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A tomato MADS-box transcription factor, *SlMADS1*, acts as a negative regulator of fruit ripening

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ABSTRACT

MADS-box genes encode a highly conserved gene family of transcriptional factors which regulate numerous developmental processes in plants. In this study, a tomato MADS-box gene, \textit{SlMADS1}, was cloned and its tissue-specific expression profile was analyzed. The Real-time PCR results showed that \textit{SlMADS1} highly expressed in sepals and fruits, and its expression level was increased with the development of sepals, while the transcript of \textit{SlMADS1} decreased significantly in accordance with fruit ripening. To further explore the function of \textit{SlMADS1}, an RNAi expression vector targeting \textit{SlMADS1}, was constructed and transformed into tomato plants. Shorter ripening time of fruit was observed in \textit{SlMADS1}-silenced tomatoes. The accumulation of carotenoid and expression of \textit{PSY1} were enhanced in RNAi fruits. Besides, ethylene biosynthetic genes, including \textit{ACS1A, ACS6, ACO1} and \textit{ACO3}, and ethylene responsive genes \textit{E4} and \textit{E8} which involved in fruit ripening were also up-regulated in silenced plants. \textit{SlMADS1} RNAi fruits showed approximately 2- to 4-fold increase in ethylene production compared with wild type. Furthermore, \textit{SlMADS1}-silenced seedlings displayed shorter hypocotyls and were more sensitive to ACC than wild type. Additionally, yeast two-hybrid assay revealed a clear interaction between \textit{SIMADS1} and \textit{SIMADS-RIN}. These results suggest that \textit{SlMADS1} plays an important role in fruit ripening as a repressive modulator.
INTRODUCTION

The ripening of fleshy fruit is a developmental biochemical process including numerous metabolic changes, such as changes in color, flavor, aroma and nutrition. These changes not only make fruit assist in seed dispersal, but also provide essential nutrition for human and animal diets (Ampomah-Dwamena et al., 2002; Giovannoni, 2004; Goff and Klee, 2006). In climacteric fruits such as tomatoes, bananas, apples and pears, ethylene plays an important role in triggering the onset of ripening and is an essential factor for the ripening process (Abeles et al., 1992; Hiwasa et al., 2003). There are two key biosynthetic enzymes in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate synthase (ACS) and ACC oxidase (ACO) (Yang and Hoffman, 1984; Kende, 1993; Zarembinski and Theologis, 1994; Oetiker et al., 1997). It has been revealed that ethylene production and fruit ripening are strongly inhibited in SlACS2 RNAi transgenic tomato fruits (Alexander and Grierson, 2002), and the expression level of SlACS2 is notably induced by exogenous ethylene (Olson et al., 1991; Lincoln et al., 1993; Barry et al., 1996; Barry et al., 2000). Furthermore, the expression of SlACO1 and SlACO3 are both significantly increased at the onset of tomato fruit ripening (Barry et al., 1996). Previous studies also indicate that RNAi inhibition of SlACO1 delays ripening of climacteric fruits (Hamilton et al., 1990; Giovannoni, 2001; Blume and Grierson, 2003). These findings suggest that the normal function of ethylene biosynthesis is required for ripening process.

