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Overexpression of a novel MADS-box gene *SIFYFL* delays senescence, fruit ripening and abscission in tomato

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MADS-domain proteins are important transcription factors involved in many biological processes of plants. In our study, a tomato MADS-box gene, *SIFYFL*, was isolated. *SIFYFL* is expressed in all tissues of tomato and significantly higher in mature leaf, fruit of different stages, AZ (abscission zone) and sepal. Delayed leaf senescence and fruit ripening, increased storability and longer sepals were observed in *35S:FYFL* tomato. The accumulation of carotenoid was reduced, and ethylene content, ethylene biosynthetic and responsive genes were down-regulated in *35S:FYFL* fruits. Abscission zone (AZ) did not form normally and abscission zone development related genes were declined in AZs of *35S:FYFL* plants. Yeast two-hybrid assay revealed that *SIFYFL* protein could interact with *SIMADS-RIN*, *SIMADS1* and *SIJOINTLESS*, respectively. These results suggest that overexpression of *SIFYFL* regulate fruit ripening and development of AZ via interactions with the ripening and abscission zone-related MADS box proteins.

Ethylene plays important roles in many aspects of plant growth and development, including the processes of leaf senescence, fruit ripening, abscission, other programmed senescence and defense signalling. In many species exogenous ethylene can promote processes that are characteristic of leaf senescence. In these studies, chlorophyll content was used as a marker of leaf senescence¹.

Tomato (*Solanum lycopersicum*) is the primary model for climacteric fruit ripening for a combination of scientific and agricultural reasons. Its fruit plays an important role in the human diet and provides health benefits as a source of vitamins, minerals, and antioxidants (phenolics, folate, lycopene, and β -carotene)².

Fruit ripening represents a summation of physiological and biochemical processes of fleshy fruits including de-greening and accumulation of colored pigments for attraction, textural changes associated with cell wall metabolism and cell turgor variation leading to softening, and metabolic changes related to flavor and nutrient composition, generally associated with accumulation of sugars, acids and volatiles culminating in a diverse array of tastes and smells varying among species. These changes not only make fruit assisting in seed dispersal, but also provide essential nutrition for human and animal diets³.

Ripening of climacteric fruits is characterized by an autocatalytic increase in respiration and ethylene biosynthesis just prior to the initiation of ripening. Ethylene biosynthesis occurs via a pathway: the first dedicated step is the conversion of *s*-adenosyl-*l*-methionine (SAM) to aminocyclopropane-1-carboxylic acid (ACC), by the normally ratelimiting enzyme ACC synthase (ACS)⁴. ACC is subsequently converted by ACC oxidase (ACO), the so-called ethylene forming enzyme, to ethylene⁵.

Besides ethylene synthesis, the ability of ethylene perception and response are necessary for fruit ripening. The expression of *E4* in fruit is rapidly induced following exogenous ethylene induction⁶. Meanwhile, the transcripts of *E4* in fruit are suppressed through ethylene biosynthesis inhibition⁷. *E8* is a tomato ripening-associated and fruit-specific expression gene⁸. *PG*, transcriptionally activated during fruit ripening, is a major cell wall polyuronide degrading enzyme, catalyzes the depolymerization of pectins⁹. Unraveling the regulation of these gene activities is important to understand the processes of ripening, senescence, abscission, and response to stress¹⁰.

To date, 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. They are useful in understanding of the transcriptional control system underlying tomato ripening. The *rin* mutant displays inhibited fruit ripening and enlarged sepals. This mutant phenotype has been attributed to functions of two MADS-box transcriptional factors, *SIMADS-RIN* and *SIMADS-MC*. *SIMADS-RIN* regulates fruit ripening and *SIMADS-MC* involves in sepal development and formation of abscission zones^{11,12}. MADS-box proteins have been found playing different and



important biological roles in tomato, such as the regulation of inflorescence and fruit ripening¹³. *TDR4* (*FUL1*) and *SLMBP7* (*FUL2*) both of which have high sequence similarity to *Arabidopsis FRUITFULL* have been demonstrated to act in fruit ripening via forming MADS-box transcription factor complexes with RIN^{14,15}. The expression of *FUL1* is up-regulated during ripening^{15,16}. *TM6* transcripts mainly accumulate in the carpel primordial and young fruits in tomato and have been considered to be involved in fruit ripening^{17,18}. RNAi suppression of *TAG1* in tomato leads to misshapen fruits and homeotic conversion of stamens into petaloid organs¹⁸. The antisense suppression of *TAGL1* results in ripening inhibition and pericarp thickness reduction¹⁹. Interestingly, all of those MADS-box proteins play as positive regulators of ripening.

Recently, we found that a tomato MADS-box transcription factor, *SIMADS1* acted as a negative regulator of fruit ripening and interacted with *SIMADS-RIN*²⁰. An *APETALA2* transcription factor (*SIAP2a*), belonging to the AP2/ERF (Ethylene Response Element) family and encoding putative transcription factors²¹, was identified through transcriptional profiling of fruit maturation. RNAi repression of *SIAP2a* results in fruits that over-produce ethylene, ripen early and modify carotenoid accumulation profiles by altering carotenoid pathway flux²². These suggest that *SIMADS1* and *SIAP2a* function as modulators of ripening and act to balance the activities of positive ripening regulators.

Therefore, there are positive and negative regulation factors to balance fruit ripening process. While only one negative regulator of fruit ripening has been reported in MADS-box family^{20,23}. Although previous researches have done a lot of contributions to tomato fruit ripening, the developmental mechanisms undoubtedly need further study.

Abscission in plants is a crucial process used to shed organs such as leaves, flowers, and fruits when they are senescent, damaged, or mature. Abscission occurs at predetermined positions called abscission zones (AZs), which have several layers of small, densely cytoplasmic cells at the junction of organ and the main body of plant^{24,25}. Control of abscission in fruit and grain crops is a key agricultural concern. For example, during cereal crop domestication, mutants that reduce seed shattering have been preferentially selected, because shattering is a major limiting factor for yield²⁶. Defective AZs in fruit pedicel facilitates large-scale harvesting of tomato by saving time removing the calices, because when the jointless fruit is harvested, the calyx remains attached to the plant, not to the fruit²⁷.

