A Tomato MADS-Box Transcription Factor, SlMADS1, Acts as a Negative Regulator of Fruit Ripening[1][C][W]

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MADS-box genes encode a highly conserved gene family of transcriptional factors that regulate numerous developmental processes in plants. In this study, a tomato (*Solanum lycopersicum*) MADS-box gene, *SlMADS1*, was cloned and its tissue-specific expression profile was analyzed. The real-time polymerase chain reaction results showed that *SlMADS1* was highly expressed in sepals and fruits; its expression level was increased with the development of sepals, while the transcript of *SlMADS1* decreased significantly in accordance with fruit ripening. To further explore the function of *SlMADS1*, an RNA interference (RNAi) expression vector targeting *SlMADS1* was constructed and transformed into tomato plants. Shorter ripening time of fruit was observed in *SlMADS1*-silenced tomatoes. The accumulation of carotenoid and the expression of *PHYTOENE SYNTHETASE1* were enhanced in RNAi fruits. Besides, ethylene biosynthetic genes, including *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE1A*, *1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE6*, *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE1*, and *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE3*, and the ethylene-responsive genes *E4* and *E8*, which were involved in fruit ripening, were also up-regulated in silenced plants. *SlMADS1* RNAi fruits showed approximately 2- to 4-fold increases in ethylene production compared with the wild type. Furthermore, *SlMADS1*-silenced seedlings displayed shorter hypocotyls and were more sensitive to 1-aminocyclopropane-1-carboxylate than the wild type. Additionally, a yeast two-hybrid assay revealed a clear interaction between SlMADS1 and SlMADS-RIN. These results suggest that *SlMADS1* plays an important role in fruit ripening as a repressive modulator.

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 tomato (*Solanum lycopersicum*), banana (*Musa* spp.), apple (*Malus domestica*), and pear (*Pyrus communis*), ethylene plays an important role in triggering the onset of ripening and is an essential factor for the ripening process (Abeles et al., 1973; Hiwasa et al., 2003). There are two key biosynthetic enzymes in ethylene biosynthesis, 1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE (ACS) and 1-AMINOCYCLOPROPANE-1-CARBOXYLATE 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* RNA interference (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, 2000). Furthermore, the expression of both *SlACO1* and *SlACO3* is significantly increased at the onset of tomato fruit ripening (Barry et al., 1996). Previous studies also indicate that RNAi inhibition of *SlACO1* delays the ripening of climacteric fruits (Hamilton et al., 1990; Blume and Grierson, 1997; Giovannoni, 2001). These findings suggest that the normal function of ethylene biosynthesis is required for the ripening process.

Besides the functional ethylene synthesis, the abilities of ethylene perception and response are also necessary for ripening. *E4* and *E8* are two classical genes that are induced by ethylene (Lincoln et al., 1987). The expression of *E4* in fruit is rapidly induced following exogenous ethylene induction (Lincoln and Fischer, 1988a). Meanwhile, the transcripts of *E4* in fruit are suppressed through ethylene biosynthesis inhibition (Tigchelaar et al., 1978; Lincoln and Fischer, 1988b). 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 (Sandhu et al., 2000; Krasnyanski et al., 2001; Kesanakurti et al., 2012).

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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 the function of two MADS-box transcriptional factors, SlMADS-RIN and SlMADS-MC. SlMADS-RIN regulates fruit ripening and SlMADS-MC is involved in sepal development (Vrebalov et al., 2002). Besides SlMADS-RIN and SlMADS-MC, other MADS-box proteins also have been investigated in tomato. A prior study indicates 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, TOMATO AGAMOUS1 (TAG1), TOMATO AGAMOUS-LIKE1 (TAGL1), TOMATO MADS BOX4 (TM4 [TDR4, FUL1]), and TM6 have been investigated and identified to be associated with the development of fruits (Giovannoni, 2007). RNAi suppression of the *TAG1* gene in tomato leads to misshapen fruits and homeotic conversion of stamens into petalloid 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 the Arabidopsis (*Arabidopsis thaliana*) *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 have been considered to be involved in fruit ripening (Pnueli et al.,

