed to conduct the Y1-H screening. The pAbAi vector was utilized to construct the bait plasmid by inserting the short fragment, –750 bp upstream of "ATG" in the promoter of *MaNAC029*, followed by linearization. The construct was transposed into the Y1-H strain, resulting in the generation of a reporter strain specific to the bait, followed by the examination of a cDNA library of ethylene-treated bananas. The DNA–protein interactions were assessed by analyzing the maturation potential of co-transformants on an SD/-Leu medium fortified with Aureobasidin A (AbA), following the standardized protocols.

Electrophoretic mobility shift assay (EMSA)

The coding sequence (CDS) of *MaSPL16* was cloned into the pGEX-4 T-1 vector with the appropriate reading frame and GST tag. Next, *Glutathione S-transferase-Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16* (*GST-MaSPL16*) was expressed in the *Escherichia coli* strain, BM Rosetta and the fusion protein was purified using glutathione-sepharose 4B beads. The probes were derived from the fragments of the *MaNAC029* promoter that were amplified and then labeled with biotin at the 5' end. The EMSA kit (Thermo Scientific, Waltham, MA, USA) was employed, as published earlier (Wei et al. 2023). The recombinant GST-MaSPL16 protein was incubated with biotin-labeled probes, and the unbound probes were separated using a native acrylamide gel. The unlabeled probes were employed as the competitors, and GST protein as the negative control.

Assay of promoter activity and subcellular localization

The tobacco BY-2 protoplasts were attributed to conducting the assay of promoter activity and subcellular localization (Shan et al. 2020). The promoter of *MaSPL16* was cloned into the pGreenII 0800-*LUC* reporter vector to activate the Firefly luciferase (*LUC*) expression. The protoplasts were transformed with the CaMV35S-*REN*/*MaSPL16pro-LUC* construct through the PEG-mediated approach and were treated with either 0 or 10 μL/L ethylene. A dual LUC assay kit (Promega, Madison, WI, USA) was utilized to detect the activities of LUC and renilla luciferase (REN).

The CDS of *MaSPL16* was inserted into the pBI221-*GFP* vector to ascertain the subcellular location of MaSPL16 while employing the nucleus-targeted mCherry as a nuclear biomarker. The protoplasts were cotransfected with *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16-Green fluorescent protein* (*MaSPL16-GFP*) and *nuclear localization signal-monomer cherry red fluorescent protein* (*NLS-mCherry*) based on a previously described method. An Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) was employed to study the fluorescence signals of GFP and mCherry.

Assay of transcriptional activation in yeast

A yeast two-hybrid (Y2-H) system (Clontech, Mountain View, CA, USA) was used to analyze the transcriptional activation of *MaSPL16*. The CDS of *MaSPL16* was subcloned into the vector. The lithium acetate method was utilized to individually transform the cells of the Y2HGold yeast strain with the fusion (pGBKT7 + *MaSPL16*), positive control (pGBKT7-p53 + pGADT7-T-antigen), and negative control (pGBKT7) vector plasmids. The yeast cells were cultured on a selective medium, and the possible autoactivation was assessed by following the growth conditions and determining the α-galactosidase activity.

Dual-luciferase transient expression assay

The entire CDS of *MaSPL16* was cloned into the pGreenII 62SK-BD vector (effector) to evaluate its capacity for transcriptional activation. The reporter vector was constructed with a dual-reporter system comprising the firefly *LUC* regulated by five

GAL4-binding elements. Based on a previous report, the tobacco leaves were cotransformed with the effector and recombinant reporter constructs (Wei et al. 2023). The activities of LUC and REN were quantified after 60 h following *Agrobacterium. tumefaciens* injection as described in the promoter activity assay.

To evaluate the influence of MaSPL16 on the activity of the promoter of *MaNAC029*, the pGreenII 62-SK effector vector was utilized for the cloning of *MaSPL16*, and the pGreenII 0800-*LUC* reporter vector for the colonization of the promoter of *MaNAC029* (Hellens et al. 2005). The *A. tumefaciens* strain, EHA105 (pSoup), was employed for the cotransformation of tobacco leaves with the effector and reporter plasmids. The ratio of LUC to REN reflected the transcriptional activation of the *MaNAC029* promoter. Each pair was measured six times.

Transient overexpression in banana fruits

An analysis of the transient overexpression of *MaSPL16* in banana fruits was performed as previously outlined (Shan et al. 2020). The open reading frame was cloned into the pCXUN-HA vector, which was used to transform the *A. tumefaciens* cells that were then used to inoculate the pulp of banana fruits. The transformed fruits were supplemented with 100 μL/L ethylene on the first-day post-inoculation and incubated at 20°C for seven days. Samples were collected to ascertain the gene expression levels. Fruit firmness, ethylene construction, color index, starch content, and chlorophyll levels were monitored at each predetermined sampling point, adopting a previously reported method (Wei et al. 2022).

Statistical analysis

The statistical analysis was performed using the SPSS software (ver. 19.0). The data were reported as the mean ± standard error (SE) from three or six replicates. The statistical significance of the variations between the specimens was evaluated through the Student's *t*-test or the analysis of variance (ANOVA) as required.

Primers

Table S1 lists the primers used in this study.