Besides the functional ethylene synthesis, the ability of ethylene perception and response are also necessary for fruit ripening. E4 and E8 are two classical genes which are induced by ethylene (Lincoln et al., 1987). The expression of E4 in fruit is rapidly induced following exogenous ethylene induction (Lincoln and Fischer, 1988). Meanwhile, the transcripts of E4 in fruit are suppressed through ethylene biosynthesis inhibition (Tigchelaar et al., 1978; Lincoln and Fischer, 1988). In tomato, E8 is regulated by ethylene and is activated at the onset of fruit ripening (Peñarrubia et al., 1992; Kneissl and Deikman, 1996). The promoter of E8 has been characterized and is widely used to drive the expression of exogenous genes in transgenic tomato fruits.
Tomato is generally considered to be a model plant for studying fruit ripening. To date, a wide range of studies have been performed to uncover the mechanism of fruit ripening of tomato, and a lot of ripening-deficient mutants, such as ripening inhibitor (rin), never ripe (Nr), nonripening (nor) and color nonripening (cnr), have been found and investigated in tomato (Tigchelaar et al., 1973; Mizrahi et al., 1982; Wilkinson et al., 1995; Vrebalov et al., 2002). The rin mutant displays enlarged sepals and inhibited fruit ripening. This mutant phenotype has been attributed to a function of two MADS-box transcriptional factors, SIMADS-RIN and SIMADS-MC. SIMADS-RIN regulates fruit ripening and SIMADS-MC involves in sepal development (Vrebalov et al., 2002). Besides SIMADS-RIN and SIMADS-MC, other MADS-box proteins also have been investigated in tomato. Prior study indicate that at least 36 MADS-box proteins have been found playing different and important biological roles in tomato, such as the determination of inflorescence and fruit ripening (Hileman et al., 2006). Among them, TAG1 (TOMATO AGAMOUS1), TAGL1 (TOMATO AGAMOUS-LIKE1), TM4 (TOMATO MADS-BOX 4, TDR4, FUL1) and TM6 (TOMATO MADS-BOX 6), have been identified and investigated to be associated with the development of fruits (Giovannoni, 2007). RNAi suppression of TAG1 gene in tomato leads to misshapen fruits and homeotic conversion of stamens into petaloid organs (Pnueli et al., 1994; Pan et al., 2010). While TAGL1 plays an important role in regulating fruit ripening. The antisense suppression of TAGL1 results in ripening inhibition and pericarp thickness reduction. Furthermore, overexpression of TAGL1 leads to ripening-like sepals and enhanced lycopene fruits (Itkin et al., 2009; Vrebalov et al., 2009; Giménez et al., 2010). TM4 is a homolog of Arabidopsis FRUITFULL (FUL) gene and has also been reported to be related to fruit ripening (Busi et al., 2003). The expression of TM4 is repressed in the rin, cnr and nor mutants (Seymour et al., 2002; Fujisawa et al., 2012). Additionally, TM6 transcripts mainly accumulate in carpel primordial and young fruits in tomato and has been considered to be involved in fruit ripening (Pnueli et al., 1994; Busi et al., 2003). Interestingly, these reported genes of MADS-box family all function as positive regulators of ripening. In general,
there are some inhibitors regulate these positive regulatory factors or directly involve in the regulation of fruit ripening in other ways, out of consideration of balance of the activities of these positive ripening regulators (Chung et al., 2010). It is reported that *SlAP2a* plays a role in fruit ripening as a negative regulator (Chung et al., 2010). Recently, *SlERF6* was reported to influence carotenoid biosynthesis and additional ripening phenotypes as an inhibitor (Lee et al., 2012). However, to date no inhibitor of fruit ripening in MADS-box family has been reported in tomato.

Here, we cloned a MADS-box gene, *SIMADS1* (GenBank No. AY294329), which has been reported as an inhibitor in vitro (Gaffe et al., 2011). SIMADS1 protein belongs to the SEP subfamily (Hileman et al., 2006). Prior report indicates that *SIMADS1* transcripts mainly accumulate in fruits and the accumulation decreases as fruits develop and ripen (Gaffe et al., 2011). However, *SIMADS1* has not been functionally analyzed in tomato to date. In this study, RNAi repression of *SIMADS1* was performed to investigate the exact role of *SIMADS1* in tomato and the results certify our supposition that *SIMADS1* acts as an inhibitor in regulating fruit ripening.

**RESULTS**

*SIMADS1* Transcripts Accumulate at High Levels in Sepals and Fruits

Based on the sequence in GenBank (accession No. AY294329), full-length cDNA of *SIMADS1* was cloned from tomato of AC++. In order to explore its tissue-specific expression profile, real-time PCR was performed to analyze the accumulation of *SIMADS1* transcripts in roots, stems, leaves, flowers, and a series of stages of fruits including normal and nonripening mutant fruits (*Nr* and *rin*). Low level of *SIMADS1* was observed in seedlings, stems and a series of stages of leaves (Fig. 1A). Nearly no transcripts accumulated in roots (Fig. 1A). In tissues of flowers, low level of *SIMADS1* was detected in stamen, while high in carpel and petals, and displayed its maximum in sepals of flowers (Fig. 1A). Additionally, the *SIMADS1* gene was highly expressed in IMG and MG fruits and a rapid declining trend was observed as fruit ripen (Fig. 1A, B). Similar expression trend were observed in *Nr* and *rin* fruits indicating that *SIMADS1*
expression is not impacted by the single locus \( SlMADS-RIN \) and \( Nr \) (Fig. 1B). To further detect the expression of \( SlMADS1 \) in sepals, its transcripts was analyzed in different developmental stages of sepals. \( SlMADS1 \) mRNA was highly accumulated in flower sepals and increased with the development of sepals (Fig. 1C), which hinted that \( SlMADS1 \) may play a role during the development of sepals.