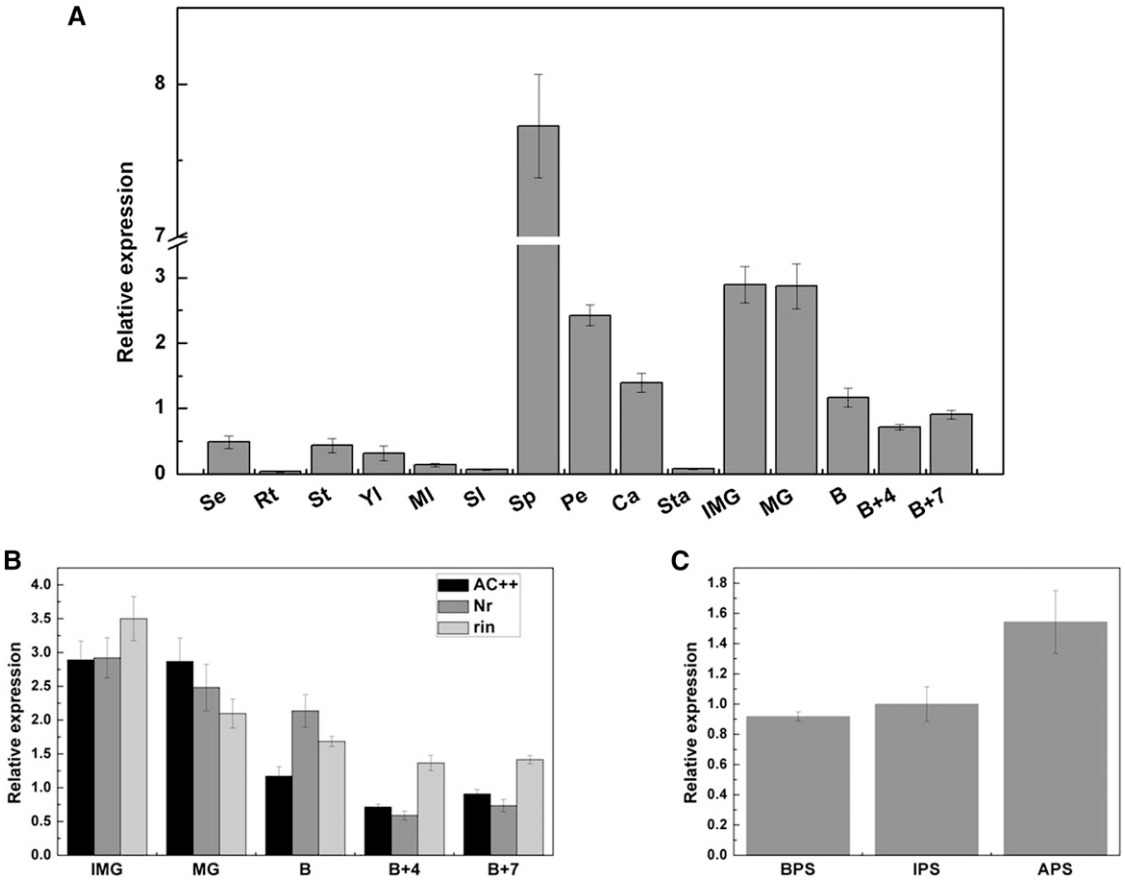

Figure 1. Expression profile of *SlMADS1* in tissues of cv Ailsa Craig and nonripening mutant fruits. A, Expression of *SlMADS1* in cv Ailsa Craig as indicated: Se, seedlings; Rt, roots; St, stems; Yl, young leaves; Ml, mature leaves; Sl, senescent leaves; Sp, sepals of flower in anthesis; Pe, petals 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 d after breaker fruits; B+7, 7 d after breaker fruits. B, Expression of *SlMADS1* in cv Ailsa Craig (AC^{++}), *Nr*, and *rin* fruits. C, Expression of *SlMADS1* in sepals of cv Ailsa Craig. BPS, Sepals of flowers before pollination; IPS, sepals of flowers in pollination; APS, sepals of flowers after pollination.

1994; Busi et al., 2003). Interestingly, these reported genes of the MADS-box family all function as positive regulators of ripening. In general, some inhibitors regulate these positive regulatory factors or are directly involved in the regulation of fruit ripening in other ways, out of consideration of the 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 the MADS-box family has been reported in tomato.

Here, we cloned a MADS-box gene, *SlMADS1* (GenBank accession no. AY294329), which has been reported as an inhibitor in vitro (Gaffe et al., 2011). The SlMADS1 protein belongs to the SEPALLATA (SEP) subfamily (Hileman et al., 2006). A prior report indicates that *SlMADS1* transcripts mainly accumulate in fruits and that the accumulation decreases as fruits develop and ripen (Gaffe et al., 2011). However, *SlMADS1* has not been functionally analyzed in tomato to date. In this study, RNAi repression of *SlMADS1* was performed to investigate the exact role of *SlMADS1* in tomato, and the results certify our supposition that *SlMADS1* acts as an inhibitor in regulating fruit ripening.

RESULTS

SlMADS1 Transcripts Accumulate at High Levels in Sepals and Fruits

Based on the sequence in GenBank, full-length complementary DNA (cDNA) of *SlMADS1* was cloned from tomato of cv Ailsa Craig. In order to explore its tissue-specific expression profile, real-time PCR was performed to analyze the accumulation of *SlMADS1* transcripts in roots, stems, leaves, flowers, and a series of stages of fruits including normal and nonripening mutant fruits (*Nr* and *rin*). A low level of *SlMADS1* was observed in seedlings, stems, and a series of stages of leaves (Fig. 1A). Almost no transcripts accumulated in roots (Fig. 1A). In tissues of flowers, a low level of *SlMADS1* was detected in stamen, high levels were seen in carpel and petals, and the maximum level was displayed in sepals of flowers (Fig. 1A). Additionally, the *SlMADS1* gene was highly expressed in immature green and mature green fruits, and a rapid declining trend was observed as fruit ripened (Fig. 1, A and B). A similar expression trend was observed in *Nr* and *rin* fruits, indicating that *SlMADS1* 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 were 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. Real-time quantitative PCR (qPCR) 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. 2A and Supplemental Fig. S1). The expression of other members of the MADS-box family, including two SEP genes, *SlMADS-RIN* and *SlMBP21*, an AGAMOUS gene, *TAGL1*, and a FUL gene, *TDR4*, was also detected. *TAGL1* and *SlMADS-RIN* were up-regulated, while the expression of *TDR4* had no obvious change in *SlMADS1*-silenced fruits compared with the wild type (Supplemental Fig. S2, A, C, and D). In particular, *SlMBP21*, a homolog of *SlMADS1* (Leseberg et al., 2008), was not impacted in *SlMADS1*-silenced lines (Supplemental Fig. S2B). These results indicated that the RNAi construct of *SlMADS1* is specific and does not target to other MADS-box genes. Subsequently, three transgenic lines, RNAi-03, RNAi-16, and RNAi-20, were selected for further investigation.