Results

MaSPL16 directly targets the MaNAC029 promoter

MaNAC029 contributed to the biosynthesis of ethylene and determining fruit quality throughout the postharvest ripening period in banana fruits (Wei et al. 2022). The proteins interacting with the promoter of *MaNAC029* were explored to determine upstream regulators by screening the Y1-H libraries. The cDNA fragment (*Ma07_g24410*) of an SPL-encoding gene was detected. A phylogenetic tree indicated that Ma07_g24410 was evolutionarily highly related to the rice OsSPL16 (Fig. S1a) and hence was designated MaSPL16. The Y1-H assay was employed to validate the interaction between MaSPL16 and the promoter of *MaNAC029* by utilizing the complete CDS of *MaSPL16* as prey. Even the basal activity of the *MaNAC029* promoter could not be detected in yeast in the presence of AbA (Fig. 1a). In contrast, the yeast cells that express MaSPL16 exhibited enhanced resistance to AbA due to elevated gene expression attributable to the higher activity of the *MaNAC029* promoter. They displayed strong growth on a medium containing AbA, confirming the physical interaction between MaSPL16 and the *MaNAC029* promoter.

An analysis of the sequence of the promoter of *MaNAC029* indicated the presence of the "GTAC" core sequence (Text S1), a key element recognized by the SPLs (Kropat et al. 2005). Hence, an EMSA was conducted to ascertain the probable direct interaction between the MaSPL16 and the *MaNAC029* promoter. The recombinant protein, GST-MaSPL16, was expressed in *E. coli* and purified (Fig. S2). The core "GTAC" sequence, which comprises the DNA segments derived from the promoter of *MaNAC029*, was employed as the probe. The results indicate that the recombinant protein, GST-MaSPL16 directly interacted with certain regions of the promoter of *MaNAC029*, thereby inducing evident changes in mobility (Fig. 1b). Additionally, the administration of increasing quantities of unlabeled probes with the same sequence caused a significant reduction in the displacement of bands. However, including mutants as competitors did not result in a similar decline, and no significant alterations in mobility were detected upon incubation with identical probes containing only GST. Collectively, these results suggest that MaSPL16 directly targets the *MaNAC029* promoter *via* the "GTAC" core.

Ethylene and ripening induce the expression of MaSPL16

An analysis of the expression patterns throughout the ripening process was conducted to assess the possible association between *MaSPL16* and FR. Bananas are climacteric fruits, and their ripening was triggered by ethylene, which was delayed by 1-MCP. A peak in ethylene production throughout the natural FR process occurred after 18 days of storage, with complete ripening on day 20. The ethylene treatment expedited FR, characterized by the appearance of an ethylene peak on day three and complete maturation on day five. Treatment with 1-MCP delayed the ethylene peak until day 30 and full ripening until day 35 (Zhu et al. 2020). Herein, the association between *MaSPL16* and FR in bananas was explored by examining the expression patterns of *MaSPL16* throughout the natural, ethylene-induced, and 1-MCP-delayed ripening processes. Figure 2a – c illustrated that the

