A Tomato MADS-Box Transcription Factor, SlMADS1, Acts as a Negative Regulator of Fruit Ripening

Tingting Dong, Zongli Hu, Lei Deng, Yi Wang, Mingku Zhu, Jianling Zhang, and Guoping Chen*

Key Laboratory of Bioresourceal Science and Technology, Ministry of Education, Bioengineering College, Chongqing University, Chongqing 400044, People’s Republic of China

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, SIMADS1, was cloned and its tissue-specific expression profile was analyzed. The real-time polymerase chain reaction results showed that SIMADS1 was highly expressed in sepalas and fruits; its expression level was increased with the development of sepalas, while the transcript of SIMADS1 decreased significantly in accordance with fruit ripening. To further explore the function of SIMADS1, an RNA interference (RNAi) expression vector targeting SIMADS1 was constructed and transformed into tomato plants. Shorter ripening time of fruit was observed in SIMADS1-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. SIMADS1 RNAi fruits showed approximately 2- to 4-fold increases in ethylene production compared with the wild type. Furthermore, SIMADS1-silenced seedlings displayed shorter hypocotyls and were more sensitive to 1-aminocyclopropene-1-carboxylate than the wild type. Additionally, a yeast two-hybrid assay revealed a clear interaction between SIMADS1 and SIMADS-RIN. These results suggest that SIMADS1 plays an important role in fruit ripening as a repressive modulator.

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* Address correspondence to chenguoping@cqu.edu.cn.

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 SIACS2 RNA interference (RNAi) transgenic tomato fruits (Alexander and Grierson, 2002), and the expression level of SIACS2 is notably induced by exogenous ethylene (Olson et al., 1991; Lincoln et al., 1993; Barry et al., 1996, 2000). Furthermore, the expression of both SIACO1 and SIACO3 is significantly increased at the onset of tomato fruit ripening (Barry et al., 1996). Previous studies also indicate that RNAi inhibition of SIACO1 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).
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, SIMADS-RIN and SIMADS-MC. SIMADS-RIN regulates fruit ripening and SIMADS-MC is involved in sepal development (Vrebalov et al., 2002). Besides SIMADS-RIN and SIMADS-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 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 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., 2002).

Figure 1. Expression profile of SIMADS1 in tissues of cv Ailsa Craig and nonripening mutant fruits. A, Expression of SIMADS1 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 SIMADS1 in cv Ailsa Craig (AC++), Nr, and rin fruits. C, Expression of SIMADS1 in sepals of cv Ailsa Craig. BPS, Sepals of flowers before pollination; IPS, sepals of flowers in pollination; APS, sepals of flowers after pollination.
近期，SIERF6被报道为一种负调节因子（Chung et al., 2010）。最近，突变果实（属于番茄不同成熟度的果实）的根、茎、叶中转录组都被分析，以检测其成熟过程中的积累情况（SlMADS1基因属于MADS-box家族的SPELF6亚家族（Hileman et al., 2006）。之前的研究报道了SlMADS1基因（GenBank存取号AAY294329），该基因已被鉴定为番茄中成熟过程的调节因子。然而，到目前为止，还没有发现一种能抑制SlMADS1的抑制因子。在其他植物中，MADS-box家族的转录因子已被报道作为成熟过程中的正调节因子或直接调节成熟过程的果实。一般而言，一些抑制因子调节MADS-box家族的成员基因，但其积累会随着果实的发展而减少（Lee et al., 2012）。然而，到目前为止，还没有在番茄中功能分析过SlMADS1。在本研究中，RNAi沉默SlMADS1，在番茄中创建了拟南芥T-DNA的沉默株系（Supplemental Fig. S1）。其他MADS-box家族成员，包括两个SEP基因，SiMADS-RIN和SiMBP21，一个AGAMOUS基因，TAGL1，和一个FUL基因，TDR4，也被检测到了。TAGL1和SiMADS-RIN被上调调节，而TDR4没有在SiMADS1沉默果实中发生改变，其果实与野生型相比没有任何变化（Fig. 2A和Supplemental Fig. S1）。其他MADS-box家族成员中，SiMADS1（Leseberg et al., 2008）在SiMADS1沉默株系（Supplemental Fig. S2B）中没有被影响。这些结果表明，RNAi沉默SlMADS1是特定的，不针对其他MADS-box家族成员。随后，三个转基因株系，RNAi-03，RNAi-16，和RNAi-20，被选择进行进一步的研究。