In tomato, *JOINTLESS* encodes a short vegetative phase group MADS-box transcription factor, and its mutant phenotype is called *jointless*, which is characterized by defective AZs in fruit pedicels^{25,27}. The mutant *lateral suppressor* (*ls*) suppress the development of pedicel AZs²⁸. *SLMADS-MC* previously identified as a sepal size regulator, physically interacts with *JOINTLESS* to regulate the development of tomato pedicel AZs^{12,25}. Transcriptome analyses of pedicels at the preabscission stage reveal that *MC* and *JOINTLESS* regulate the expression of *LeWUS*, *Bl*, *GOB* and *Ls*, which are homologs of *WUSCHEL*, *REGULATOR OF AXILLARY MERISTEMS*, *CUP-SHAPED COTYLEDON*, and *LATERAL SUPPRESSOR* in *Arabidopsis*, respectively, and these transcription factors play key roles in pedicel AZ development¹². In addition, ethylene-induced abscission is correlated with an increase in poly-galacturonase (PG) and endo- β -1, 4-D-glucanase (cellulase) activity in tomato^{29,30}. *TAPG1* and cell wall hydrolase *Cel2* are also required for abscission at the AZ³⁰. Although the regulation of fruit abscission is essential for agriculture, the developmental mechanisms remain unclear.

Here, we describe a novel tomato MADS-box transcription factor *SIFYFL* (GenBank No. KF709444), an ortholog of *Arabidopsis FYF* (*FOREVER YOUNG FLOWER*), AtAGL42, which acts as a repressor controlling floral organ senescence and abscission³¹. In this study, *35S:FYFL* plants were generated to investigate the exact role of *SIFYFL* in tomato, and the results revealed that overexpression of

SIFYFL can delay fruit ripening, leaf and sepal senescence and the development of fruit pedicel AZs.

Results

***SIFYFL* isolation and expression pattern analyse.** Based on the sequence in GenBank (accession No.KF709444), full-length cDNA of *SIFYFL* was cloned with specific primers SIFYFL-F and SIFYFL-R (Table 1S) from tomato (*Solanum lycopersicon* Mill. cv. Ailsa Craig) and sequenced. Phylogenetic and amino acid homology analysis showed that *SIFYFL* was highly homologous to AtFYF (AtAGL42) (Fig. 1 A) and belonged to a very conservative MADS-box transcription factor family (Fig. 1 B). Quantitative real-time PCR technology was performed to analyze the expression of *SIFYFL*. The results showed that the expression level of *SIFYFL* was high in mature leave, AZ, sepal and immature green fruit than other tissues, and its expression decreased slightly after the onset of ripening (Fig. 1 C). During the development of fruit abscission zones in tomato, the *SIFYFL* expression increased gradually (Fig. 1 D). In flower sepals, the *SIFYFL* mRNA was highly accumulated in young sepals and increased to the highest level in mature sepals, then decreased slightly in senescence sepals (Fig. 1 E), which expression pattern was similar to that in leaves (Fig. 1 C). These results indicate that *SIFYFL* may play an important role in fruit ripening and development process of AZ, leaf and sepal.

***35S:FYFL* plant delayed leaf and sepal senescence.** Mature leaves were detached and incubated on wet filter paper under air condition. Five days later, the detached leaves from wild type exhibited senescence, while that from *35S:FYFL* plant remained green. The detached leaves from the wild type and transgenic plants both became yellowing after 5 days of ethylene treatment, but wild type leaves exhibited more yellowing than transgenic lines (Fig. 2 A). The chlorophyll contents of wild type leaves were dramatically reduced after 5 days of treatment with air or ethylene, whereas that of transgenic plant had no significant difference (Fig. 2 B). In addition, 120 days after colonization, the wild type leaves next to the first inflorescence were completely yellow, while only edge of transgenic leaves exhibited senescence (Fig. 2 C, D). We also observed that sepal senescence was delayed in transgenic plants. At B + 14 stage, wild-type sepals have become senescent, while transgenic sepals were still green (Fig. 2 E). The chlorophyll content of wild-type sepal decreased dramatically at B + 14 stage, while that in transgenic lines still remained at high level (Fig. 2 F).

Ethylene biosynthetic genes were expressed at low levels in the leaves and sepals of transgenic plant. As ethylene plays an important role in senescence, we examined the expression of ethylene biosynthesis genes, such as *ACO1*, *ACO3*, *ACS1A*, *ACS2* and *ACS6* in leaf and sepal of wild-type and transgenic plants. The results showed that these five ethylene biosynthesis genes were down-regulated significantly in leaf of transgenic plant (Fig. 3 A). In sepal of transgenic plant, except *ACO3*, expression level of other four genes were all decreased by 50–80% (Fig. 3 B). These results suggest that reduced expression of ethylene biosynthesis genes might affect the ethylene biosynthesis, thus delay the senescence of transgenic leaves and sepals.

***35S:FYFL* fruit ripening was delayed.** During the process of fruit development, we measured the time from pollination to ripening. We observed that the color of *35S:FYFL* fruits changed later than the wild type (Fig. 2 E), and its ripening time was delayed 3 to 5 days (Table 2S). It was reported that the dramatic change of pigmentation in ripening tomato fruits was caused by accumulation of carotenoids³². Thus, the carotenoids in transgenic and wild type fruits at B (the colour change from green to yellow), B + 7 (7 days after B) and B + 14 (14 days after B) stage were extracted and determined. As shown in Fig. 2 G, the accumulation of carotenoid