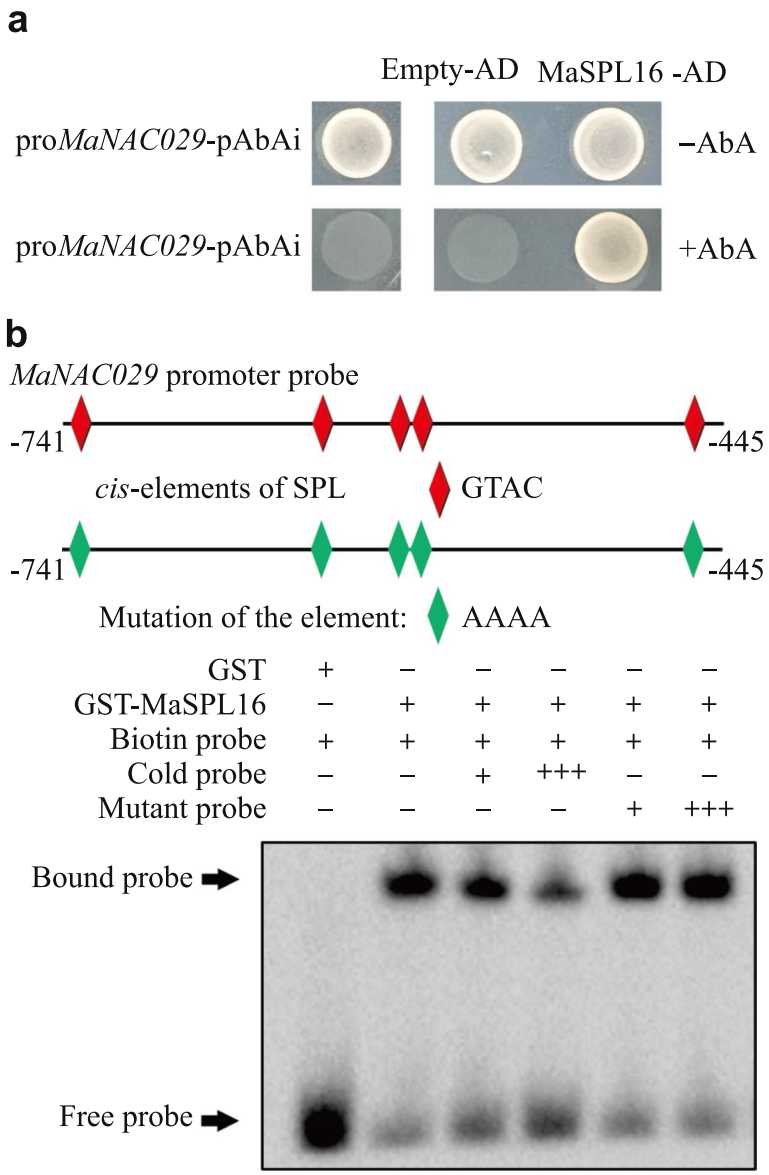

Fig. 1 *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16* (*MaSPL16*) binds to the promoter of *Musa acuminata NAC* (*NAM, ATAF, CUC*) *029* (*MaNAC029*). (**a**) The effects of MaSPL16 on the *MaNAC029* promoter ascertained through a Y1-H approach. Left: the *MaNAC029* promoter was not expressed in yeast cultivated on SD media lacking Leu but supplemented with 700 ng/mL Aureobasidin A (AbA). Right: the yeast growth assay of Y1-H strains was performed after transformation with either the empty pGADT7 plasmid (negative control) or the one containing a cassette expressing the MaSPL16 effector. Their interaction was evaluated based on the capacity of transformed yeast cells to grow when cultivated on a Leu-deficient SD medium supplemented with AbA. (**b**) The binding of MaSPL16 to the *MaNAC029* promoter was ascertained *via* EMSA. The GST or GST-MaSPL16 protein was incubated with the probes. Subsequently, the DNA–protein complexes obtained were segregated on native polyacrylamide gels. The competitors used comprised of unlabeled wild-type and mutated probes. Fig. S3 presents the uncropped and full-length blot for EMSA

expression of *MaSPL16* was remarkably induced by ethylene, with its transcript levels showing a tendency to rise markedly and then decrease during the three ripening processes, which was per ethylene production.

Additionally, a transient protoplast assay was employed to identify the impact of ethylene on the activity of the *MaSPL16* promoter. The *MaSPL16* promoter was isolated, fused with *LUC*, and cloned into the dual luciferase reporter vector. The 35S promoter was used to drive the expression of *REN* in the identical vector, which served as an internal control (Fig. 2d). The results revealed that the activity of the *MaSPL16* promoter was induced by

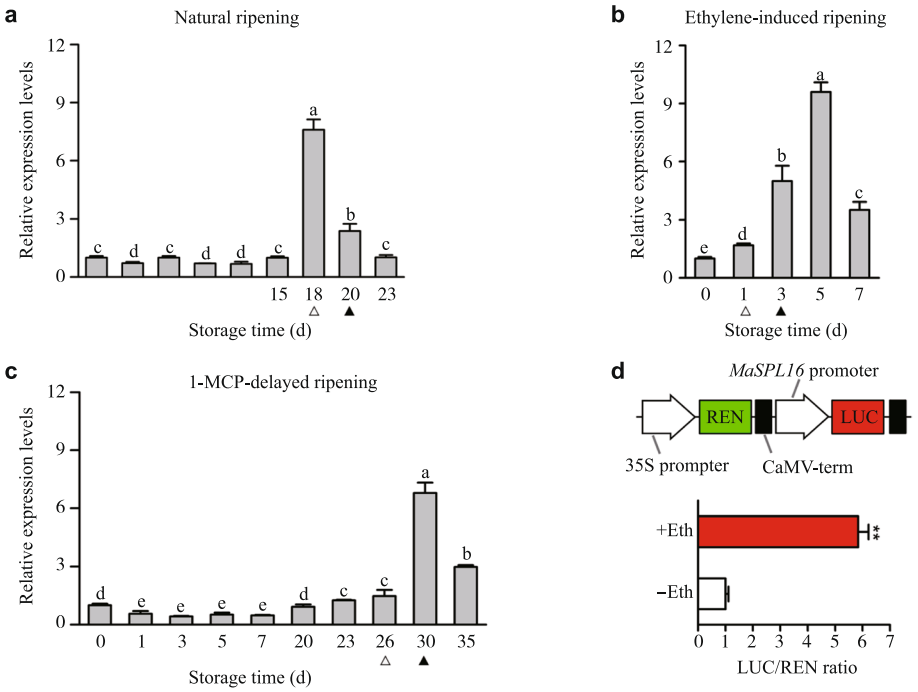

Fig. 2 The expression of *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16* (*MaSPL16*) is induced by ethylene and ripening. (**a**) The expression of *MaSPL16* in banana fruits was examined in natural ripening (**b**) Ethylene-induced ripening (**c**) Ripening delayed by 1-MCP treatment. The relative expression levels at each time point were referred to as the gene transcript levels in the control at the 0 day. The physiological data about fruit ripening and softening have been expounded upon in a prior investigation (Zhu et al. 2020). The open and closed triangles symbolized the initiation and culmination of ethylene production at different time points for each treatment, respectively. The data presented in this study represent the mean ± SE obtained from three independent replicates. The lowercase letters indicated the statistically significant differences observed among the different groups, as determined by a one-way ANOVA followed by Tukey's test ($P < 0.05$). **(d)** The activity of the *MaSPL16* promoter in response to ethylene was investigated. The reporter construct was transiently introduced into the tobacco BY-2 protoplasts and subsequently tested for induction by ethylene. The error bars in the graph indicate the SE calculated from six replicates (Student's *t*-test, $^{**}P < 0.01$). REN, Renilla luciferase; LUC, Firefly luciferase; CaMV-term, cauliflower mosaic virus terminator; Eth, ethylene

ethylene in tobacco BY-2 protoplasts transfected with CaMV35S-*REN*/*MaSPL16* promoter-*LUC*, which was confirmed by a remarkable rise in the ratio of LUC/REN. In summary, these findings revealed that *MaSPL16* was induced by ethylene and ripening.

Molecular characterization of MaSPL16

An analysis of the 154 – 169 amino-acid sequence of MaSPL16 indicated the existence of a nuclear localization signal (NLS) (Fig. S1b). The subcellular location of MaSPL16 was determined using a transient expression of GFP tagged with MaSPL16 in the tobacco BY-2 protoplasts. Subsequently, the nucleus-targeted mCherry (NLS-mCherry) was coexpressed as a control to observe the nucleus. The GFP-fluorescence was detected in both the cytoplasm and nucleus in the control group (Fig. 3a). The nuclear localization of MaSPL16 was confirmed by the colocalization of the green fluorescent signal emitted by MaSPL16-GFP and the red fluorescent signal emitted by NLS-mCherry (Fig. 3a).

