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Research article

Suppression of the MADS-box gene *SlMBP8* accelerates fruit ripening of tomato (*Solanum lycopersicum*)

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ABSTRACT

MADS-box genes encode important transcription factors that are involved in many biological processes of plants, including fruit ripening. In our research, a MADS-box gene, *SlMBP*8, was identified, and its tissue-specific expression profiles were analysed. *SlMBP8* was highly expressed in fruits of the B+4 stage, in senescent leaves and in sepals. To further characterize its function, an RNA interference (RNAi) expression vector of *SlMBP8* was constructed and transferred into tomato. In the transgenic plants, the ripening of fruits was shortened by 2–4 days compared to that of wild type. At the same time, carotenoids accumulated to higher levels and the expression of phytone synthase 1 (*PSY1*), phytoene desaturase (*PDS*) and ς-carotene desaturase (*ZDS*) was enhanced in RNAi fruits. The transgenic fruits and seedlings showed more ethylene production compared with that of the wild type. Furthermore, *SlMBP8*-silenced seedlings displayed shorter hypocotyls due to higher endogenous ethylene levels, suggesting that *SlMBP8* may modulates the ethylene triple response negatively. A yeast two-hybrid assay indicated that SlMBP8 could interact with SlMADS-RIN. Besides, the expression of ethylene-related genes, including *ACO1*, *ACO3*, *ACS2*, *ERF1*, *E4* and *E8*, was simultaneously up-regulated in transgenic plants. In addition, *SlMBP8*-silenced fruits showed higher ethylene production, suggesting that suppressed expression of *SlMBP8* promotes carotenoid and ethylene biosynthesis. In addition, the fruits of transgenic plants displayed more rapid water loss and decreased storability compared to wild type, which was due to the significantly induced expressions of cell wall metabolism genes such as *PG*, *EXP*, *HEX*, *TBG4*, *XTH5* and *XYL*. These results suggest that *SlMBP8* plays an important role in fruit ripening and softening.

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1. Introduction

Fruit ripening involves obvious complex biochemical and physiological changes, such as pigment accumulation for bright colouring, cell wall degradation for fruit softening and accumulation of volatiles for aromas. In climacteric fruits, ripening is accompanied by a peak in respiration and a concomitant burst of ethylene biosynthesis. ACS (1-Aminocyclopropane-1-carboxylate synthase) and ACO (1-Aminocyclopropane-1-carboxylate oxidase) are two key biosynthetic enzymes (Adams and Yang, 1979; Yang and Hoffman, 1984). The expression levels of both *SlACO1* and *SlACO3* are significantly increased at the onset of tomato fruit ripening (Barry et al., 1996). It has been revealed that the expression of *SlACO3* is transiently induced at the breaker stage, while *SlACO1* is sustained during ripening (Barry et al., 1996). Previous studies also have indicated that *SlACS2* is an important factor in the transition between ethylene synthesis system 1 to ethylene synthesis system 2, and RNA interference (RNAi) inhibition of *SlACS2* strongly inhibits ethylene biosynthesis and fruit ripening (Barry et al., 2000). In addition to ethylene synthesis, the ability to perceive and respond to ethylene is necessary for fruit ripening. *E4* and *E8* are two classical ethylene-response genes that are involved in fruit ripening (Lincoln and Fischer, 1988; Kesanakurti et al., 2012).

Fruit softening is type of fruit quality, but excessive softening limits fruit shelf-life and storage. The expression of cell wall-modifying genes has been used to investigate the role of particular activities of fruit softening during ripening and in the manufacture of processed fruit products (Brummell and Harpster, 2001; Vicente et al., 2007). Ripening-specific or postharvest-softening-

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; WT, Wild-type; B, breaker; IMG, immature green; MG, mature green; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction.

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specific genes encoding cell wall-modifying enzymes have been characterized and cloned. Polygalacturonase (*PG*), which is transcriptionally activated during fruit ripening, is a major cell wall polyuronide-degrading enzyme involved in the fruit softening process (Montgomery et al., 1993).

Tomato (*Solanum lycopersicum*) is generally considered to be a model plant for studying climacteric fruit ripening and the mechanism of tomato fruit ripening has been uncovered through a wide range of studies. Molecular and biochemical studies on tomato ripening mutants such as *ripening inhibitor* (*rin*), *never ripe* (*Nr*), *nonripening* (*nor*) and *color nonripening* (*cnr*) have been conducted (Wilkinson et al., 1995; Vrebalov et al., 2002). In terms of floral organ development, a considerable number of MADS-box genes have been identified in *Arabidopsis thaliana* and *Antirrhinum majus* (Causier et al., 2003; Urbanus et al., 2009; Becker and Ehlers, 2016). In addition, regulation of fruit development, embryogenesis and vegetative organ development have been successively revealed in various species, which suggests a diverse role of these MADS-box transcriptional factors (Yanofsky et al., 1990; Sommer et al., 1990). SlMADS-RIN is a classical MADS-box protein involved in tomato fruit ripening, this protein is the essential positive regulator. *FRUITFULL*(*FUL*) plays an important role in dry and fleshy fruit development by regulating colour development and anthocyanin-related gene expression. The *TM4* gene (Tomato MADS-box 4), which is homologous to *Arabidopsis FUL*, is repressed in the *rin*, *cnr* and *nor* mutants, indicating that the function of *TM4* is associated with fruit development (Seymour et al., 2002; Fujisawa et al., 2012). It was revealed that FUL1 and FUL2, which are highly similar to FRUITFULL in *Arabidopsis*, bind to the same genomic sites as RIN (Fujisawa et al., 2011; Giovannoni, 2007). Different from other identified ripening regulators, *FUL1* and *FUL2* generally regulate fruit ripening in an ethylene-independent manner (Shima et al., 2013). In addition, *SlMADS1* negatively regulates fruit ripening, and overexpression of the novel MADS-box gene *SlFYFL* delays fruit ripening (Dong et al., 2013; Xie et al., 2014). Moreover, SlMADS1 interacts with RIN, similar to the behaviour of TOMATO AGAMOUS-LIKE1 (*TAGL1*), *TAGL11* and *FUL2*. In addition, there may be many other unidentified MADS-box genes that play important roles in tomato fruit ripening.