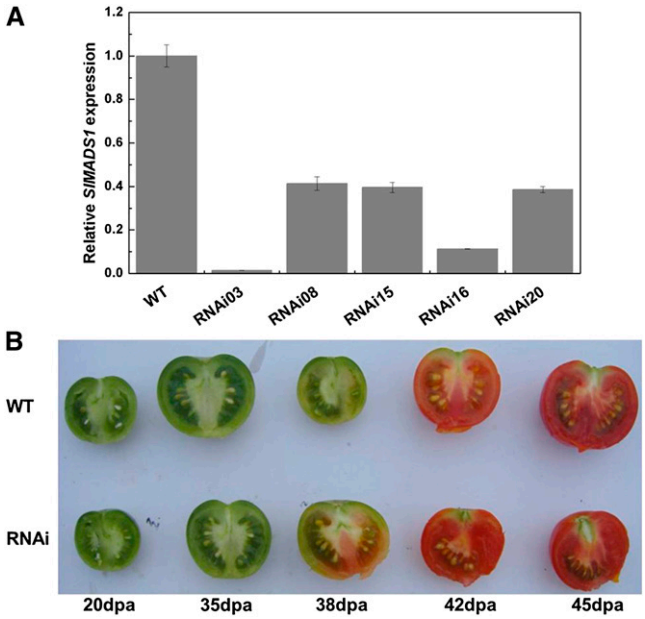

Figure 2. *SlMADS1* repression phenotypes. A, Expression of *SlMADS1* in RNAi lines and wild type (WT). RNAs were extracted for qPCR assay from breaker fruits of RNAi lines and the wild type. Three replications for each sample were performed. B, Genotypes are *SlMADS1* RNAi lines (RNAi) and the wild type. The color of *SlMADS1*-silenced fruits changed earlier than in the wild type. [See online article for color version of this figure.]

Table I. *Days from anthesis to breaker stage for control and SlMADS1-silenced lines*

Tomato Line	Days
Wild type	38.0 ± 0.50
RNAi-03	31.8 ± 0.45
RNAi-16	33.6 ± 0.48
RNAi-20	34.6 ± 0.48

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 fruits (Fig. 2B), and their ripening time was accelerated 3 to 6 d compared with the wild type (Table I). It has been shown that the dramatic change of pigmentation in ripening tomato fruits is caused by the accumulation of carotenoids (Fraser et al., 1994). In this study, the carotenoids in transgenic and wild-type fruits at 38 and 42 DPA were extracted and determined. As shown in Figure 3A, the accumulation of carotenoid in RNAi lines was much higher than in the wild type. Real-time PCR analysis results indicated that *PHYTOENE SYNTHETASE1* (*PSY1*) was up-regulated in RNAi fruits both at 38 and 42 DPA (Fig. 3B).

Ethylene-Related and Ripening-Related Genes Are Significantly Up-Regulated in *SlMADS1*-Silenced Fruits

To further characterize the molecular regulation mechanism of *SlMADS1* in fruit ripening, a set of ethylene-related and ripening-related genes in wild-type and transgenic tomato fruits were examined. Two ethylene biosynthetic genes, *ACS2* and *ACO3*, were dramatically up-regulated in breaker + 4 d fruits of *SlMADS1*-silenced lines (Fig. 4, A and C), and the transcripts of another ethylene biosynthesis gene, *ACO1*, was also increased significantly in *SlMADS1*-silenced fruits at all stages (Fig. 4B). Furthermore, the expression of two ripening-related genes that responded specifically to ethylene, *E4* and *E8*, was markedly increased in *SlMADS1*-silenced fruits at the breaker + 4 d stage (Fig. 4, D and E). These results indicated that *SlMADS1* 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 associated with defense responses, were also analyzed. Dramatic increases were also detected in transgenic fruits at the mature green stage (Fig. 4, F and G), suggesting that *SlMADS1* might play a role in the stress response.

More Ethylene Is Produced by *SlMADS1*-Silenced Lines

To further investigate the relationship between *SlMADS1* and ethylene, we measured ethylene production during fruit development and ripening. *SlMADS1* RNAi lines exhibited a rapid and massive increase in ethylene production at the breaker + 3 d, stage like the wild type, but *SlMADS1* RNAi fruits produced approximately 2- to 4-fold more ethylene than the wild type during fruit ripening and remained at high levels even at breaker + 14 d (Fig. 5).

To ascertain if the high level of ethylene production in fruit tissues of *SlMADS1* RNAi lines persisted in non-fruit tissues, an ethylene triple response assay was performed. Wild-type and *SlMADS1*-silenced seeds were germinated on Murashige and Skoog (MS) medium supplemented with or without the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), which could be taken up by the roots and converted rapidly to ethylene. The elongation of hypocotyls and roots was detected 7 d after sowing. The results showed that the average length of hypocotyl elongation of RNAi lines was significantly shorter than that of the wild type both in the absence (0 μM) and presence (5.0 μM) of ACC (Fig. 6, A and B), while the root elongation of wild-type and RNAi lines was nearly identical in the above two conditions (Fig. 6, A and C).