The capacity of MaSPL16 for transcriptional activation was evaluated through a yeast reporter system responsive to GAL4. *MaSPL16* was fused with *GAL4-binding domain* (*GAL4BD*) and then expressed in Y2HGold yeast. The yeast cells that were successfully transformed with the DBD-p53, AD-T-antigen (positive control), and DBD-*MaSPL16* vectors exhibited normal growth even in the absence of Trp (Fig. 3b). These cells exhibited α-Gal activity and were able to survive even in the absence of Trp, His and Ade. On the other hand, the yeast transformed with the negative control did not exhibit any growth (Fig. 3b). These findings suggest that MaSPL16 can activate self-transcription in yeast. Utilizing a dual-luciferase reporter system with 5 × GAL4 DNA-binding components, the transcriptional activation capacity of MaSPL16 was additionally investigated *in planta*. VP16, a robust transcriptional activator, was used as a positive control (Fig. 3c). In comparison to the empty BD vector, MaSPL16 enhanced the LUC/REN ratio significantly by 6.1-fold (Fig. 3c), thereby serving as additional evidence supporting the transcriptional

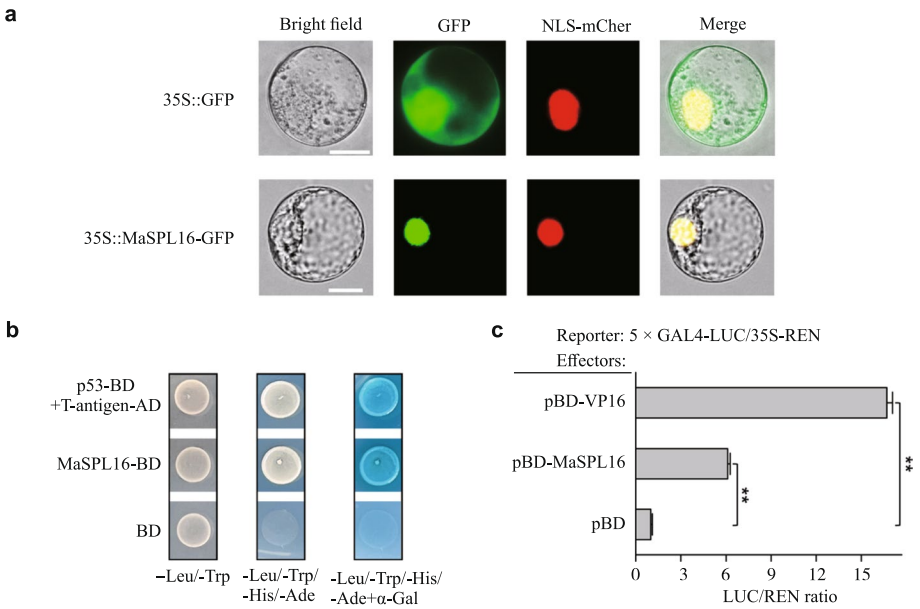

Fig. 3 Molecular characteristics of *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16* (*MaSPL16*). (**a**) The localization of MaSPL16 in the tobacco BY-2 protoplasts. The nuclear marker, the nucleus-targeted mCherry (NLS-mCherry) was employed in this study, bars = 25 μm. (**b**) Stimulation of the transcription of *MaSPL16* in yeast cells. The pGBKT7 vector from BD was used as a negative control, while p53-BD + T-antigen-AD was used as a positive control. (**c**) The transcriptional activity of *MaSPL16* in tobacco leaves. VP16 was employed as a positive control to establish a baseline. The ratio of LUC to REN activity in the empty pBD vector was normalized to 1, which served as the negative control. Error bars represent the SE from six replicates (Student's *t*-test, $^{**}P < 0.01$). GFP, Green fluorescent protein; LUC, Firefly luciferase; REN, Renilla luciferase

activation potential of MaSPL16. The data presented suggested that MaSPL16 may possess the potential to act as a transcriptional activator contributing to FR in bananas.

MaSPL16 directly activates the transcription of MaNAC029

Since MaSPL16 exhibited the capability of transcriptional activation and was directly bound to the *MaNAC029* promoter (Figs. 1, 3), a transient dual luciferase assay was conducted to examine the potential role of MaSPL16 in inducing the transcription of *MaNAC029*. The *MaNAC029* promoter was fused with *LUC*, while the 35S promoter-driven *REN* was utilized as an internal control using the same vector for both (Fig. 4a). The effector construct contained the 35S promoter-driven *MaSPL16* cDNA (Fig. 4a). The activity of the promoter of *MaNAC029* enhanced remarkably upon cotransfection with the 35S::*MaSPL16* effector, as compared to the empty effector (Fig. 4b), suggesting that MaSPL16 possessed the ability to transactivate the promoter of *MaNAC029* as indicated by the relatively greater LUC/REN ratio.