Previous reports have indicated that *SlMBP8* transcripts increase during the process of fruit development and ripening, which suggests that the function of *SlMBP8* might be related to fruit ripening(Hileman et al., 2006). To further reveal its regulatory function in fruit, RNA interference of *SlMBP8* (GenBank accession XM_004252664) was performed, and darker red fruits were observed on transgenic plants, together with earlier ripening and more carotenoid accumulations, which supports our hypothesis that *SlMBP8* plays an important role in regulating fruit ripening.

2. Materials and methods

2.1. Plant material and growth conditions

Tomato plants (*Solanum lycopersicum Mill. cv. Ailsa Craig AC*$^{++}$) were used as the wild type (WT) in our experiments. Transgenic and wild-type plants were grown in greenhouse conditions (16-h-day/8-h-night cycle, 25/18 °C day/night temperature, 80% humidity, and 250 μmol m^{-2} s^{-1} light intensity). There were two generations used in the experiments: T_0 originated from tissue culture, and T_1 originated from seedlings. Flowers were tagged at anthesis, and the fruits were harvested according to the number of days post-anthesis (dpa) and fruit colour. In wild type, we defined MG (Mature green) as fruits of 35 dpa, B (Breaker stage) as fruits of 38 dpa in which the colour changed from green to yellow, and B+4 and B+7 as fruits of 4 days after break and 7 days after break, respectively. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C for further analyses.

2.2. Isolation and sequence analysis of SlMBP8

Total RNA was extracted from all plant tissues for three biological repeats with the Trizol reagent (Invitrogen, USA). Then, a 1 μg aliquot of RNA treated with RNAiso Plus (TaKaRa) was used for synthesis of first-strand cDNA by reverse transcription polymerase chain reaction (M-MLV reverse transcriptase, TaKaRa, China) with tailed oligo $d(T)_{18}$ primers (5′ GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG TTT TTT TTT TTT TTT TTT 3′). An amount of 1−2 μl of cDNA was used to clone the complete sequence of the coding region of the *SlMBP8* gene with the primers *SlMBP8*-F (5′ CAA CAA TGG GAG AAC TGC TAC A 3′) and dT-R (5′GCT GTC AAC GAT ACG CTA CGT AAC G 3′) through high-fidelity PCR (Prime START^{M} HS DNA polymerase, TaKaRa). The PCR procedure was performed at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. In addition, the amplified products were then subcloned into the pMD18-T vector (TaKaRa) and confirmed by sequencing (Invitrogen). Multiple sequence alignment of SlMBP8 with other MADS-box proteins was conducted using the DNAMAN 5.2.2 programs.

2.3. Construction of the SlMBP8 RNAi vector and plant transformation

TO repress the expression of the *SlMBP8* gene, a vector (pBIN19RNAi) was constructed (Supplementary Fig. S2). The 423-bp *SlMBP8*-specific DNA fragment used in the hairpin was amplified using the primers *SlMBP8*-F (5′CAA CAA TGG GAG AAC TGC TAC A 3′) and *SlMBP8*-R (5′AGA AAC AAG AAC AAG GAT GAA TA 3′) with *Hind* III, *Kpn* I, *EcoR* I and *Xba* I restriction sites at the 5′ end. The resultant construct was used to transform tomato cotyledon explants with *Agrobacterium tumefaciens* (strain LBA4404) (Zhu et al., 2014). Transformed lines were selected for kanamycin (80 mg l^{-1}) resistance and then analysed by PCR with the primers NPTII-F (5′GAC AAT CGG CTG CTC TGA 3′) and NPTII-R (5′AAC TCC AGC ATG AGA TCC 3′) to determine the presence of T-DNA.

2.4. Quantitative real-time PCR analysis

qRT-PCR was performed using the SYBR® Premix Ex Taq II kit (TaKaRa, China) in a 10-μl total sample volume (5.0 μl of 2 × SYBR Premix Ex Taq, 1.0 μl of primers, 1.0 μl of cDNA, 3.0 μl of ddH2O), and all reactions were performed using the CFX96™ Real-Time System (Bio-Rad) under the following conditions: 95 °C for 3 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. To verify that the PCR master mixes were free of contamination and to remove the template from the environment, a no-template control (NTC) and a no-reverse-transcription control (NRT) were included with each assay. In addition, three technical replicates and three biological replicates for each sample were used. The tomato clathrin adaptor complexes medium subunit (*CAC*) gene was selected as an internal standard (Exposito-Rodriguez et al., 2008). The expression levels of *SlMBP8* in WT, *Nr*, *rin* and transgenic lines were determined with *SlMBP8* (RT)-F and *SlMBP8* (RT)-R primers (Supplementary Table S1). The relative gene expression levels were detected using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Furthermore, the mRNA expression levels of other genes, including fruit ripening-related genes (*E4*, *E8*, *PG*, *Pti4*, *LOXA* and *LOXB*), carotenoid biosynthesis genes (*PSY1*, *PDS* and *ZDS*), ethylene biosynthesis pathway genes (*ACO1*, *ACO3*, *ACS2* and *ERF1*) and cell-wall-modifying genes (*PE*, *CEL2*, *EXP*, *HEX*, *MAN*, *TBG4*, *XTH5* and *XYL*), were determined simultaneously, their primers are shown in

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