**Creation of \( SlMADS1 \)-silenced Lines**

To gain further insight into the function of \( SlMADS1 \), an RNAi construct targeting the specific fragment of \( SlMADS1 \) was created and transformed into wild type tomato plants via \( Agrobacterium tumefaciens \)-mediated T-DNA transfer. Five independent transgenic lines confirmed for transgene integration were selected for characterization. Quantitative real-time PCR results showed that \( SlMADS1 \) transcripts were significantly reduced in the transgenic lines compared with the wild type, and the most silenced \( SlMADS1 \) line, named RNAi-03, had a 99% reduction in breaker fruits and about 80% in seedlings (Fig. 4A, S2). The expression of other members of MADS-box family including two SEP genes, \( SlMADS-RIN \) and \( SlMBP21 \), an AGAMOUS gene, \( TAGL1 \) and a FUL gene, \( TDR4 \) were also detected. \( TAGL1 \) and \( SlMADS-RIN \) were up-regulated, while the expression of \( TDR4 \) had no obvious change in \( SlMADS1 \)-silenced fruits compared with wild type (Fig. S1A, C and D). In particular, \( SlMBP21 \), a homolog of \( SlMADS1 \) (Leseberg et al., 2008), was not impacted in \( SlMADS1 \)-silenced lines (Fig. S1B). The results indicated that the RNAi construct of \( SlMADS1 \) is specific and do not target to other MADS-box genes. Subsequently three transgenic lines, RNAi-03, 16 and 20, were selected for further investigation.

**\( SlMADS1 \) Impacts Fruit Ripening**

During the process of fruit development, we measured the time from anthesis to ripening, and observed that the color of \( SlMADS1 \)-silenced fruits changed earlier than wild type (Fig. 2B), and its ripening time was accelerated 3 to 6 days compared with wild type (Table II). It has been shown that the dramatic change of pigmentation in ripening tomato fruits is caused by accumulation of carotenoids (Fraser et al., 1994). In this study, the carotenoids in transgenic and wild type fruits at 38 dpa (38 days after anthesis) and 42 dpa (42 days after anthesis) were extracted and determined. As shown
in Fig. 3A, the accumulation of carotenoid in RNAi lines was much higher than wild type. Real-time PCR analysis results indicated that *PSY1* (Phytone synthase1) was up-regulated in RNAi fruits both at 38 dpa and 42 dpa (Fig. 3B).

**Ethylene-related and Ripening-related Genes are Significantly Up-regulated in *SIMADS1*-silenced Fruits**

To further characterize the molecular regulation mechanism of *SIMADS1* in fruit ripening, a set of ethylene-related and ripening-related genes in wide type and transgenic tomato fruits were examined. Fig.4 showed that two ethylene biosynthetic genes, *ACS2* and *ACO3*, were dramatically up-regulated in B+4 fruits of *SIMADS1*-silenced lines (Fig. 4B, D), and the transcripts of another ethylene biosynthesis gene *ACO1* was also increased significantly in *SIMADS1*-silenced fruits at all stages (Fig. 4C). Furthermore, the expression of two ripening related genes which responded specifically to ethylene, *E4* and *E8*, were markedly increased in *SIMADS1*-silenced fruits at B+4 stage (Fig. 4E, F). These results indicated that *SIMADS1* might inhibit fruit ripening by directly or indirectly impacting ethylene biosynthesis or ethylene response.

Additionally, two ethylene-responsive genes, *ERF1* and *Pti4*, which have been reported to be factors associate with defense responses, were also analyzed. Dramatic increases were also detected in transgenic fruits at MG stage (Fig. 4G, H), suggesting that *SIMADS1* might play a role in stress response.

**More Ethylene is Produced by *SIMADS1*-silenced Lines**

To further investigate the relationship between *SIMADS1* and ethylene, we measured the ethylene production during fruit development and ripening. *SIMADS1* RNAi lines exhibited a rapid and massive increase in ethylene production at B+3 stage as the wild type did, but *SIMADS1* RNAi fruits produced approximately 2- to 4-fold more ethylene than wild type during fruit ripening and remained at high levels even at B+14 (Fig. 5).

To ascertain if the high level of ethylene production in fruit tissues of *SIMADS1* RNAi lines persisted in non-fruit tissues, ethylene triple response assay was performed. The wild type and *SIMADS1*-silenced seeds were germinated on MS medium supplemented with or without the ethylene precursor ACC, which could be taken up by
the roots and converted rapidly to ethylene. The elongation of hypocotyls and roots were detected 7 days after sowing. The results showed that the average length of hypocotyl elongation of RNAi lines were significantly shorter than that of wild type both in the absence (0 µM) and presence (5.0 µM) of ACC (Fig. 6A, B), while the root elongation of wild type and RNAi lines were nearly identical in above two conditions (Fig. 6A, C).