Overexpression of MaSPL16 enhances the MaNAC029-based activation of ripening-related genes

MaNAC029 activated the genes involved in ethylene biosynthesis and the degradation of cell walls, starch, and chlorophyll throughout the FR process in bananas (Wei et al. 2022). As MaSPL16 bound directly to the promoter of *MaNAC029* and induced its expression (Figs. 1, 4), it is reasonable to assume that MaSPL16 enhanced the MaNAC029-based transactivation of ripening-related genes. To verify this hypothesis, *MaSPL16* was transiently overexpressed in banana fruits (Fig. 5a, b) which was confirmed by qRT-PCR (Fig. 5b). In contrast to the empty vector (control), the transient overexpression of *MaSPL16* enhanced FR in banana, resulting in a faster yellowing (ripening) phenotype (Fig. 5a). In *MaSPL16*-over-expressing fruits, the decline in the color index was advanced by two days during maturation, and concomitantly, the ethylene production increased dramatically, while the firmness, chlorophyll content, and starch levels of fruits reduced throughout the ripening process (Fig. 5c). More importantly, the overexpression of *MaSPL16*, in turn, induced the enhanced expression of *MaNAC029* in fruits throughout ripening (Fig. 5d). In parallel, the expression of target genes downstream of *MaNAC029*, were induced markedly in the *MaSPL16*-overexpressing fruits. These included *Musa acuminata 1-amino-cyclopropane-1-carboxylic acid synthase 1* (*MaACS1*), *Musa acuminata 1-amino-cyclopropane-1-carboxylic acid oxidase 1* (*MaACO1*) and *MaACO13*, involved in ethylene biosynthesis

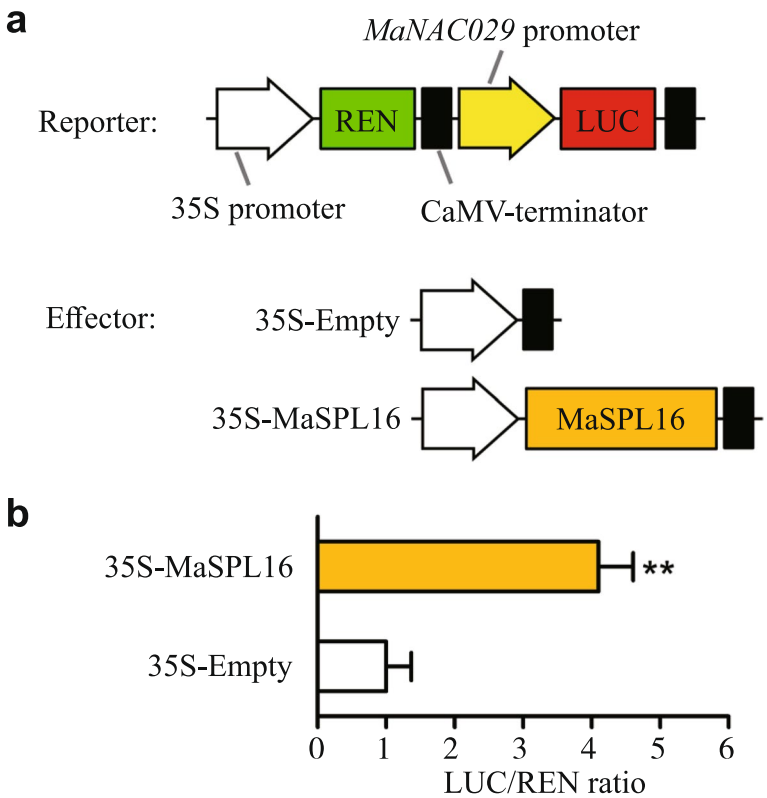

Fig. 4 *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) *16* (*MaSPL16*) activates the promoter of *Musa acuminata NAC* (*NAM, ATAF, CUC*) *029 MaNAC029*. (**a**) Diagram illustrating the reporter and effector constructs. (**b**) MaSPL16 enhanced the activity of the MaNAC029 promoter. The Firefly luciferase (LUC) to Renilla luciferase (REN) ratio of an empty vector containing the *MaNAC029* promoter was employed as a calibrator and designated 1. Error bars represent the SE from six replicates (Student's *t*-test, $^{**}P < 0.01$). CaMV-term, cauliflower mosaic virus terminator

(Jourda et al. 2014); *Musa acuminata Expansins 2* (*MaEXP2*), *MaEXP15*, *Musa acuminata xyloglucan endotransglycosylase/hydrolases 28* (*MaXTH28*) and *MaXTH30*, associated with cell wall degradation (Han et al. 2016); *Musa acuminata α-glucan water dikinase 1* (*MaGWD1*) related with starch degradation (Xiao et al. 2018); and *Musa acuminata Stay-Green 1* (*MaSGR1*) and *Musa acuminata Pheophytinase* (*MaPPH*), involved in chlorophyll catabolism (Wei et al. 2023) (Fig. 6). These findings suggest that MaSPL16 induced the expression of *MaNAC029*, thereby enhancing the MaNAC029-based transcriptional activation of genes involved in ethylene biosynthesis, enhancement in fruit quality, promoting FR, and determination of quality in banana.

Discussion

Typically, bananas are harvested upon reaching the mature green phase and ripened using ethylene. This process results in bananas attaining a desirable golden yellow color and optimal edible quality during the postharvest phase (Maduwanthi and Marapana 2019). Therefore, postharvest ripening determined the commodity value of banana fruits. The participation of several TFs precisely modulates the process of FR (Li et al. 2021), such as MaNAC029, which regulated the biosynthesis of ethylene and fruit quality in bananas throughout postharvest ripening (Wei et al. 2022). However, the regulatory mechanisms upstream of *MaNAC029* are still not precise. Herein, MaSPL16, a transcription regulator upstream of *MaNAC029*, was identified, and the molecular mechanisms by which the MaSPL16-MaNAC029 transcriptional cascade module regulated banana postharvest ripening were discussed.