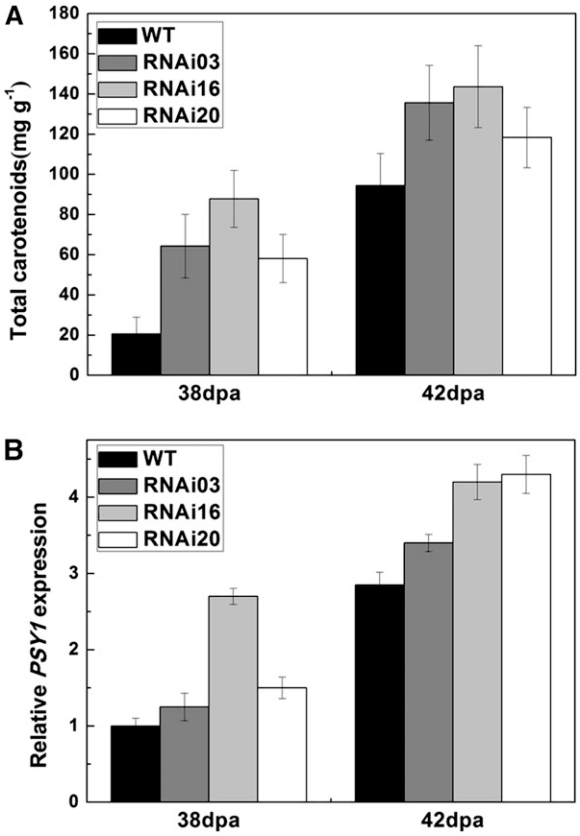

Figure 3. Carotenoid accumulation and expression of *PSY1* in *SlMADS1*-silenced and wild-type (WT) fruits. A, Analysis of carotenoid accumulation in 38- and 42-DPA fruits of transgenic *SlMADS1* RNAi lines and the wild type. SE is indicated for a minimum of three fruits per sample. B, Expression of *PSY1* in 38- and 42-DPA fruits of transgenic *SlMADS1* lines and the wild type.

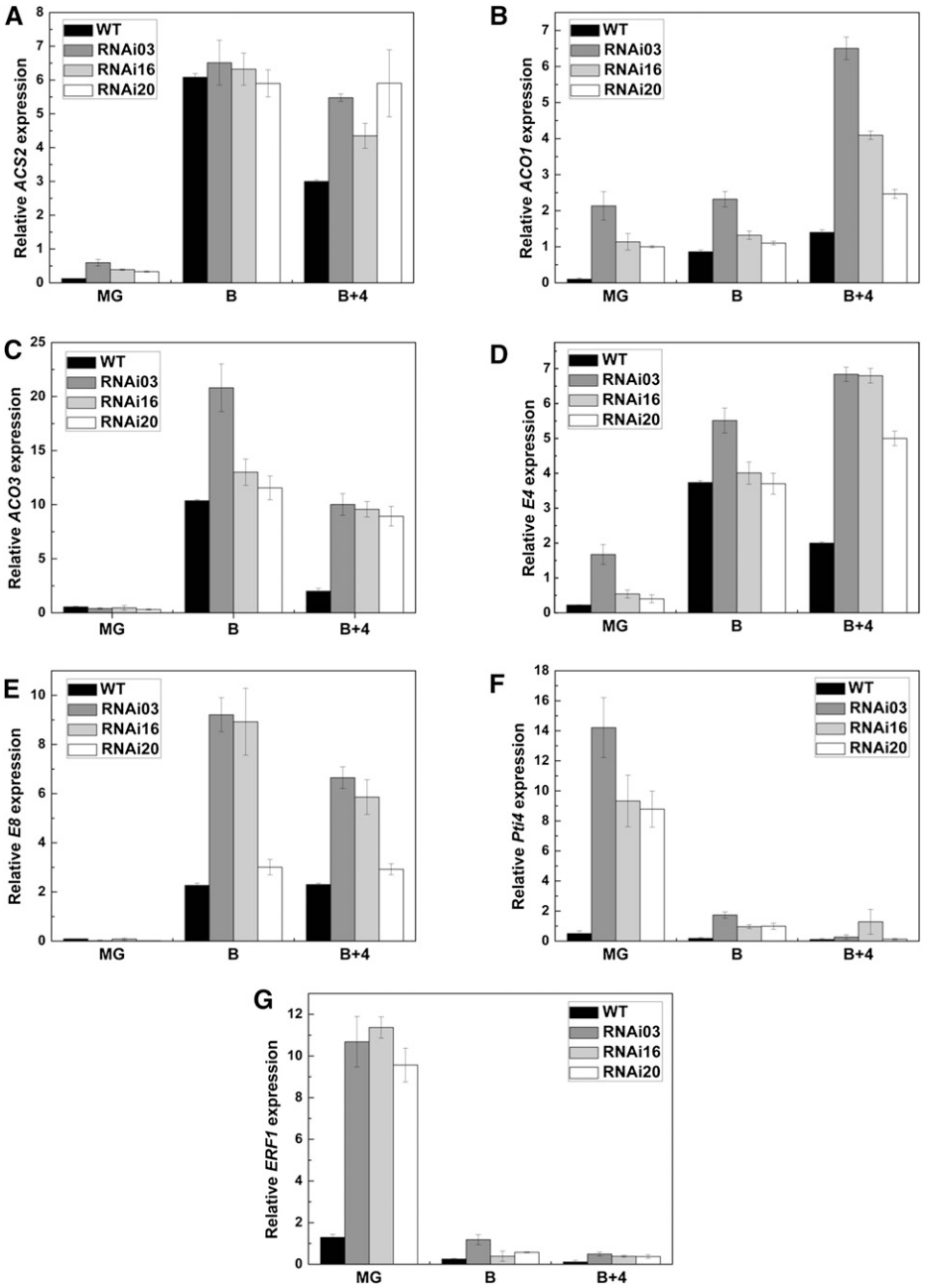

Figure 4. Ripening- and ethylene-related gene expression in *SlMADS1*-silenced and wild-type (WT) fruits. RNAs were extracted for qPCR assay from mature green (MG), breaker (B), and breaker + 4-d (B+4) fruits of RNAi lines and the wild type. Three replications for each sample were used. A, Expression of *ACS2* in RNAi lines and the wild type. B, Expression of *ACO1* in RNAi lines and the wild type. C, Expression of *ACO3* in RNAi lines and the wild type. D, Expression of *E4* in RNAi lines and the wild type. E, Expression of *E8* in RNAi lines and the wild type. F, Expression of *Pti4* in RNAi lines and the wild type. G, Expression of *ERF1* in RNAi lines and the wild type.