To verify the triple response exhibited by silenced lines, the expression of \textit{SlMADS1} in RNAi and wild type seedlings was detected. The result suggested that \textit{SlMADS1} expression was reduced at least 60% (Fig. S2). Expression of \textit{ACS1A}, \textit{ACS2}, \textit{ACS6} and \textit{ACO1} were also detected by qPCR, in order to further explore the triple response mechanism of \textit{SlMADS1}-silenced seedlings. The results displayed that \textit{ACS1A}, \textit{ACS6} and \textit{ACO1} were all up-regulated significantly in seedlings of RNAi lines in the absence of ACC (Fig. 6D), which suggested that silencing \textit{SlMADS1} could activate the expression of ethylene biosynthesis genes. While the transcripts of \textit{ACS2} were slightly increased in transgenic lines (Fig. 6D). The expression of \textit{SlMADS1} in AC++ seedlings decreased dramatically after the treatment of ACC and slow declining trend was observed with the increased density of ACC (Fig. 6E), which suggested that \textit{SlMADS1} might be impacted by ACC or ethylene.

**Yeast Two-hybrid Assay Demonstrate \textit{SlMADS1} Interacts with \textit{SIMADS-RIN}**

An essential regulator of tomato fruit ripening SIMADS-RIN was preferentially selected for yeast two-hybrid assay. The open reading frame of \textit{SlMADS1} was amplified and cloned into pGBK7 as the bait. Self-activation of pGBK7-MADS1 was tested, and the result was minus (Fig.7). The open reading frame of \textit{SIMADS-RIN} was amplified and cloned into pGADT7 as the prey. An empty prey and bait vector were used as negative controls with each bait and prey construct, respectively. Fig. 7 showed that the yeast grew on selective media and turned blue on X-α-gal indicator plate, suggesting that there exist an interaction between \textit{SIMADS1} and SIMADS-RIN in vivo.

**DISCUSSION**

\textit{SlMADS1} Inhibits Ethylene Biosynthesis and Impacts Fruit Ripening as an
Inhibitor

In higher plants ethylene biosynthesis pathway has been well studied (Bleecker and Kende, 2000). Two modes of ethylene synthesis, system 1 and system 2, have been defined (McMurchie et al., 1972; Barry et al., 2000). System 1 contributes to providing basal ethylene in vegetative tissues and unripe fruits. System 2 produces a large amount of ethylene at the onset of fruit ripening (Yang and Oetiker, 1994; Nakatsuka et al., 1998). Two kinds of rate-limiting enzymes (ACC synthase and ACC oxidase) in ethylene biosynthesis have been reported. ACC synthase catalyzes the conversion of S-adenosyl-L-methionine (AdoMet) to ACC and the conversion of ACC to ethylene is carried out by ACC oxidase (Kende, 1993). At least nine ACS genes (ACS1A, ACS1B, ACS2, ACS3, ACS4, ACS5, ACS6, ACS7 and ACS8) and five ACO genes (ACO1 to 5) have been identified in tomato (Zarembinski and Theologis, 1994; Barry et al., 1996; Oetiker et al., 1997; Nakatsuka et al., 1998; Shiu et al., 1998; Sell and Hehl, 2005). It has been proposed that SlACS1A and SlACS6 are involved in system 1 and present in tomato fruits before the onset of ripening (Barry et al., 2000). Prior studies have reported that SlACS2 was an important factor to transit System 1 to System 2 (Nakatsuka et al., 1998; Barry et al., 2000). The fruit of RNAi repression of SlACS2 could not ripen normally (Oeller et al., 1991). Moreover, two ACC oxidases (SlACO1 and SlACO3) have been reported to contribute to triggering fruit ripening (Alexander and Grierson, 2002). The expression of SlACO3 is induced but transitory at the breaker stage while SlACO1 expression is sustained during ripening (Barry et al., 1996; Nakatsuka et al., 1998).