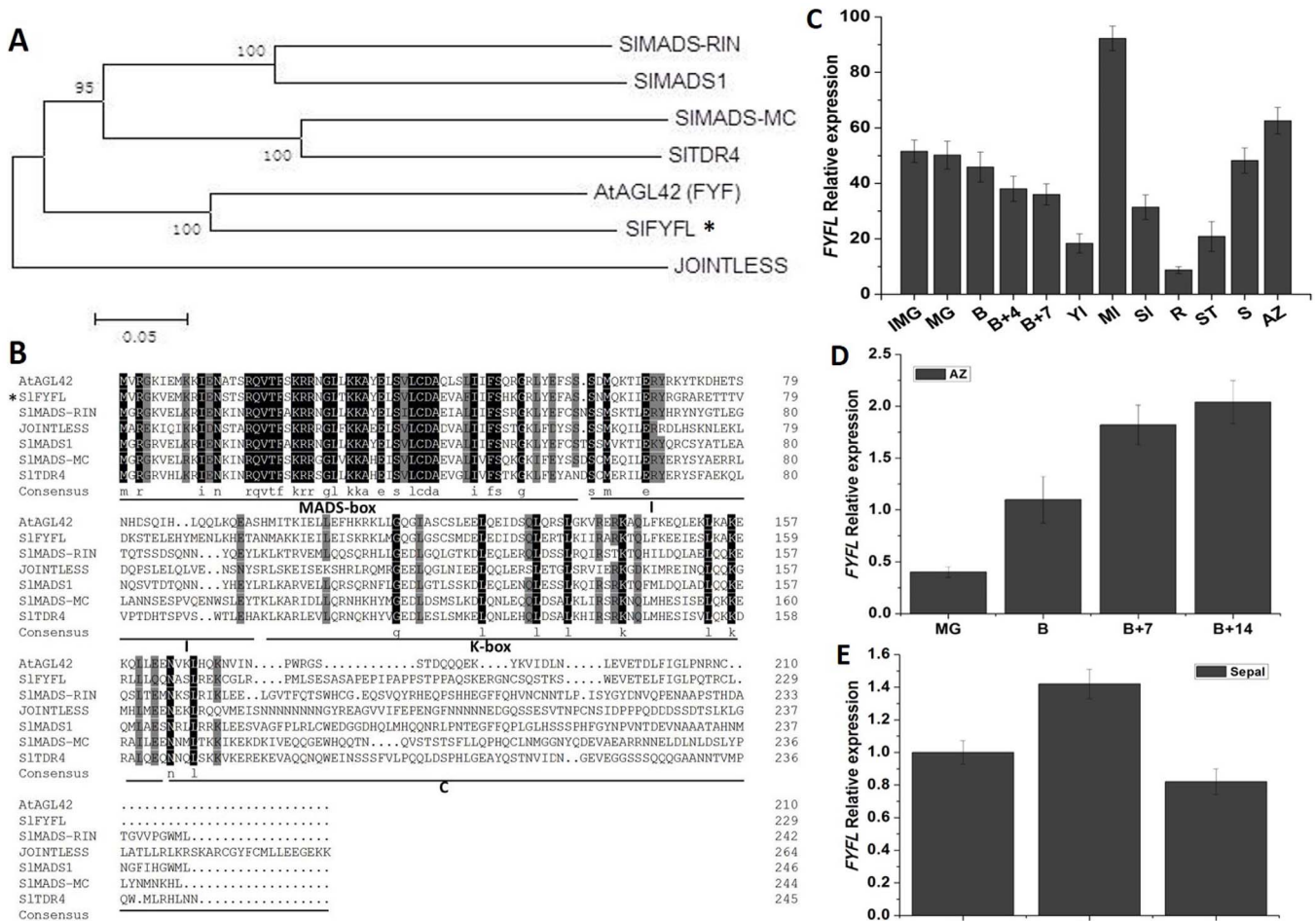


Figure 1 | *SIFYFL* sequence and expression analysis in AC (WT). (A) Phylogenetic analysis of *FYFL* and other MADS-Box proteins was constructed by the neighbor-joining method, bootstrap analysis of 1000 replicates. *FYFL* is marked with asterisk. Accession numbers for the proteins listed are as follows: SIMADS-MC (NP_001234665), SITDR4 (FUL1) (NM_001247244), SIMADS-RIN (NM_001247741.1), SIMADS1 (NP_001234380), AtAGL42 (AY141213), SIFYFL (KF709444), JOINTLESS (AAG09811). (B) Multiple sequence alignment of *FYFL* and other MADS-Box proteins. SIFYFL is marked with asterisk. Identical amino acids are shaded in black, and similar amino acids are shaded in gray. (C) The relative expression patterns of *FYFL* in AC. IMG, immature green fruit; MG, mature green fruit; B, breaker fruit; B + 4, 4 days after breaker fruit; B + 7, 7 days after breaker fruit; YL, young leaf; ML, mature leaf; SL, senescent leaf; R, root; ST, stem; S, sepal of flower in anthesis; AZ, abscission zone of flower in anthesis. The relative expression of *FYFL* in different stages AZ (D) and different stages sepal (E) of AC. MG, mature green fruit; B, breaker fruit; B + 4, 4 days after breaker fruit; B + 7, 7 days after breaker fruit; B + 14, 14 days after breaker fruit. Ys, young sepal; Ms, mature sepal; Ss, senescent sepal. The data represent mean from three replicates with three biological repeats. Error bars indicate SE.

in transgenic lines was down-regulated by 30–40% than wild type. Real-time PCR analysis results revealed that expression level of *PSY1* (Phytone synthase1), *PDS* (phytone desaturase) and *ZDS* (ζ -carotene desaturase) were down-regulated by 35–50% in transgenic lines compared with wild type at the stages of fruit ripening (Fig. 2 H, I and G). These results indicate that overexpression of *SIFYFL* gene affects the fruit ripening of tomato.

Ethylene-related and ripening-related genes were significantly down-regulated in 35S:*FYFL* fruits. To further characterize the molecular regulation mechanism of *SIFYFL* in fruit ripening, a set of ethylene-related and ripening-related genes in wide type and transgenic tomato fruits were examined. Three ethylene biosynthesis genes, *ACS2*, *ACO1* and *ACO3*, and two ripening-related genes, *E4* and *E8*, which responded specifically to ethylene, were down-regulated to different degrees in 35S:*FYFL* fruits at B, B + 4, B + 7 and B + 14 stages (Fig. 3 C–G). *SIMADS-RIN* mRNA level was 30–75% lower in 35S:*FYFL* fruits than that in wild type (Fig. 3 H). Expression of *PG* was decreased by 20–60% in 35S:*FYFL* fruits at B, B + 4 and B + 7 stages (Fig. 3 I). These results indicated that

overexpression of *SIFYFL* might inhibit fruit ripening by impacting ethylene biosynthesis or ethylene response. Additionally, ethylene-responsive factor *ERF1* associated with defense responses, was also down-regulated in transgenic fruits at all stages (Fig. 3 J), suggesting that *SIFYFL* might play a role in stress response.