To verify the triple response exhibited by silenced lines, the expression of *SlMADS1* in RNAi and wild-type seedlings was detected. The result suggested that *SlMADS1* expression was reduced at least 60% (Supplemental Fig. S1). The expression of *ACS1A*, *ACS2*, *ACS6*, and *ACO1* was also detected by quantitative PCR, in order to further explore the triple response mechanism of *SlMADS1*-silenced seedlings. The results demonstrated that *ACS1A*, *ACS6*, and *ACO1* were all up-regulated significantly in seedlings of RNAi lines in the absence of ACC (Fig. 6D), which suggested that silencing *SlMADS1* could activate the expression of ethylene biosynthesis genes, while the transcripts of *ACS2* were slightly increased in transgenic lines (Fig. 6D). The expression of *SlMADS1* in cv Ailsa Craig seedlings decreased dramatically after the ACC treatment, and a slow declining trend was observed with the increased density of ACC (Fig. 6E), which suggested that *SlMADS1* might be impacted by ACC or ethylene.

The Yeast Two-Hybrid Assay Demonstrates That SlMADS1 Interacts with SlMADS-RIN

An essential regulator of tomato fruit ripening, SlMADS-RIN was preferentially selected for yeast two-hybrid assay. The open reading frame of *SlMADS1* was

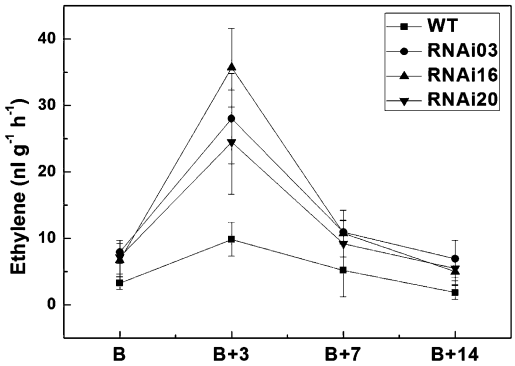

Figure 5. Production of ethylene in control and *SlMADS1*-silenced lines. Fresh fruits of breaker (B), breaker + 3 d (B+3), breaker + 7 d (B+7), and breaker + 14 d (B+14) were sealed in air-tight vials, and 1 mL of gas was sampled from the headspace after 24 h. Values represent means of at least three individual fruits. Error bars represent SE. WT, Wild type.

amplified and cloned into pGBKT7 as the bait. Self-activation of pGBKT7-MADS1 was tested, and the result was negative (Fig. 7). The open reading frame of *SlMADS-RIN* was amplified and cloned into pGADT7 as the prey. An empty prey and bait vector was used as a negative control with each bait and prey construct, respectively. Figure 7 shows that the yeast grew on selective medium and turned blue on the 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal) indicator plate, suggesting that there exists an interaction between SlMADS1 and SlMADS-RIN in vivo.

DISCUSSION

SlMADS1 Inhibits Ethylene Biosynthesis and Impacts Fruit Ripening as an Inhibitor

In higher plants, the ethylene biosynthesis pathway is 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 (ACS and ACO) in ethylene biosynthesis have been reported. ACS catalyzes the conversion of *S*-adenosyl-L-Met to ACC, and the