The three groups of TFs, namely, MADS, SPL, and NAC, have been acknowledged for their significant contribution to FR (Giovannoni et al. 2017; Li et al. 2019; Liu et al. 2022). An analysis of the genes encoding TFs revealed that NACs and MADSs form a dual-loop circuit, modulating FR in bananas (Lv et al. 2018). However, the correlation between the regulatory effects of SPLs and NACs is still not understood. The current investigation illustrated that MaSPL16 specifically interacted with the promoter of *MaNAC029*, resulting in the activation of its expression (Figs. 1, 4), indicating that MaSPL16 and MaNAC029 constituted a transcriptional cascade

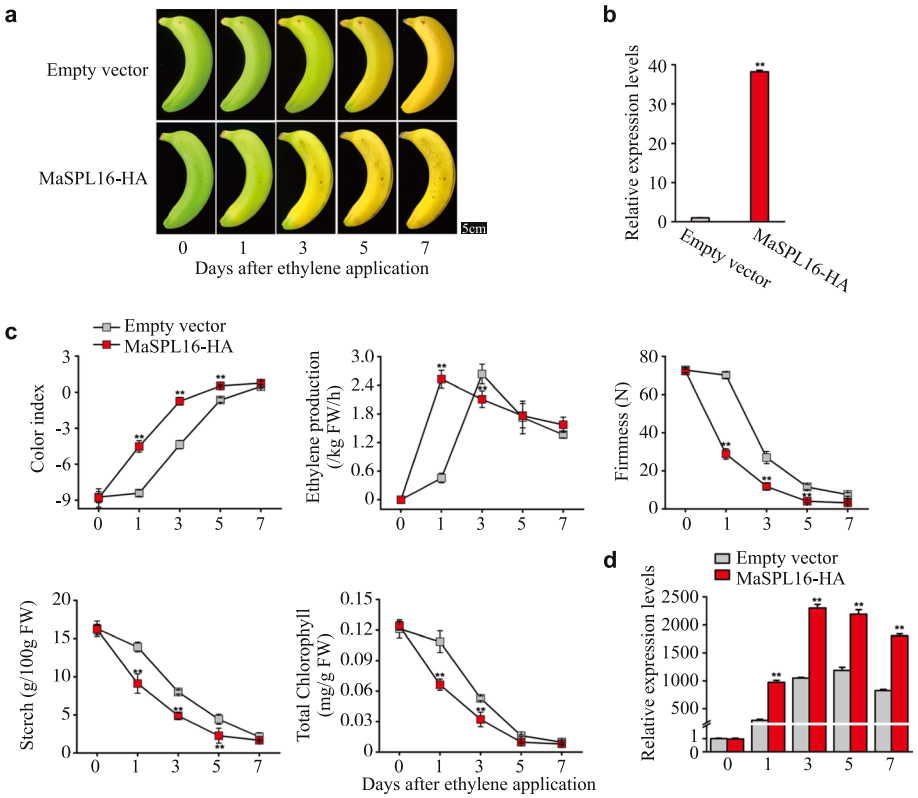

Fig. 5 Transient upregulation of *Musa acuminata SPL* (*SQUAMOSA promoter binding protein-like*) 16 (*MaSPL16*) in banana fruits accelerates fruit ripening and determination of quality. (**a**) The appearance of banana fruits that transiently overexpressed *MaSPL16* or the empty vector throughout ripening. (**b**) qRT-PCR indicating the levels of *MaSPL16* mRNAs in these banana fruits. (**c**) Color index, ethylene production, firmness, starch levels, and total chlorophyll content in bananas as shown in (**a**). (**d**) Relative expression of *Musa acuminata NAC* (*NAM, ATAF, CUC*) *029* (*MaNAC029*) in bananas as shown in (**a**). Error bars in (**b**), (**c**) and (**d**) represent the SE from six and three replicates, respectively (Student's *t*-test, $^{**}P < 0.01$)

involved in banana ripening. These data enrich the knowledge of the interrelationship between ripening-correlated TFs and assist in building a transcriptional modulatory network for FR.

Banana, being a climacteric fruit, requires ethylene for its ripening. The investigation of the modulatory pathways of ethylene biosynthesis during FR in bananas has long been a prominent area of research (Tang et al. 2023). The direct modulation of the genes involved in ethylene biosynthesis throughout the FR process was attributed to the NAC TFs in several fruits, including tomatoes (Gao et al. 2018), kiwifruits (Wu et al. 2020), and peaches (Dai et al. 2023). The NAC TF, MaNAC029, induced by ripening and ethylene, directly influenced the expression of *MaACS1*, *MaACO1* and *MaACO13* (Wei et al. 2023). Nevertheless, the precise mechanism through which ethylene enhances the expression of *MaNAC029* remains ambiguous. This study demonstrated that ethylene stimulated the expression of *MaSPL16* by enhancing the activity of its promoter (Fig. 2). Moreover, MaSPL16 induced the MaNAC029-involved activation of *MaACS1*, *MaACO1* and *MaACO13*, which in turn promoted ethylene production (Figs. 5, 6), that may serve as a feedback regulatory mechanism.