Ethylene production was reduced significantly in 35S:*FYFL* fruit. To further investigate the relationship between *SIFYFL* and ethylene, we measured the ethylene production during the fruit ripening process. 35S:*FYFL* fruit exhibited a climacteric rise with the peak of ethylene production occurring at day 3 after ripening was initiated and declined at day 7 when fruits entered the senescence stage as the wild type did, but 35S:*FYFL* fruit produced only about half level of the ethylene as wild type did during fruit ripening (Fig. 3 K).

The storability of 35S:*FYFL* fruits were improved. Fruits of wild-type and transgenic lines were harvested at B + 7 stage and stored under the same conditions. Fourteen days after harvested, wild type tomatoes began to soften, darken, yet transgenic fruits remained hardness and lighter in color. Thirty-two days after harvested,

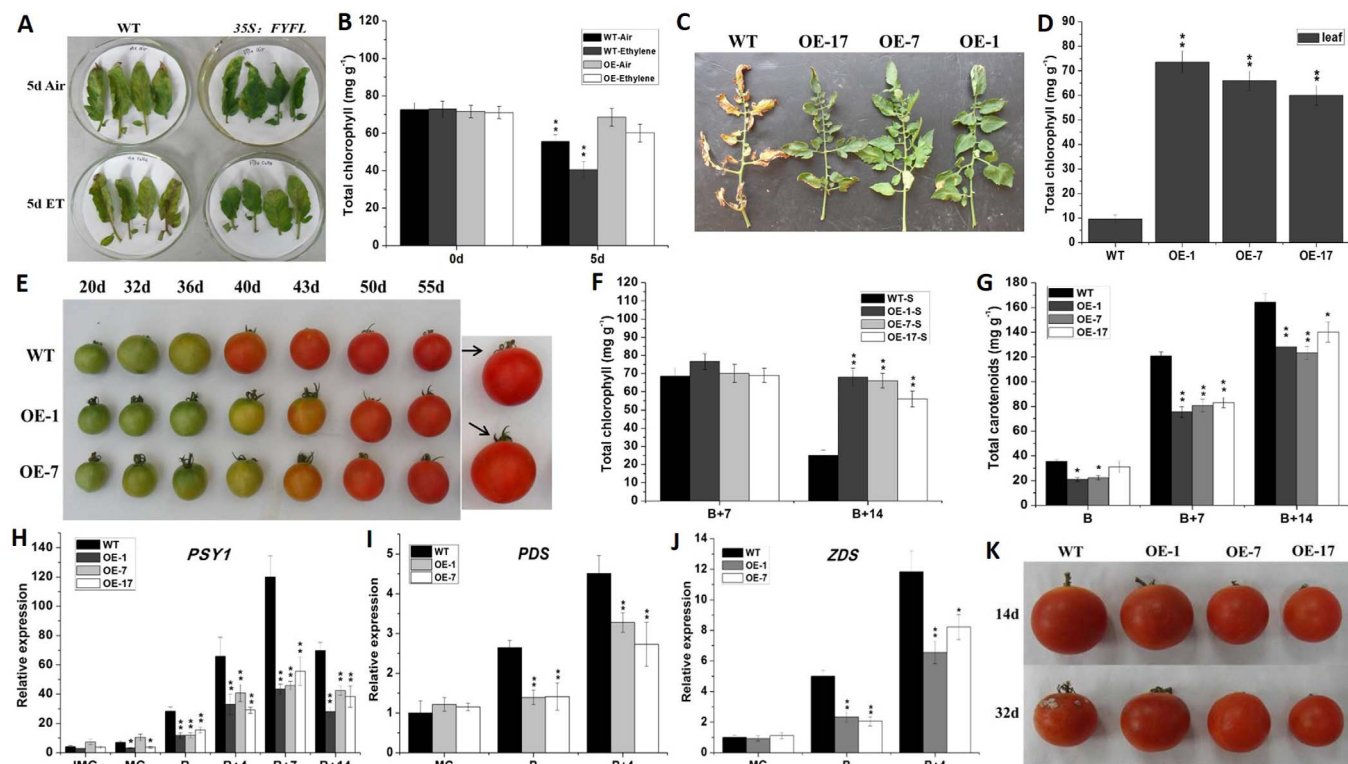


Figure 2 | Phenotype and physiological indices, and related genes expression of wild-type and *35S:FYFL* lines. (A) Leaf phenotype of wild type and transgenic lines after 5 day treatment of ethylene (ET) and the air (Air). (B) Total chlorophyll content of leaves of wild type and transgenic lines before and after 5 day treatment of ethylene (ET) and the air (Air). Leaf phenotype (C) and total chlorophyll content of leaves (D) of wild type and transgenic lines of 120 days old. The leaves next to the first inflorescence. (E) Fruits phenotype of wild-type and *35S:FYFL* lines. 20 d–55 d, statistical time starting from the pollination. *35S:FYFL* lines displayed 3–5 days delayed fruit ripening and longer and younger sepals than wild type. (F) Total chlorophyll content of different stages sepal of wild-type and transgenic lines. (G) Total carotenoid content of wild-type and *35S:FYFL* lines. (H) to (J) respectively represent expression analysis of *PSY1*, *PDS*, *ZDS* in different stages fruits of wild-type and transgenic lines. (K) Fruits storability phenotype of wild-type and transgenic lines. 14 d and 32 d, post-harvest storage time. WT, wild type; OE-1, OE-7, OE-17, different transgenic lines. IMG, immature green fruit; MG, mature green fruit; B, breaker fruit; B + 4, 4 days after breaker fruit; B + 7, 7 days after breaker fruit; B + 14, 14 days after breaker fruit. The data represent mean from three replicates with three biological repeats. *, indicate $P < 0.05$, **, indicate $P < 0.01$ between the wild type and others by *t*-test. Error bars indicate SE.

wild-type tomatoes were soft, dehydrated and moldy, while transgenic tomatoes just began to soften (Fig. 2 K).