In this study, we tested the expression of ACS2 in SIMADS1-silenced fruits and ACS1A, ACS6 in SIMADS1-silenced seedlings. Results showed that expression levels of all these ACC synthase genes were noticeably higher in RNAi lines than in wild type (Fig. 4B and 6D). Furthermore, the accumulation of the ACC oxidase (ACO1, ACO3) transcripts in transgenic fruit were much higher than wild type (Fig. 4C, D and 6D). These results indicate that SIMADS1 might inhibit the expression of ethylene biosynthesis genes, then impact the ethylene biosynthesis in tomatoes, which was confirmed by ethylene determination of fruit and triple response assay. SIMADS1 RNAi fruits produce more ethylene (Fig. 5). And the hypocotyl elongation of RNAi lines were
shorter than wild type in the absence of ACC and the RNAi seedlings were more sensitive to ACC than wild type (Fig. 6A, B), which indicated that more ethylene were probably produced in the RNAi transgenic plants than wild type. These results suggest that \textit{SlMADS1} impacts ethylene biosynthesis both in vegetative organs and fruits.

\textit{E4} and \textit{E8} are well known to be important ethylene responsive genes during fruit ripening. \textit{E8} influences ethylene biosynthesis both in fruit and flower (Kneissl and Deikman, 1996). The expression of \textit{E4} is suppressed when high-level ethylene biosynthesis is inhibited by mutations that block fruit ripening (Tigchelaar et al., 1978). Our study showed that both of the two genes expressed highly in the transgenic fruits compared with wild type (Fig. 4E, F).

For \textit{SIMADS-RIN}, TDR4 (TM4, FUL1) and TAGL1, three MADS-box proteins, are necessary for the completion of fruit ripening (Vrebalov et al., 2002; Vrebalov et al., 2009). Their expression levels were significantly up-regulated in \textit{SlMADS1}-silenced fruits (Fig. S1A, C and D). \textit{PSY1}, a major regulator of metabolic flux toward downstream carotenoids, is induced by ethylene during fruit ripening (Fray and Grierson, 1993). In our study, expression of \textit{PSY1} was notably increased in transgenic fruits (Fig. 3B). Furthermore, phenotype analysis demonstrated that \textit{SlMADS1}-silenced fruits ripen in advance (Fig. 2B and table II). These results suggest that suppressing expression of \textit{SlMADS1} promotes the expression of ripening-related genes and accelerates the rate of ripening, which indicate that \textit{SlMADS1} acts as an inhibitor in fruit ripening.

\textbf{\textit{SlMADS1} Might Weaken the Activity of \textit{SIMADS-RIN}}

In recent years, more and more MADS-box genes have been identified and revealed to play positive roles in fruit ripening. Hetero- or homo-dimers or higher-order complexes have been detected in MADS-domain proteins (Favaro et al., 2002; Shchennikova et al., 2004; De Folter et al., 2006). \textit{SIMADS-RIN} is a classical and essential positive regulator of tomato fruit ripening among the MADS-box proteins, and associate with ethylene biosynthesis, ethylene perception and ethylene response. As previously reported, \textit{ACS2} and \textit{ACS4} are bound by \textit{SIMADS-RIN} (Ito et al., 2008; Martel et al., 2011; Fujisawa et al., 2012). \textit{ACO1} is influenced by \textit{SIMADS-RIN} through a home-box gene \textit{HB1} which interacts with the promoter of \textit{ACO1} (Lin et al., 2008; Martel et al., 2011). \textit{E8} is
identified as a novel direct target of SIMADS-RIN, which can be rapidly induced following ethylene induction and during normal fruit ripening (Martel et al., 2011; Qin et al., 2012). In our study, ACO1, ACS2 and E8 are up-regulated markedly in SIMADS1-silenced lines, which suggest that these genes are negatively regulated by SIMADS1 (Fig. 4). Moreover, the yeast two-hybrid assay indicates that there is an interaction between SIMADS1 and SIMADS-RIN (Fig. 7). These results imply that SIMADS1 might bind to SIMADS-RIN and depress its activity, subsequently influence the expression of ethylene biosynthesis and response genes such as ACO1, ACS2 and E8, then reduce biosynthesis of ethylene and inhibit fruit ripening.

In summary, SIMADS1 plays an important role in fruit ripening as a repressive modulator through regulating ethylene biosynthesis directly or impacting ethylene biosynthesis and response indirectly by interacting with SIMADS-RIN. Although higher levels of a developmental regulatory cascade of this gene remain need to be discovered, as a repressive regulator, SIMADS1 acts an important role in balancing the activities of positive ripening regulators and adds a new component to the emerging mechanisms regulating fleshy fruit ripening.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

In experiments we used plants of Solanum lycopersicon Mill. cv. Ailsa Craig (AC++), a near-isogenic tomato line, as the wild type plants. The plants were planted in greenhouse and watered daily. Transgenic cultures grew under standard 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). Two generations of tomato plants were used in experiments. The plants of first generation (T0) came from tissue culture and plants of the second generation (T1) were from seedlings. Flowers were tagged at anthesis. The ripening stages of tomato fruits were divided according to days after anthesis (dpa) and fruit colour. In wild type, IMG (Immature green) were defined as 20 dpa. MG (Mature green) were defined as 35 dpa and were characterized as being green and shiny.
with no obvious colour change. B (Breaker) fruits were defined as fruits of 38 dpa and the colour change from green to yellow. B+4 and B+7 fruits were respectively defined as fruits of 4 days after breaker and 7 days after breaker. All plant samples were immediately frozen with liquid nitrogen, mixed, and stored at –80 °C until further use.