Fruit quality is a composite trait involving flavor, aroma, color, and texture that finally determines the commercial value of fruit (Gapper et al. 2013; Hu et al. 2019). Several TFs from various families have been identified to play a role in the modulation of FR in bananas *via* the direct control of the expression of genes associated with fruit quality. For instance, *Musa acuminata* basic helix-loop-helix 6 (MabHLH6) and *Musa acuminata* MYB (v-myb avian myeloblastosis viral) 3 (MaMYB3) directly regulated the genes correlated with starch degradation, where MabHLH6 promoted the degradation of starch during FR, while MaMYB3 inhibited it (Fan et al. 2018; Xiao et al. 2018). MaMYB60 and *Musa acuminata* ethylene insensitive 3-like 9 (MaEIL9) serve as upstream regulators of chlorophyll degradation and carotenoid biosynthesis, respectively, during the ripening of bananas (Wei et al. 2023; Zhu et al. 2023). Nevertheless, the upstream transcriptional modulators of the genes encoding these

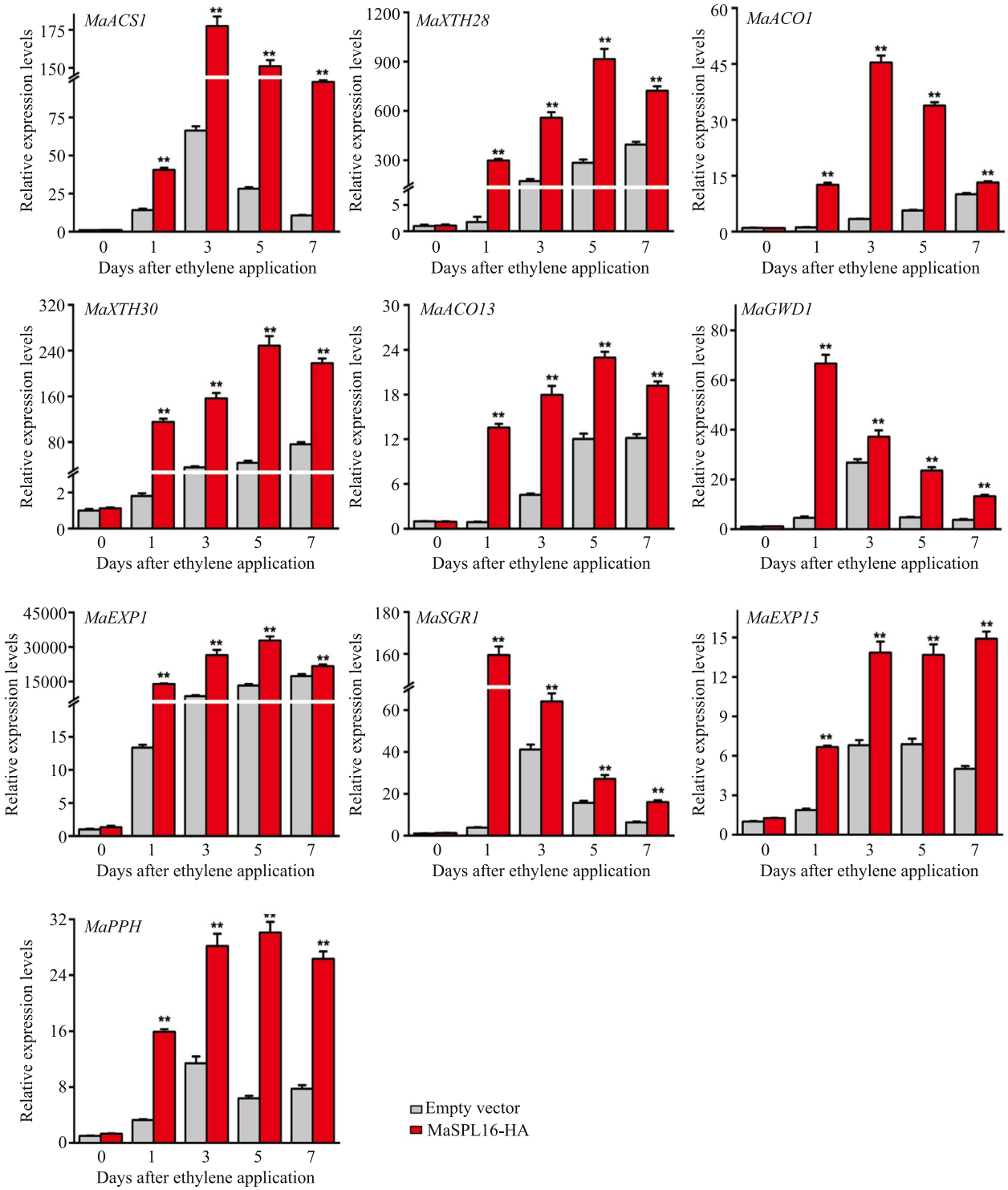

Fig. 6 The expression of *MaACS1*, *MaACO1*, *MaACO13*, *MaEXP2*, *MaEXP15*, *MaXTH28*, *MaXTH30*, *MaGWD1*, *MaSGR1* and *MaPPH* in the *MaSPL16*-overexpressing and control banana fruits. Error bars represent the SE from three replicates (Student's *t*-test, $^{**}P < 0.01$). *MaACS1*, *Musa acuminata 1-amino-cyclopropane-1-carboxylic acid synthase 1*; *MaACO1*, *Musa acuminata 1-amino-cyclopropane-1-carboxylic acid oxidase 1*; *MaEXP2*, *Musa acuminata Expansins 2*; *MaXTH28*, *Musa acuminata xyloglucan endotransglycosylase/hydrolases 28*; *MaGWD1*, *Musa acuminata α-glucan water dikinase 1*; *MaSGR1*, *Musa acuminata Stay-Green 1*; *MaPPH*, *Musa acuminata Pheophytinase*