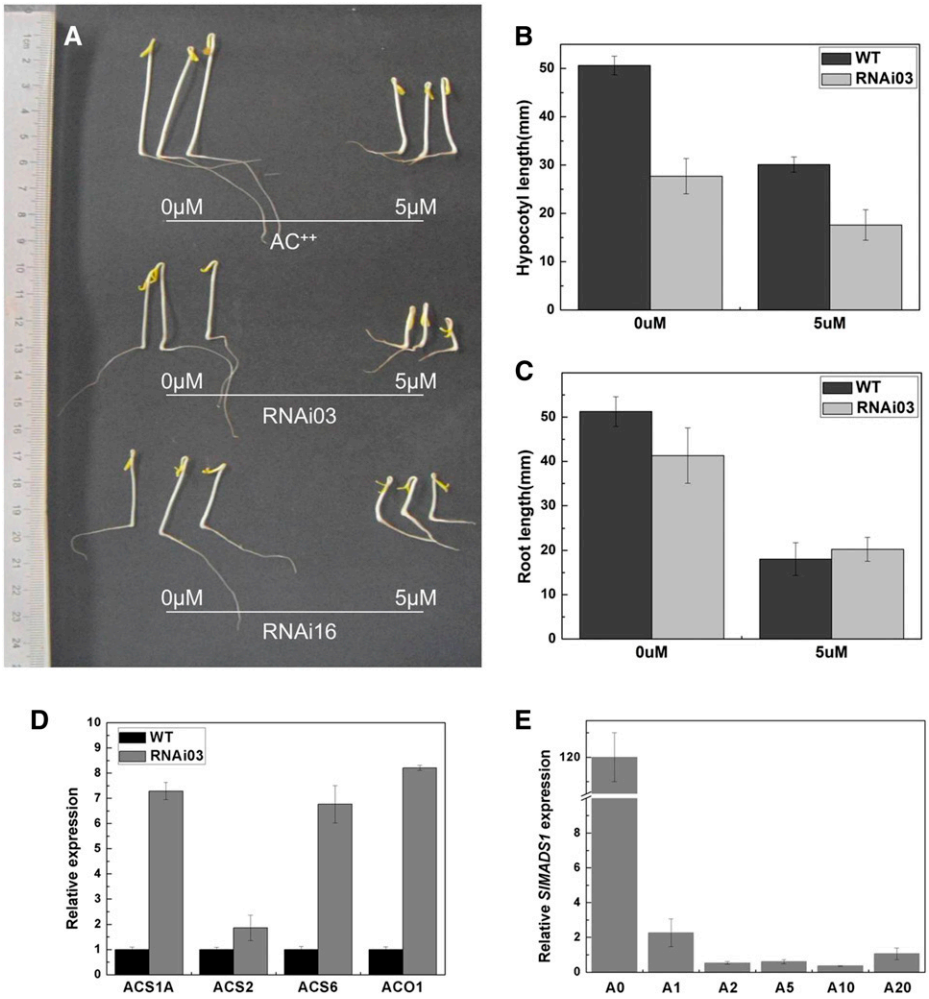

Figure 6. Ethylene triple response assay. A, Seedlings of wild-type Ailsa Craig (AC^{++}) and RNAi lines (RNAi-03 and RNAi-16) treated with 0 and 5.0 μM ACC. B and C, Elongation of hypocotyl (B) and root (C) growth on different concentrations of ACC. Error bars represent ± SE. D, Expression of *ACS1A*, *ACS2*, *ACS6*, and *ACO1* in seedlings of RNAi lines and the wild type (WT). E, Expression of *SlMADS1* in seedlings of the wild type treated with 0 (A0), 1.0 (A1), 2.0 (A2), 5.0 (A5), 10.0 (A10), and 20.0 (A20) μM ACC. [See online article for color version of this figure.]

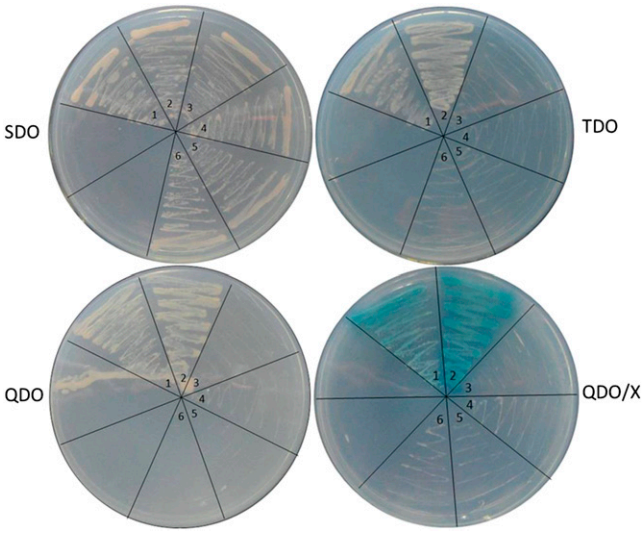

Figure 7. Yeast two-hybrid assay for SlMADS1 and SlMADS-RIN proteins. SDO, SD medium without Trp; TDO, SD medium without Trp, His, and adenine; QDO, SD medium without Trp, Leu, His, and adenine; QDO/X, SD medium without Trp, Leu, His, and adenine with X-α-Gal. Numbered wedges are as follows: 1, pGBKT7-MADS1 and pGADT7-RIN (interaction of SlMADS1 and SlMADS-RIN); 2, pGBKT7-53 and pGADT7-T (positive control); 3, pGBKT7-Lam and pGADT7-T (negative control); 4, pGBKT7-MADS1 (autoactivation assay); 5, pGBKT7 and pGADT7-RIN (empty bait vector); 6, pGBKT7-MADS1 and pGADT7 (empty prey vector). [See online article for color version of this figure.]

conversion of ACC to ethylene is carried out by ACO (Kende, 1993). At least nine *ACS* genes (*ACS1A*, *ACS1B*, *ACS2*, *ACS3*, *ACS4*, *ACS5*, *ACS6*, *ACS7*, and *ACS8*) and five *ACO* genes (*ACO1*–*ACO5*) 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 from RNAi repression of *SlACS2* could not ripen normally (Oeller et al., 1991). Moreover, two ACO genes (*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 *SlMADS1*-silenced fruits and *ACS1A* and *ACS6* in *SlMADS1*-silenced seedlings. The results showed that expression levels of all these ACS genes were noticeably higher in RNAi lines than in the wild type (Figs. 4A and 6D). Furthermore, the accumulation of the ACO transcripts (*ACO1* and *ACO3*) in transgenic fruit was much higher than in the wild type (Figs. 4, B and C, and 6D). These results indicate that *SlMADS1* might inhibit the expression of ethylene biosynthesis genes, then impact the ethylene biosynthesis in tomatoes, which was confirmed by ethylene determination of fruit and the triple response assay. *SlMADS1* RNAi fruits produce more ethylene (Fig. 5). Also, the hypocotyl elongation of RNAi lines was shorter than in the wild type in the absence of ACC, and the RNAi seedlings were more sensitive to ACC than the wild type (Fig. 6, A and B), which indicated that more ethylene was probably produced in

Table II. *Details of primers for qPCR amplification*

Primer Name	Primer Sequence (5′–3′)	Product
		bp
SlCAC	CCTCCGTTGTGATGTAACTGG ATTGGTGGAAAGTAACATCATCG	173
SlEF1α	ACCTTTGCTGAATACCCTCCATTG CACACTTCACTTCCCCTTCTTCTG	150
SlMADS1	GTGTAGCTGGATTTCCACTTCG GCCGCTGCATTCACCTCAT	175
E4	AGGGTAACAACAGCAGTAGCA CCCAACCTCCGTCTTCAC	167
E8	GGCACCATTCAACATACCG CTTTCACCGAAGAAGCACG	242
PSY1	AGAGGTGGTGGAAAGCAA TCTCGGGAGTCATTAGCAT	298
ACO1	ACAAACAGACGGGACACGAA CTCTTTGGCTTGAAACTTGA	181
ACO3	CAAGCAAGTTTATCCGAAAT CATTAGCTTCCATAGCCTTC	113
ACS2	GAAAGAGTTGTTATGGCTGGTG GCTGGGTAGTATGGTGAAGGT	107
ERF1	TTTTAGTATCGGATGGACG GGCGGAGAAACAGAAGTA	102
Pti4	CTCTAAGCGTCGGATGGTC AATGTCTTCCTTTCGGTGTTT	150

the RNAi transgenic plants than the wild type. These results suggest that *SlMADS1* impacts ethylene biosynthesis both in vegetative organs and fruits.