RESULTS

SlMADS1 Transcripts Accumulate at High Levels in Sepals and Fruits

基于GenBank的序列，SlMADS1的全长cDNA被从cv Ailsa Craig的番茄中克隆。为了探索其组织特异性表达模式，实时PCR被用来分析SlMADS1在不同发育阶段的根、茎、叶和花中的转录水平。SlMADS1在不同发育阶段的果实中没有被检测到（Fig. 1A）。几乎所有的转录本都集中在根中（Fig. 1A）。在花器官中，Low level of SlMADS1在未成熟的绿叶中被检测到，成熟阶段的绿叶也观察到类似情况（Fig. 1A）。另外，SlMADS1基因的表达在未成熟的绿色和成熟绿色果实中被高表达，并且随着果实的成熟而快速下降（Fig. 1C），这表明SlMADS1可能在成熟过程中发挥作用。 Creazation of SlMADS1-Silenced Lines

为了进一步了解SlMADS1的功能，一个针对SlMADS1的RNAi表达载体被创建，并导入拟南芥的野生型株系中。三个transgenic lines，RNAi-03，RNAi-16，和RNAi-20，被选择进行进一步的研究。
**SIMADS1 Impacts Fruit Ripening**

During the process of fruit development, we measured the time from anthesis to ripening and observed that the color of *SIMADS1*-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 *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 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 *SIMADS1*-silenced lines (Fig. 4, A and C), and the transcripts of another ethylene biosynthesis gene, *ACO1*, was also increased significantly in *SIMADS1*-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 *SIMADS1*-silenced fruits at the breaker + 4 d stage (Fig. 4, D and E). 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 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 *SIMADS1* might play a role in the stress response.

**More Ethylene Is Produced by *SIMADS1*-Silenced Lines**

To further investigate the relationship between *SIMADS1* and ethylene, we measured ethylene production during fruit development and ripening. *SIMADS1* RNAi lines exhibited a rapid and massive increase in ethylene production at the breaker + 3 d, stage like the wild type, but *SIMADS1* 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 *SIMADS1* RNAi lines persisted in non-fruit tissues, an ethylene triple response assay was performed. Wild-type and *SIMADS1*-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).
To verify the triple response exhibited by silenced lines, the expression of SIMADS1 in RNAi and wild-type seedlings was detected. The result suggested that SIMADS1 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 SIMADS1-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 SIMADS1 could activate the expression of ethylene biosynthesis genes, while the transcripts of ACS2 were slightly increased in transgenic lines (Fig. 6D). The expression of SIMADS1 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 SIMADS1 might be impacted by ACC or ethylene.

The Yeast Two-Hybrid Assay Demonstrates That SIMADS1 Interacts with SIMADS-RIN

An essential regulator of tomato fruit ripening, SIMADS-RIN was preferentially selected for yeast two-hybrid assay. The open reading frame of SIMADS1 was
amplified and cloned into pGBK7 as the bait. Self-activation of pGBK7-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-β-d-galactopyranoside (X-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 5. Production of ethylene in control and SlMADS1-silenced lines.](image)

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 st. WT, Wild type.

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 st. WT, Wild type.

![Figure 6. Ethylene triple response assay.](image)

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 ± st. 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.]
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 transient 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, 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 tomato fruits before ripening.

Table II. Details of primers for qPCR amplification

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<tr>
<td></td>
<td>ATGGTGGAAGAATACACATCGG</td>
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</tr>
<tr>
<td>SIEF1a</td>
<td>ACCTTTGCGGATACCCCATG</td>
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<td>SIMADS1</td>
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<tr>
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the RNAi transgenic plants than the wild type. These results suggest that *SIMADS1* 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 SIMADS-RIN, TDR4 (TM4, FULL), 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 SIMADS1-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 SIMADS1-silenced fruits ripen in advance (Fig. 2B; Table I). These results suggest that suppressing the expression of SIMADS1 promotes the expression of ripening-related genes and accelerates the rate of ripening, indicating that SIMADS1 acts as an inhibitor in fruit ripening.