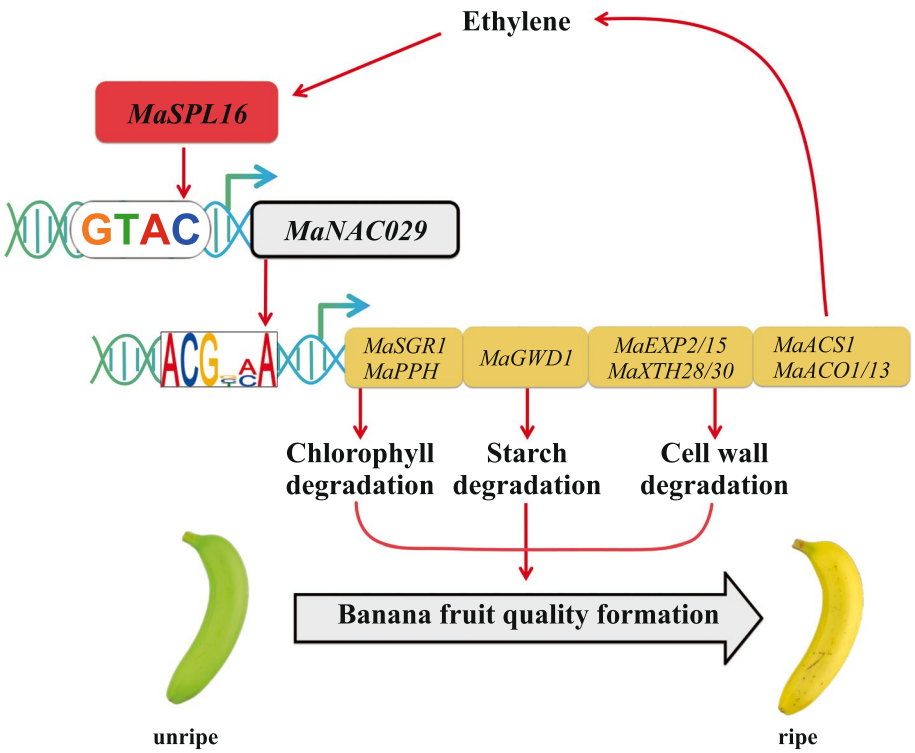

Fig. 7 A model proposed to explain the function of the transcriptional cascade, *Musa acuminata* SPL (SQUAMOSA promoter binding protein-like) 16-*Musa acuminata* NAC (NAM, ATAF, CUC) 029 (MaSPL16-MaNAC029) in banana ripening. *MaSGR1*, *Musa acuminata Stay-Green 1*; *MaPPH*, *Musa acuminata Pheophytinase*; *MaGWD1*, *Musa acuminata α-glucan water dikinase 1*; *MaEXP2/15*, *Musa acuminata Expansins 2/15*; *MaXTH28/30*, *Musa acuminata xyloglucan endotransglycosylase/hydrolases 28/30*; *MaACS1*, *Musa acuminata 1-amino-cyclopropane-1-carboxylic acid synthase 1*; *MaACO1/13*, *Musa acuminata 1-amino-cyclopropane-1-carboxylic acid oxidase 1/13*

TFs and the transcriptional cascade-associated interconnections among them remain unidentified. MaNAC029 was recently reported to directly impact the expression of genes involved in the cell wall, starch, and chlorophyll degradation (Wei et al. 2022). In this study, MaSPL16 directly induced the expression of *MaNAC029*, which in turn upregulated the downstream target genes associated with the degradation of the cell wall, starch, and chlorophyll, ultimately promoting FR and enhancing the quality (Figs. 1, 4, 5 , 6).

Moreover, the possibility of MaSPL16 directly regulating the activation of the fruit quality-related genes requires further study. In summary, the MaSPL16-MaNAC029 transcriptional cascade acted as an upstream regulatory module for establishing quality in banana fruits. These results enrich the understanding of the regulatory mechanisms underlying fruit quality and provide excellent gene resources for breeding banana varieties with high-quality fruits.

Relying on the current and past research conducted by our research group, a functional model explaining the mechanism through which MaSPL16 operates in banana ripening was proposed (Fig. 7). The activation of *MaSPL16* was observed throughout the process of postharvest maturation. MaSPL16 directly bound to the promoter of *MaNAC029*, resulting in the activation of its expression, which in turn promoted the transcriptional activation of ethylene biosynthesis-related genes, resulting in the feedback regulation of ethylene biosynthesis. In addition, the quality-related structural genes were also activated, stimulating the production of good-quality banana fruits throughout the FR processes.

Conclusions

In summary, a transcriptional regulatory cascade, specifically the MaSPL16-MaNAC029 pathway was demonstrated, which linked ethylene biosynthesis to fruit quality. These findings provide novel perspectives on the gene-transcriptional mechanisms behind FR. These factors could potentially influence the advancement of molecular techniques that seek to augment the quality and durability of fleshy fruits.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s44281-023-00013-4.

Additional file 1: Fig. S1. Phylogenetic analysis of MaSPL16. **Fig. S2.** Coomassie blue-stained SDS-PAGE shows the prokaryote-expressed and purified GST-MaSPL16 recombinant protein. **Fig. S3.** Uncropped version of the blots presented in Fig. 1b. **Text S1.** The SPL TF recognition sequences (GTAC) in the nucleotide sequences of *Musa acuminata NAC* (*NAM, ATAF, CUC*) *029* (*MaNAC029*) promoter. **Table S1.** Summary of primers used in this study.

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Not applicable.

Authors' contributions
The study was conceived and designed by WS and WW who participated as a collaborator in conducting the experiments. The process of data analysis was conducted by a collaborative team which was comprised by WW, Y-Y Y, C-J W, J-F K and WS. The process of manuscript composition and revision was undertaken by J-Y C, WS and WW. All authors actively engaged in discussions related to the manuscript.

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Availability of data and materials
Data will be available on request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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