SIMADS1 Isolation

Total RNA of tomato was extracted using Trizol (Invitrogen, USA) according to the manufacturer’s instructions. Then 1µg total RNA was used to synthesis first strand cDNA through reverse transcription polymerase chain reaction (M-MLV reverse transcriptase, Takara, China) with tailed Oligo d(T)_{18} primer (5’ GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG TTT TTT TTT TTT TTT TTT 3’). 1-2 µl cDNA was used to clone the full length of SIMADS1 gene with primers of SIMADS1-F (5’ ATG GGA AGA GGA AGA GTT G 3’) and dT-r (5’GCT GTC AAC GAT ACG CTA CGT AAC G 3’) through high fidelity PCR (Prime START\textsuperscript{M} HS DNA polymerase, Takara, China). The amplified products were tailed by using DNA A-Tailing kit (Takara, China) and linked with pMD18-T vector (Takara, China). Positive clones were picked out via Escherichia coli JM109 transformation and confirmed by sequencing (Invitrogen, China).

Construction of SIMADS1 RNAi Vector and Plant Transformation

In order to down-regulate the expression of SIMADS1 gene, an RNA interference (RNAi) vector was constructed. A 515-bp specific DNA fragment of SIMADS1 was amplified with primers SIMADS1i-F (5’ CGG GGT ACC AAG CTT GAT TAC TCC GTA GAA A 3’) and SIMADS1-R (5’ CGG CTC GAG TCT AGA CAA TGA TAC AAA AAA TAC C 3’) which have been tailed with Hind III, Kpn I and Xho I, Xba I restriction site at the 5’ end respectively. Then the amplified products digested with Hind III / Xba I and Kpn I / Xho I, and linked into pHANNIBAL plasmid at Hind III / Xba I restriction site in the sense orientation and at Kpn I / Xho I restriction site in the antisense orientation. Finally, the double-stranded RNA expression unit, containing the cauliflower mosaic virus (CaMV) 35S promoter, SIMADS1 fragment in antisense orientation, PDK intron, SIMADS1 fragment in sense orientation and OCS terminator, was purified and inserted into the plant binary vector pBIN 19 with Sac I and XbaI restriction site.
The generated binary plasmids were translated into *Agrobacterium* LBA4404 strain and *Agrobacterium*-mediated transformation was performed following the protocols described by Chen et al. (2004). The transgenic plants were detected with primers NPTII-F (5’ GAC AAT CGG CTG CTC TGA 3’) and NPTII-R (5’ AAC TCC AGC AGA AGA TCC 3’). The positive transgenic plants were selected and used for subsequent experiments.

**Quantitative Real-time PCR Analysis**

Total RNA of tissues of AC++ (Mill. cv. Ailsa Craig), *Nr* (*never ripe*), *rin* (*ripening inhibitor*) and transgenic lines were extracted using Trizol (Invitrogen, USA) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using the SYBR® Premix Ex Taq II kit (TaKARA, China) in a 10 μl total sample volume (5.0 μl 2xSYBR Premix Ex Taq, 1.0 μl primers, 1.0 μl cDNA, 3. μl ddH2O). To remove the effect of genomic DNA and the template from environment, NTC (no template control) and NRT (no reverse transcription control) were performed. Additionally, three replications for each sample were used and standard curves were run simultaneously. Tomato *SlCAC* (Expósito-Rodríguez et al., 2008) gene and *SlEF1α* (Expósito-Rodríguez et al., 2008) gene were used as internal standard. The primers *SlMADS1(RT)-F* and *SlMADS1(RT)-R* (Table I) were used to determine the expression level of *SlMADS1* in wild type, *Nr*, *rin* and transgenic lines. Furthermore, the expression levels of other MADS-box genes, including *SlMADS-RIN* (Vrebalov et al., 2002), *SlMBP21* (Leseberg et al., 2008), *TAGL1* (Busi et al., 2003; Vrebalov et al., 2009) and *TDR4* (*TM4, FUL1*) (Seymour et al., 2002; Bemer et al., 2012) and fruit ripening-related, carotenoid biosynthesis and ethylene biosynthesis and responded genes, such as *E4* (Lincoln et al., 1987; Peñarrubia et al., 1992), *E8* (Kneissl and Deikman, 1996), *ACO1, ACO3, ACS2* (Griffiths et al., 1999; Alexander and Grierson, 2002), *PSY1* (Fray and Grierson, 1993), *Pti4* (Chakravarthy et al., 2003), and *ERF1* (Li et al., 2007) were determined simultaneously. Primers were shown in table I and table S1.