**SIMADS1 Might Weaken the Activity of SIMADS-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). SIMADS-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 SIMADS-RIN (Ito et al., 2008; Martel et al., 2011; Fujisawa et al., 2012). ACOT is influenced by SIMADS-RIN through the homeobox gene *HB1*, which interacts with the promoter of *ACOT* (Lin et al., 2008; Martel et al., 2011). *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, *ACOT*, *ACS2*, and *E8* are up-regulated markedly in SIMADS1-silenced lines, which suggests 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 influencing the expression of ethylene biosynthesis and response genes such as *ACOT*, *ACS2*, and *E8*, and then reduce the biosynthesis of ethylene and inhibit fruit ripening.

In summary, SIMADS1 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 SIMADS-RIN. Although higher levels of a developmental regulatory cascade of this gene remain to be discovered, as a repressive regulator, SIMADS1 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’ AC1), 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.

**SIMADS1 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 tagged oligo(dT)_{18} primer (5′-GCTGTCACAGATACGATACGCACTAGTTT-3′). One to 2 μl of cDNA was used to clone the full-length SIMADS1 gene with primers of SIMADS1-F (5′-ATGGGAAAGAAAAGTGGTTTGGTTTTT-3′) and dT-R (5′-GCTGTCACAGATACGCACTAGTTT-3′) and dT-R (5′-GCTGTCACAGATACGCACTAGTTT-3′) through high-fidelity PCR (Prime START HS DNA polymerase; Takara). The amplified products were tagged by using the DNA A-Tailing kit (Takara) and linked with pMD18-T vector (Takara). The generated binary plasmid was translated into the plant binary vector pBIN19 with the double-stranded RNA expression unit, containing the cauliflower mosaic virus 35S promoter, SIMADS1 fragment in the sense orientation, and an RNA interference (RNAi) element. The plasmid was then transformed into the binary vector pBIN19 with the RNAi element. The generated binary plasmid was transformed into *Agrobacterium tumefaciens* strain LBA4404 and *A. tumefaciens*-mediated transformation was performed following the protocols described by Chen et al. (2004). The transgenic plants were detected with primers NPTIII-R (5′-GAACTACGGCTGCTCTGTA-3′) and NPTII-R (5′-AACCTCGAGCATGATTC-3′). The positive transgenic plants were selected and used for subsequent experiments.
Quantitative Real-Time PCR Analysis

Total RNA of tissues of cv Ailsa Craig, Nr, nr, 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 replicates for each sample were used, and standard curves were run simultaneously. Tomato SICAC (Exposito-Rodríguez et al., 2008) and SIEFA (Exposito-Rodríguez et al., 2008) were used as internal standards. The primers SIMADS1(RT)-F and SIMADS1(RT)-R (Table II) were used to determine the expression levels of SIMADS1 in the wild type, Nr and nt, and transgenic lines. Furthermore, the expression of other levels of MADS-box genes, including SIMADS-RIN (Vrebalov et al., 2002), SIMPR2 (Lesbegue et al., 2008), TAGLI (Busi et al., 2003; Vrebalov et al., 2009), and TD48 (TM4, FULL1; Seymour et al., 2002; Beren et al., 2012), as well as fruit ripening-related, carotenoid biosynthesis, and ethylene biosynthesis and response genes, such as E4 (Lincoln et al., 1987; Perturbuia 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 10-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 (μg 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 temperature 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 0.5, 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 ACS5A, ACS2, ACS6, and ACO1 in the wild type and transgenic lines was 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

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 SIMADS1 was amplified by PCR with the primer pair SIMADS1(Y)-F (′-CCCGAATTCTGAGGAGGCAGGCTTACACCGG-3′) and SIMADS1(Y)-R (′-CCGGGATTCTTAAAGCATCCATGTTTATT-3′). The product was cloned into the pGBKT7 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-Gal to judge whether SIMADS1 can interact with SIMADS-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 SIMADS1 (AY294329), E4 (S44898), E8 (X13437), P5Y1 (EF157835), ACO1 (NM_001247095), ACO3 (Z54199), ACS2 (AY326998), ERF1 (AY077626), and Pti4 (U89255).

Supplemental Data

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

Supplemental Figure S1. SIMADS1 expression in seedlings of RNAi lines and wild type.

Supplemental Figure S2. Other MADS-box gene expression in SIMADS1-silenced and wild-type fruits.

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

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