***SIFYFL* affected the development of tomato fruit stalk abscission zone.**

In *35S:FYFL* lines, another striking phenotype is that the formation of fruit stalk abscission zone (AZs) is obviously suppressed, even non-AZ-forming is observed in B + 14 fruit stalk of transgenic lines (Fig. 4 A). To clearly observe the development of fruit stalk AZs, microscopy was used to examine the morphological changes in the wild type and *35S:FYFL* AZs. At B + 7 stage, AZ cells were observed obviously at the longitudinal section plane of fruit stalk AZs (edge and central) in wild-type plant, while a smooth pedicel without obvious AZ cells was observed at the center of *35S:FYFL* plant AZs. At B + 14 stage, the AZ cells of *35S:FYFL* plants began to be observed obviously, and even non-AZ-forming was detected in some transgenic lines (Fig. 4 B). To further explore the differences in abscission between the wild type and *35S:FYFL* plants, a breakstrength meter (Fig. 2 S) was used to quantitatively measure the force needed to break fruit stalk from the AZs. As shown in Fig. 4 C, the breakstrength of the *35S:FYFL* pedicel were significantly larger than wild type at B + 7 and B + 14 stages, and the breakstrength of *35S:FYFL* pedicel at B + 14 stage were approximately equal to that of wild type at B + 7 stage (Fig. 4 C), suggesting that the timing of AZ cell separation should be delayed in the *35S:FYFL* fruit stalk AZs. In addition, a set of AZ-related genes, such as *J*, *MC*, *TAPG1*, *Cel2*, *WUS*, *Bl*, *GOB* and *Ls*, were examined in

fruit stalk abscission zone. These genes in transgenic tomato AZs were all significantly down-regulated (Fig. 5 A–H), implying that *SIFYFL* might inhibit fruit AZs development by impacting cell wall modification and degradation at fruit AZ. The ethylene biosynthesis genes, *ACO1*, *ACO3*, *ACS1A*, *ACS2* and *ACS6*, were also detected in B + 7 and B + 14 AZs of wild-type and transgenic fruits. The results showed that expression of these five ethylene biosynthesis genes were decreased by 30–80% in transgenic AZs (Fig. 5 I–M), indicating that the ethylene biosynthesis was affected, which might be one of the reasons of delaying the formation of AZs in transgenic lines.

Yeast two-hybrid assay demonstrated *SIFYFL* interacted with *SIMADS-RIN*, *SIMADS1* and *SIJOINTLESS*, respectively.

To further explore the relationship between *SIFYFL* with other MADS-box proteins, two essential ripening-related regulators of *SIMADS-RIN* and *SIMADS1* and AZs development related protein *SIJOINTLESS* were preferentially selected for yeast two-hybrid assay. The open reading frame of *SIFYFL* was amplified and cloned into pGBKT7 as the bait. Self-activation of *FYFL*-pGBKT7 was tested, and the result was minus. The open reading frame of *SIMADS-RIN*, *SIMADS1* and *SIJOINTLESS* were amplified and cloned into pGADT7 as the prey, respectively. An empty prey and bait vector were used as negative controls with each bait and prey construct, respectively. Fig. 6 showed that the yeast grew on selective media and turned blue on X- α -gal indicator plate, suggesting that there exist