**Carotenoid Extraction**

A 1.0 g sample of each line was cut from pericarp in a 5 mm wide strip around the equator of 38d pa and 42 dpa fruits, respectively. Then 10 ml of 60:40 (v/v)
hexane-acetone was added respectively and total carotenoids of wild type and RNAi lines fruits were extracted. The extract was centrifuged at 4000 g for 5 min and the absorbance of supernatant was measured at 450 nm. Carotenoid content was calculated with the following equations: total carotenoid mg ml\(^{-1}\) = 4*(OD\(_{450}\))*10 ml/l g (Fray and Grierson, 1993; Forth and Pyke, 2006). Three independent experiments were performed for each sample.

**Ethylene Measurements**

Fruits of beaker, B+3, B+7 and B+14 were harvested and placed in open 100 ml jars for 3 h to minimize the effect of wound ethylene caused by picking. Jars were then sealed and incubated at room temperate for 24 h, 1 ml of headspace gas was injected into a Hewlett-Packard 5890 series gas chromatograph equipped with a flame ionization detector (FID). Samples were compared with reagent grade ethylene standards of known concentration and normalized for fruit weight (Chung et al., 2010).

**Ethylene Triple Response Assay**

The seeds of wild type were sterilized and sown on MS media supplemented with 0, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 µM ACC, then cultured in the dark at 25 °C. Meanwhile, T1 seeds of RNAi lines were sterilized and sown on MS media supplemented with 0 and 5.0 µM ACC and cultured in the same conditions as the wild type. The hypocotyl and root elongation were measured 7 days after sowing, and at least 20 seedlings were measured for each culture. To further explore the molecular mechanism of triple response of transgenic lines, the expression of ACS1A, ACS2, ACS6 and ACO1 in wild type and transgenic lines were measured by qPCR. The expression of SIMADS1 was also detected in wild type seedlings treated with 0, 1.0, 2.0, 5.0, 10.0, and 20.0 µM ACC.

**Yeast Two-hybrid Assay**

Yeast two-hybrid was performed using the MATCHMAKER TM GAL4 Two-Hybrid System III according to the manufacturer’s protocol (Clontech). The open reading frame of SIMADS1 was amplified by PCR with the primer pairs SIMADS1(Y)-F (5’ CCG GAA TTC ATG GGA AGA GGA AGA GTT G 3’) and SIMADS(Y)-R (5’CGC GGA TCC TTA AAG CAT CCA TCC ATG AAT A 3’). The PCR products were digested using EcoR I and Sal I and cloned into the EcoR I/Sal I site of the pGBK7 bait vector to
obtain the vector pGBK7-MADS1. Then pGBK7-MADS1 vector was translated into Y2HGold. The Y2HGold with bait was plated on SD medium lacking Trp (SDO) and SD medium lacking Trp, His, Ade (TDO) to test self-activation of pGBK7-MADS1. In parallel, the open reading frame of *SIMADS-RIN* was also amplified by primers SIRIN(Y)-F (5' CCG GAA TTC ATG GGT AGA GGG AAA GTA GA 3') and SIRIN(Y)-R ( 5' CGC GGA TCC TCA TAG ATG TTT ATT CAT 3'). The product was cloned into the pGADT7 vector, and translated into Y187. Subsequently, Y2HGold with bait and Y187 with prey were cultured together in 2×YPDA medium for 24h. After that these cultures were cultured on SD medium lacking Trp, Leu (DDO) to select for diploids containing prey and bait vectors. After 2 to 5 days, fresh diploid cells were plated on SD medium lacking Trp, Leu, and His, Ade, with X-α-Gal (QDO/X) to judge whether SIMADS1 can interact with SIMADS-RIN or not. Plates were incubated for 3 to 7 days at 30°C. An empty prey and bait vector were used as negative controls with each bait and prey construct, respectively. Meanwhile, positive controls were cultured. The assays were repeated at least three times with fresh transformants.
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**Grandillo S, Giovannoni J** (2009) Fleshy fruit expansion and ripening are regulated by the tomato *SHATTERPROOF* gene *TAGL1*. The Plant Cell Online 21: 3041-3062