E4 and *E8* are well known as important ethylene-responsive genes during fruit ripening. *E8* influences ethylene biosynthesis both in fruit and flower (Kneissl and Deikman, 1996). The expression of *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 these genes were expressed highly in the transgenic fruits compared with the wild type (Fig. 4, D and E).

For SlMADS-RIN, TDR4 (TM4, FUL1), and TAGL1, three MADS-box proteins are necessary for the completion of fruit ripening (Vrebalov et al., 2002, 2009). Their expression levels were significantly up-regulated in *SlMADS1*-silenced fruits (Supplemental Fig. S2, A, C, and D). *PSY1*, a major regulator of metabolic flux toward downstream carotenoids, is induced by ethylene during fruit ripening (Fray and Grierson, 1993). In our study, the expression of *PSY1* was notably increased in transgenic fruits (Fig. 3B). Furthermore, phenotype analysis demonstrated that *SlMADS1*-silenced fruits ripen in advance (Fig. 2B; Table I). These results suggest that suppressing the expression of *SlMADS1* promotes the expression of ripening-related genes and accelerates the rate of ripening, indicating that *SlMADS1* acts as an inhibitor in fruit ripening.

SlMADS1 Might Weaken the Activity of *SlMADS-RIN*

In recent years, more and more MADS-box genes have been identified and revealed to play positive roles in fruit ripening. Heterodimers, homodimers, or higher order complexes have been detected in MADS-domain proteins (Favaro et al., 2002; Shchennikova et al., 2004; de Folter et al., 2006). SlMADS-RIN is a classical and essential positive regulator of tomato fruit ripening among the MADS-box proteins and is associated with ethylene biosynthesis, ethylene perception, and ethylene response. As reported previously, *ACS2* and *ACS4* are bound by *SlMADS-RIN* (Ito et al., 2008; Martel et al., 2011; Fujisawa et al., 2012). *ACO1* is influenced by *SlMADS-RIN* through the homeobox gene *HB1*, which interacts with the promoter of *ACO1* (Lin et al., 2008; Martel et al., 2011). *E8* is identified as a novel direct target of *SlMADS-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 *SlMADS1*-silenced lines, which suggests that these genes are negatively regulated by *SlMADS1* (Fig. 4). Moreover, the yeast two-hybrid assay indicates that there is an interaction between SlMADS1 and SlMADS-RIN (Fig. 7). These results imply that SlMADS1 might bind to SlMADS-RIN and depress its activity, subsequently influence the expression of ethylene biosynthesis and response genes such as *ACO1*, *ACS2*, and *E8*, and then reduce the biosynthesis of ethylene and inhibit fruit ripening.

In summary, *SlMADS1* plays an important role in fruit ripening as a repressive modulator by regulating ethylene biosynthesis directly or impacting ethylene biosynthesis and response indirectly by interacting with SlMADS-RIN. Although higher levels of a developmental regulatory cascade of this gene remain to be discovered, as a repressive regulator, *SlMADS1* plays 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 our experiments, we used plants of tomato (*Solanum lycopersicum* 'Ailsa Craig' AC^{++}), a near-isogenic tomato line, as the wild type. The plants were planted in a greenhouse and watered daily. Transgenic cultures grew under standard greenhouse conditions (16-h-day/8-h-night cycle, 25°C/18°C day/night temperature, 80% humidity, and 250 μmol m^{-2} s^{-1} light intensity). Two generations of tomato plants were used in the experiments. Plants of the 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 DPA and fruit color. In the wild type, immature green was defined as 20 DPA. Mature green was defined as 35 DPA and characterized as being green and shiny with no obvious color change. Breaker fruits were defined as fruits of 38 DPA with the color change from green to yellow. Other fruits of 4 d after breaker and 7 d after breaker were also used. All plant samples were immediately frozen with liquid nitrogen, mixed, and stored at –80°C until further use.

SlMADS1 Isolation

Total RNA of tomato was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Then, 1 μg of total RNA was used to synthesize first-strand cDNA through reverse transcription-PCR using Moloney murine leukemia virus reverse transcriptase (Takara) with tailed oligo$(dT)_{18}$ primer (5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTGTTTT-TTTTTTTTTTTTTT-3′). One to 2 μL of cDNA was used to clone the full-length *SlMADS1* gene with primers of SlMADS1-F (5′-ATGGGAAGAGGAAGAG-TTG-3′) and dT-r (5′-GCTGTCAACGATACGCTACGTAACG-3′) through high-fidelity PCR (Prime START HS DNA polymerase; Takara). The amplified products were tailed by using the DNA A-Tailing kit (Takara) and linked with pMD18-T vector (Takara). Positive clones were picked out via *Escherichia coli* JM109 transformation and confirmed by sequencing (Invitrogen).

Construction of the *SlMADS1* RNAi Vector and Plant Transformation

In order to down-regulate the expression of the *SlMADS1* gene, an RNAi vector was constructed. A 515-bp specific DNA fragment of *SlMADS1* was amplified with primers SlMADS1i-F (5′-CGGGGTACCAAGCTTGATTAC-TCCGTAGAAA-3′) and SlMADSi-R (5′-CCGCTCGAGTCTAGACAATGA-TACAAAAAATAC-3′), which had been tailed with *Hin*dIII/*Kpn*I and *Xho*I/*Xba*I restriction sites at the 5′ end, respectively. Then, the amplified products were digested with *Hin*dIII/*Xba*I and *Kpn*I/*Xho*I and linked into the pHANNIBAL plasmid at the *Hin*dIII/*Xba*I restriction site in the sense orientation and at the *Kpn*I/*Xho*I restriction site in the antisense orientation. Finally, the double-stranded RNA expression unit, containing the cauliflower mosaic virus 35S promoter, *SlMADS1* fragment in the antisense orientation, *PDK* intron, *SlMADS1* fragment in the sense orientation, and *OCS* terminator, was purified and inserted into the plant binary vector pBIN19 with *Sac*I and *Xba*I restriction sites.