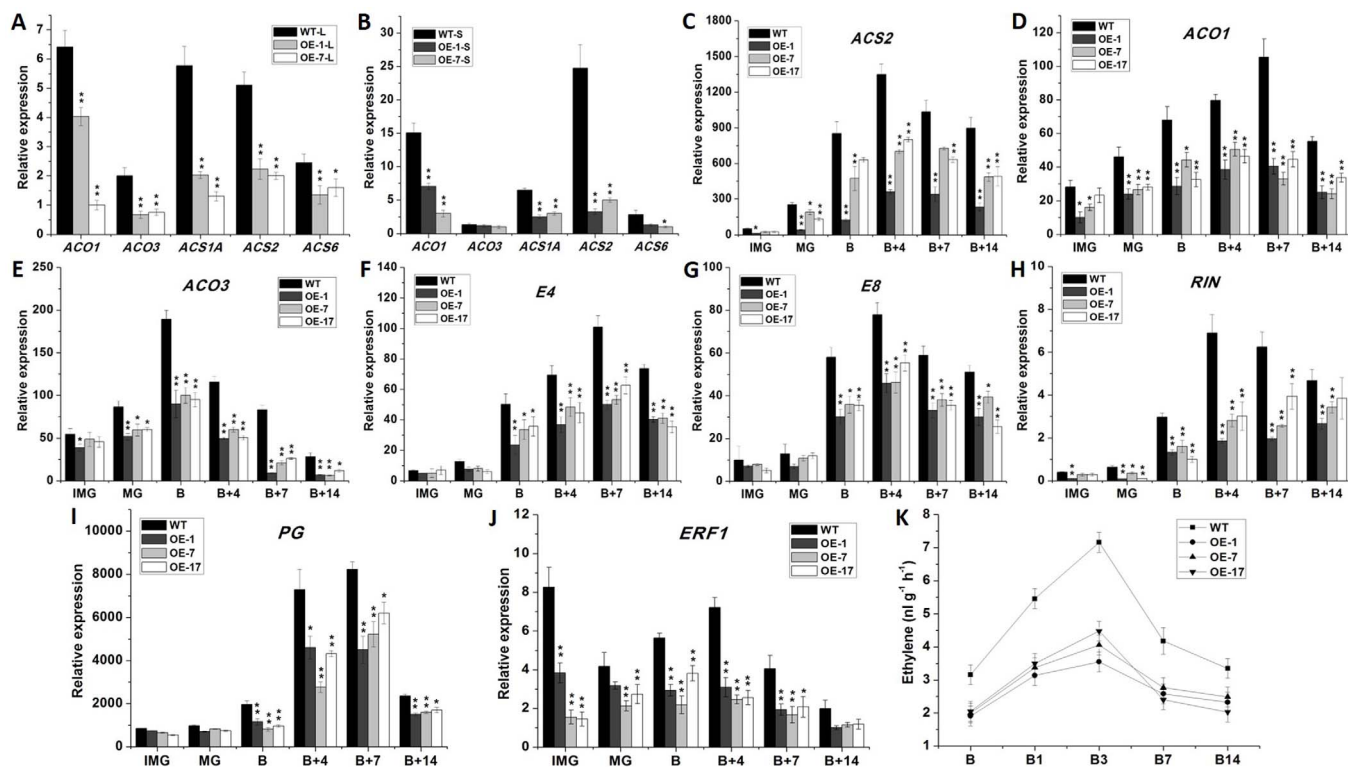


Figure 3 | Ethylene synthesis genes expression in 120 days old leaves, next to the first inflorescence, and in B + 14 stage sepals of wild-type and transgenic lines, Ethylene synthesis and Fruit ripening-related genes expression, and Ethylene content of different stages fruits of wild-type and transgenic lines. (A) Ethylene biosynthesis genes expression in 120 days old leaves, next to the first inflorescence of wild-type and transgenic lines. (B) Ethylene biosynthesis genes expression in B + 14 stage sepals of wild-type and transgenic lines. (C) to (J) respectively represent expression analysis of *ACS2*, *ACO1*, *ACO3*, *E4*, *E8*, *RIN*, *PG* and *ERF1* in different stages fruits of wild-type and transgenic lines. (K) Ethylene content in different stages fruits of wild type and transgenic lines. WT, wild type; OE-1, OE-7, OE-17, different transgenic lines. IMG, immature green fruit; MG, mature green fruit; B, breaker fruit; B + 1, 1 days after breaker fruit; B + 3, 3 days after breaker fruit; B + 4, 4 days after breaker fruit; B + 7, 7 days after breaker fruit; B + 14, 14 days after breaker fruit. The data represent mean from three replicates with three biological repeats. *, indicate $P < 0.05$, **, indicate $P < 0.01$ between the wild type and others by *t*-test. Error bars indicate SE.

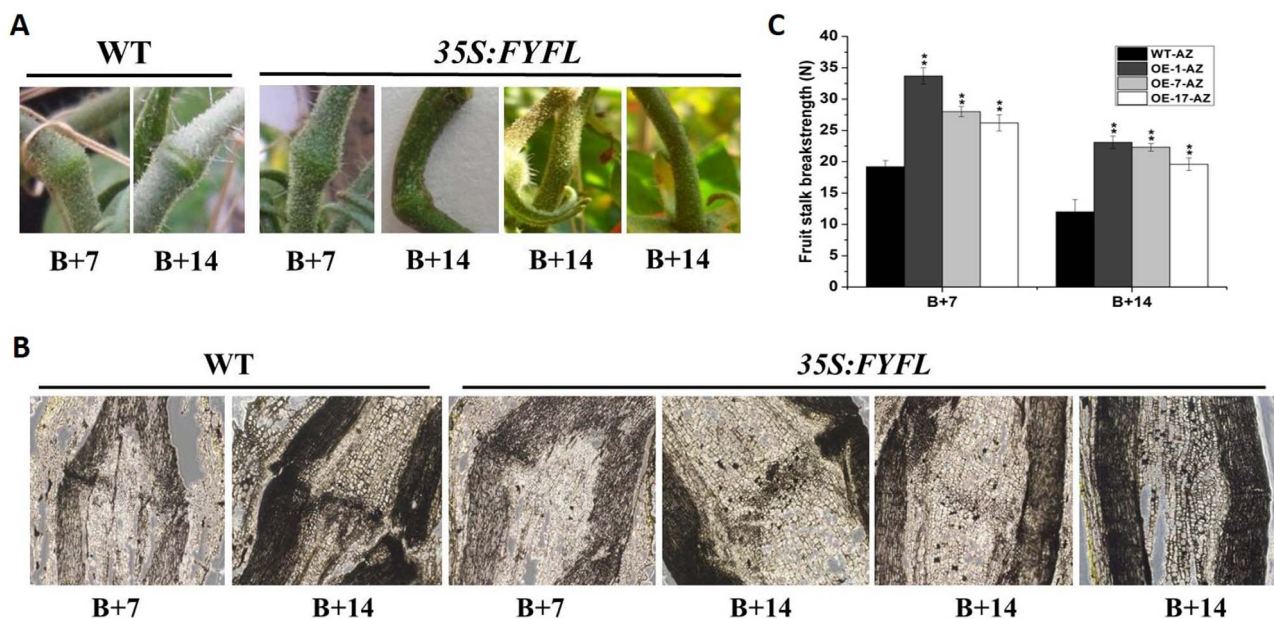


Figure 4 | Phenotype and breakstrength of AZs of wild-type and *35S:FYFL* lines. (A) AZs phenotype of wild-type and *35S:FYFL* lines. (B) AZs microscopic observation phenotype of wild type and *35S:FYFL* lines. (C) Breakstrength of different stages fruit stalk AZs of wild-type and *35S:FYFL* lines. WT, wild type; OE-1, OE-7, OE-17, different transgenic lines. B + 7, 7 days after breaker fruit; B + 14, 14 days after breaker fruit. The data represent mean from three replicates with three biological repeats. *, indicate $P < 0.05$, **, indicate $P < 0.01$ between the wild type and others by *t*-test. Error bars indicate SE.