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Figure 1. Expression profile of SlMADS1 in Tissues of AC++ and Nonripening Mutant Fruits. (A). The expression of SlMADS1 in Se, seedlings; Rt, roots; St, stems; Yl, young leaves; Mi, mature leaves; Sl, senescent leaves; Sp, sepals of flower in anthesis; Pe, petals of flower in anthesis; Ca, carpels of flower in anthesis; Sta, stamens of flower in anthesis; IMG, immature green fruits; MG, mature green fruits; B, breaker fruits; B+4, 4 days after breaker fruits; B+7, 7 days after breaker fruits in AC++. (B). The expression of SlMADS1 in AC++; Nr and rin fruits. (C). The expression of SlMADS1 in sepals of AC++. BPS, sepals of flowers before pollination; IPS, sepals of flowers in pollination; APS, sepals of flowers after pollination.

Figure 2. SlMADS1 Repression Phenotypes Genotypes are SlMADS1 RNAi lines (RNAi) and wild type (WT). The color of SlMADS1-silenced fruits changed earlier than wild type.

Figure 3. Carotenoid Accumulation and Expression of PSY1 in SlMADS1-silenced and Wild Type Fruits (A). Analysis of carotenoid accumulation at 38 dpa and 42d dpa fruits of transgenic SlMADS1 RNAi lines and wild type. Standard error is indicated for a minimum of three fruits per sample. (B). The expression of PSY1 in 38 dpa and 42 dpa fruits of transgenic SlMADS1 lines and wild type.

Figure 4. Ripening- and Ethylene-related Gene Expression in SlMADS1-silenced and Wild Type Fruits. (A). Expression of SlMADS1 in RNAi lines and wild type. RNAs were extracted for qPCR assay from breaker fruits of RNAi lines and wild type. Three replications for each sample were performed. (B)-(H). Expression of ripening- and ethylene-related gene in SlMADS1-silenced and wild type fruits. RNAs were extracted for qPCR assay from MG, B and B+4 fruits of RNAi lines and wild type. Three replications for each sample were used. (B). Expression of ACS2 in RNAi lines and wild type; (C). Expression of ACO1 in RNAi lines and wild type; (D). Expression of ACO3 in RNAi lines and wild type; (E). Expression of E4 in RNAi lines and wild type; (F). Expression of E8 in RNAi lines and wild type; (G).
Expression of *PtI4* in RNAi lines and wild type; (H). Expression of *ERF1* in RNAi lines and wild type.

**Figure 5. Production of Ethylene in Control and *SLMADS1*- silenced Lines.** Fresh fruits of B, B+3, B+7 and B+14 were sealed in airtight vials and 1ml of gas was sampled from the headspace after 24h. Values represent means of at least three individual fruits. Vertical bars represent standard error.

**Figure 6. Ethylene Triple Response Assay** (A). Seedlings of wild type and RNAi lines (RNAi03 and RNAi16) treated with 0 and 5.0 µM ACC. (B) and (C). The elongation of hypocotyl (B) and root (C) growth on different concentrations of ACC. Vertical bars represent ±SE. (D). Expression of *ACS1A, ACS2, ACS6* and *ACO1* in seedlings of RNAi lines and wild type. (E). Expression of *SLMADS1* in seedlings of wild type treated with 0 (A0), 1.0 (A1), 2.0 (A2), 5.0 (A5), 10.0 (A10), and 20.0 µM (A20) ACC.

**Figure 7. Yeast Two-hybrid Assay for *SLMADS1* and *SLMADS-RIN* Proteins**
SDO, SD medium without Trp; TDO, SD medium without Trp, His and Ade; QDO, SD medium without Trp, Leu, His and Ade; QDO/X, SD medium without Trp, Leu, His, Ade and with X-α-Gal. 1. pGBK7-MADS1 & pGADT7-RIN (interaction of *SLMADS1* and *SLMADS-RIN*); 2. pGBK7-53 & pGADT7-T (positive control); 3. pGBK7-Lam & pGADT7-T (negative control); 4. pGBK7-MADS1 (autoactivation assay); 5. pGBK7 & pGADT7-RIN (empty bait vector); 6. pGBK7-MADS1 & pGADT7 (empty prey vector)
Table I. Details of primers for qPCR amplification

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Table II. Days from anthesis to breaker stage for control and *SIMADS1*-silenced lines

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<td>RNAi20</td>
<td>34.6±0.48</td>
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</table>
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