The generated binary plasmids were translated into *Agrobacterium tumefaciens* LBA4404 strain, and *A. tumefaciens*-mediated transformation was performed following the protocols described by Chen et al. (2004). The transgenic plants were detected with primers NPTII-F (5′-GACAATCGGCTGCTCTGA-3′) and NPTII-R (5′-AACTCCAGCATGAGATCC-3′). The positive transgenic plants were selected and used for subsequent experiments.

Quantitative Real-Time PCR Analysis

Total RNAs of tissues of cv Ailsa Craig, *Nr*, *rin*, and transgenic lines were extracted using Trizol (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR Premix Ex Taq II kit (Takara) 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, and 3 μL of distilled, deionized water). To remove the effect of genomic DNA and the template from the environment, no-template control and no-reverse transcription control experiments were performed. Additionally, three replications for each sample were used, and standard curves were run simultaneously. Tomato *SlCAC* (Expósito-Rodríguez et al., 2008) and *SlEF1α* (Expósito-Rodríguez et al., 2008) were used as internal standards. The primers SlMADS1(RT)-F and SlMADS1(RT)-R (Table II) were used to determine the expression levels of *SlMADS1* in the wild type, *Nr* and *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), as well as fruit ripening-related, carotenoid biosynthesis, and ethylene biosynthesis and response genes, such as *E4* (Lincoln et al., 1987; Peñarrubia et al., 1992), *E8* (Kneissl and Deikman, 1996), *ACO1*, *ACO3*, and *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 are shown in Table II and Supplemental 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 38- and 42-DPA fruits. Then, 10 mL of 60:40 (v/v) hexane:acetone was added, and total carotenoids of wild-type and RNAi line fruits were extracted. The extract was centrifuged at 4,000*g* for 5 min, and the absorbance of the supernatant was measured at 450 nm. Carotenoid content was calculated with the following equation: total carotenoid (mg mL^{-1}) = 4×(optical density at 450 nm) × 10 mL/1 g (Fray and Grierson, 1993; Forth and Pyke, 2006). Three independent experiments were performed for each sample.

Ethylene Measurements

Fruits of beaker, beaker + 3 d, beaker + 7 d, and beaker + 14 d 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, and 1 mL of headspace gas was injected into a Hewlett-Packard 5890 series gas chromatograph equipped with a flame ionization detector. 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 plants were sterilized and sown on MS medium supplemented with 0, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 μM ACC and then cultured in the dark at 25°C. Meanwhile, T1 seeds of RNAi lines were sterilized and sown on MS medium supplemented with 0 and 5.0 μM ACC and then cultured in the same conditions as the wild type. Hypocotyl and root elongation were measured 7 d after sowing, and at least 20 seedlings were measured for each culture. To further explore the molecular mechanism of the triple response of transgenic lines, the expression of *ACS1A*, *ACS2*, *ACS6*, and *ACO1* in the wild type and transgenic lines was measured by qPCR. The expression of *SlMADS1* 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

The yeast two-hybrid assay was performed using the MATCHMAKER GAL4 Two-Hybrid System III according to the manufacturer's protocol (Clontech). The open reading frame of *SlMADS1* was amplified by PCR with the primer pair SlMADS1(Y)-F (5′-CCGGAATTCATGGGAAGAGGAAGA-GTTG-3′) and SlMADS(Y)-R (5′-CGCGGATCCTTAAAGCATCCATCCATG-AATA-3′). The PCR products were digested using *Eco*RI and *Sal*I and cloned into the *Eco*RI/*Sal*I site of the pGBKT7 bait vector to obtain the vector pGBKT7-MADS1. Then, pGBKT7-MADS1 vector was translated into Y2HGold. The Y2HGold with bait was plated on synthetic dropout (SD) medium lacking Trp and SD medium lacking Trp, His, and adenine to test the self-activation of pGBKT7-MADS1. In parallel, the open reading frame of *SlMADS-RIN* was also amplified by primers SlRIN(Y)-F (5′-CCGGAATTCATGGGTAGAGGGA-AAGTAGA-3′) and SlRIN(Y)-R (5′-CGCGGATCCTCATAGATGTTTATT-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 (yeast extract, peptone, and dextrose medium supplemented with adenine hemisulfate) medium for 24 h. After that, these cultures were cultured on SD medium lacking Trp and Leu to select for diploids containing prey and bait vectors. After 2 to 5 d, fresh diploid cells were plated on SD medium lacking Trp, Leu, His, and adenine with X-α-Gal to judge whether SlMADS1 can interact with SlMADS-RIN or not. Plates were incubated for 3 to 7 d at 30°C. An empty prey and bait vector was used as a negative control with each bait and prey construct, respectively. Meanwhile, positive controls were cultured. The assays were repeated at least three times with fresh transformants.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *SlMADS1* (AY294329), *E4* (S44898), *E8* (X13437), *PSY1* (EF157835), *ACO1* (NM_001247095), *ACO3* (Z54199), *ACS2* (AY326958), *ERF1* (AY077626), and *Pti4* (U89255).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *SlMADS1* expression in seedlings of RNAi lines and wild type.

Supplemental Figure S2. Other MADS-box gene expression in *SlMADS1*-silenced and wild-type fruits.

Supplemental Table S1. Details of other MADS-box gene primers for qPCR amplification.

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