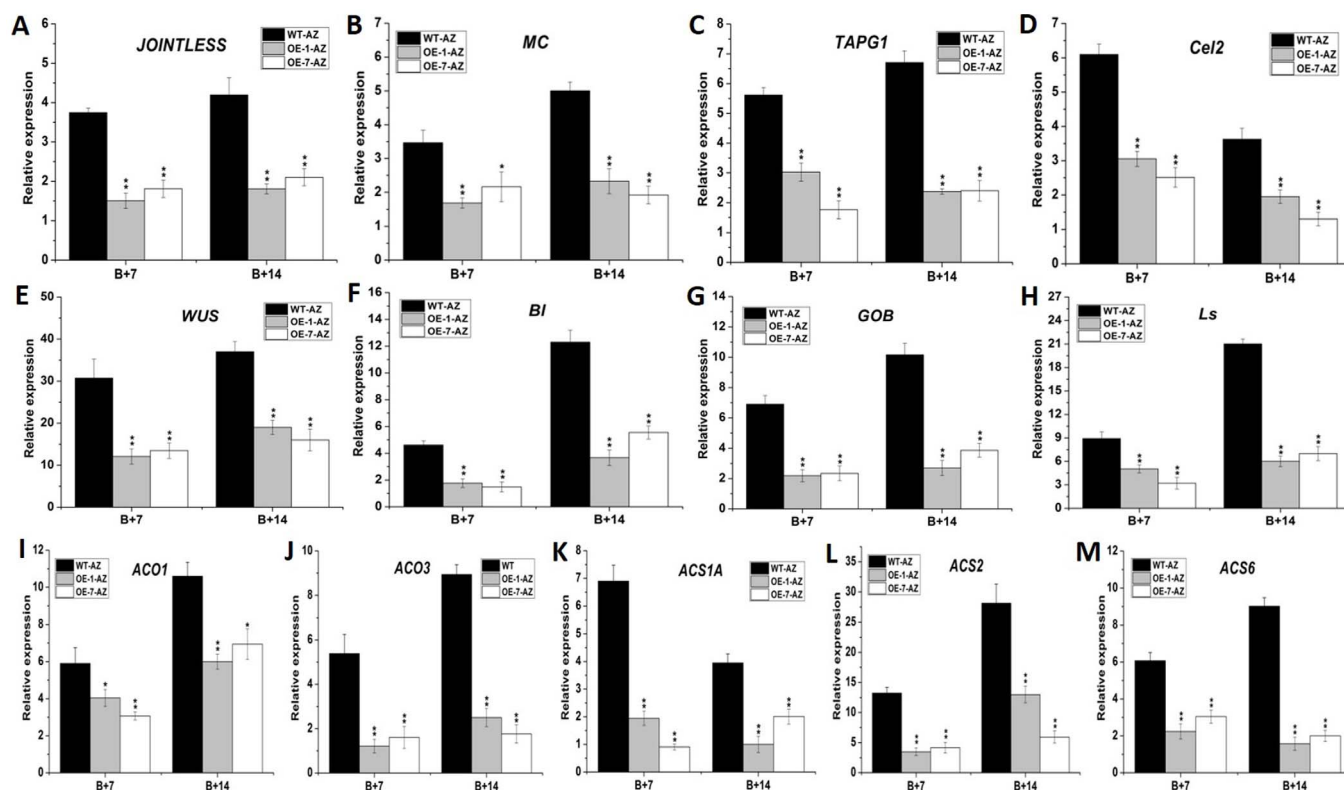


Figure 5 | Relative expression of abscission zone formation-related genes and ethylene biosynthetic genes of wild-type and *35S:FYFL* lines. (A) to (M) respectively represent expression analysis of *JOINTLESS*, *MC*, *TAPG1*, *Cel2*, *WUS*, *Bl*, *GOB*, *Ls*, *ACO1*, *ACO3*, *ACS1A*, *ACS2* and *ACS6* in different stages AZs of wild type and transgenic lines. WT, wild type; OE-1, OE-7, different transgenic lines. B + 7, 7 days after breaker fruit; B + 14, 14 days after breaker fruit. The data represent mean from three replicates with three biological repeats. *, indicate $P < 0.05$, **, indicate $P < 0.01$ between the wild type and others by *t*-test. Error bars indicate SE.

interaction between *SIFYFL* and *SIMADS-RIN*, *SJJOINTLESS* and *SIMADS1* in vivo respectively.

Discussion

Overexpression of *SIFYFL* delays sepal and plant senescence by inhibiting the biosynthesis of ethylene. Senescence is the final stage of the development of organism, which is a process with a series of recessions. Gradual loss of chlorophyll is the most obvious characteristic of plant senescence. Plant hormones are closely associated with senescence and ethylene is a typical senescence promoting hormone³³. In our study, wild-type plants and fruit sepals turn yellow earlier than transgenic lines (Fig. 2C, E). Comparing the total chlorophyll content of sepal of wild type and transgenic fruits at B + 14 stage, chlorophyll content in wild-type are significantly reduced, while that in transgenic lines still remain at high level (Fig. 2 F). Expression levels of ethylene biosynthesis genes *ACS1A*, *ACS2*, *ACS6* and *ACO1*, are decreased in leaf and sepal of transgenic plants compared with wild-type (Fig. 3 A, B). In addition, detached leaf senescence experiment results demonstrate that senescence of transgenic leaf is considerably delayed after 5 days of treatment with air or ethylene (Fig. 2A, B). These results suggest that overexpression of *SIFYFL* may decrease the ethylene biosynthesis, thereby delay the senescence of tomato plant. *SIFYFL* impacts sepal development. To date, five classes of MADS-box genes (A, B, C, D and E) determine the identities of floral organ^{34,35}. In the ABCDE model, sepal structures are specified by genes of A-class. So far, a number of MADS-box genes including *SIMADS-MC* and *TAGL1* were reported to influence sepal development. *TAGL1* overexpression induces swelling and ripening of sepals^{19,36}. The mutant *rin* displays enlarged sepals because of missing of *SIMADS-MC*¹¹. In addition, *SIMADS-MC* has been reported to be the homolog of *API*, which is a A-class

gene of *Arabidopsis*¹¹. In our study, the *35S:FYFL* lines represented longer sepals (Fig. S3 A–C), and *SIMADS-MC* was down-regulated in sepals of *35S:FYFL* lines (Fig. S3 D). These results indicated that *SIFYFL* might be a member of A-class gene and regulate the development of sepal. *SIFYFL* overexpression inhibits ethylene biosynthesis and fruit ripening. In plants, ethylene biosynthesis pathway is well studied. Two modes of ethylene synthesis, system 1 and system 2, have been defined³⁷. System 1 contributes to providing basal ethylene in vegetative tissues and unripe fruits. System 2 produces a large

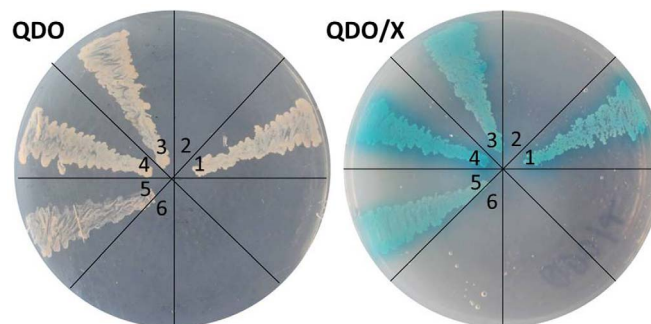


Figure 6 | Yeast Two-hybrid Assay for *SIFYFL* & *SIMADS-RIN*, *SIFYFL* & *SIMADS1* and *SIFYFL* & *SJJOINTLESS* Proteins. QDO, SD medium without Trp, Leu, His and Ade; QDO/X, QDO medium with X- α -Gal. 1.pGBKT7-53 & pGADT7-T (positive control); 2.pGBKT7-Lam & pGADT7-T (negative control); 3.pGBKT7-FYFL & pGADT7-RIN; 4.pGBKT7-FYFL & pGADT7- MADS1; 5.pGBKT7-FYFL & pGADT7-JOINTLESS; 6.Blank. Empty bait vector, empty prey vector and autoactivation assay were growth of none yeast.



amount of ethylene at the onset of fruit ripening³⁸. Transcriptional regulation of ACS is one of the major control points of ethylene biosynthesis³⁹. *SIACS2* is an important factor to transit System 1 to System 2⁴⁰. Antisense suppression of tomato *ACS2* prevents ripening in a manner recoverable with exogenous ethylene^{5,41}. *SIACS1A* and *SIACS6* are involved in system 1 and present in tomato fruits before the onset of ripening⁴⁰. In addition, *SIACO1* and *SIACO3* have been reported to contribute to triggering fruit ripening⁵. *SIACO3* is induced but transitory at the breaker stage while *SIACO1* expression is sustained during ripening³⁹. In this study, we tested the expression of *ACS2*, *ACO1* and *ACO3* in *35S:FYFL* fruits. The results showed that expression levels of these ACC synthase genes in *35S:FYFL* lines were 30–60% lower than in wild type (Fig. 3 C, D and E), and half ethylene was produced in the transgenic fruits (Fig. 3 K). These results suggest that *SIFYFL* overexpression impacts ethylene biosynthesis in fruit. *E4* and *E8* are well known to be important ethylene response factors impacting fruit ripening⁴². Our study showed that both of the two genes expressed at lower levels in the transgenic fruits compared with wild type (Fig. 3 F and G). In addition, *PSY1*, a major regulator of metabolic flux toward downstream carotenoids, induced by ethylene during fruit ripening⁴³, and other two carotenoid biosynthesis enzymes *PDS* and *ZDS*, downstream of *PSY1*, were notably decreased and carotenoid contents were 20–45% lower in transgenic fruits (Fig. 2 G–J). Fig. 2 E also showed the ripening of *35S:FYFL* fruits were delayed. These results suggest that overexpression of *SIFYFL* inhibits tomato fruit ripening. *SIFYFL* overexpression downregulates the expression of *SIMADS-RIN* and might affect its activity. Recently, hetero- or homo-dimers or higher-order complexes have been detected in MADS-domain proteins⁴⁴. SLMADS-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 responsiveness. As previously reported, *ACS2* is bound by *SIMADS-RIN*^{16,45}. *ACO1* is indirectly influenced by *SIMADS-RIN* through a homeobox gene *HBI*^{16,46}. *E8* is identified as a novel direct target of *SIMADS-RIN*, which can be rapidly induced following ethylene induction and during normal fruit ripening^{16,47}. In our study, *ACO1*, *ACS2*, *E8* and *SIMADS-RIN* are all down-regulated by 30–80% in *35S:FYFL* lines, which suggest that these genes are negatively regulated by *SIFYFL* (Fig. 3 C, D, G and H). Moreover, the yeast two-hybrid assay indicates that there is an interaction between *SIFYFL* and *SIMADS-RIN* (Fig. 6), implying that *SIFYFL* might bind to *SIMADS-RIN* and affect its activity. Furthermore, *SIMADS1* was reported as a negative regulator of fruit ripening and was interacted with *SIMADS-RIN*²⁰. In our study, *SIFYFL* interacts with *SIMADS1* in vivo (Fig. 6). Therefore, we suspect that *SIFYFL* and *SIMADS1* may form heterodimers with *SIMADS-RIN* respectively or together to regulate the expression of ripening related genes, thereby affecting the fruit ripening (Fig. 7). *SIFYFL* overexpression increases fruits storability. Tomato fruits become senescent and soft quickly after ripening. Therefore, tomato storability is an extremely important quality trait. There are many indicators to measure the storability of tomato fruits, such as fruit color, fruit firmness and pericarp thickness, etc. Ethylene and poly-galacturonic acid enzyme (PG) are two key regulatory factors in the process of tomato fruit ripening⁴⁸. In our study, ethylene production was reduced and the transcript level of PG was decreased by 30–60% in *35S:FYFL* fruits (Fig. 3 I, K), and *35S:FYFL* fruits displayed slower rotting and dehydration than wild type (Fig. 2 K). These results indicate that overexpression of *SIFYFL* improves the storability of tomato fruit, and could be used as a molecular tool to improve fruit storability through modulating the expression of *RIN*. *SIFYFL* overexpression inhibits abscission zone development. Abscission is a key agricultural concern and an important trait for tomato to commercial products. The mutant of *JOINTLESS* has defective AZs in fruit pedicels²⁵, which phenotype facilitates large-scale harvesting of tomato fruit by saving time removing the calices²⁷. Another mutant *lateral suppressor (ls)*,

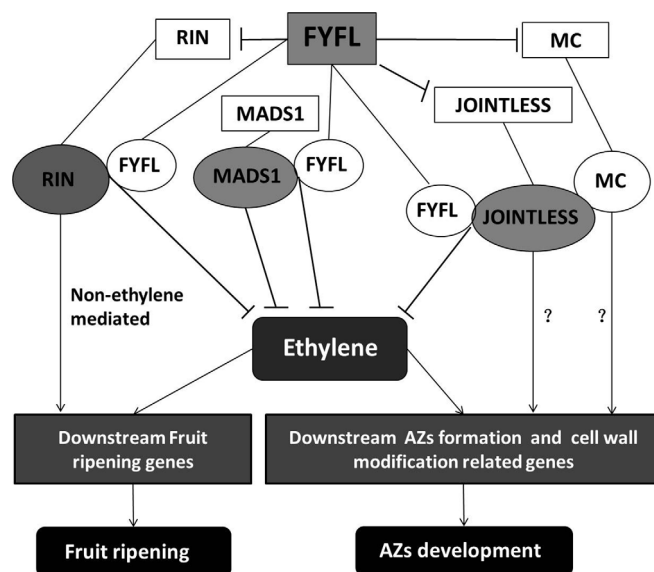


Figure 7 | Patterns exist in tomato fruit ripening and abscission zone formation network. Connection between different regulatory factors based on expression data analysis and yeast two-hybrid test in this study. The overexpression of *FYFL* inhibits the expression of ripening-related regulatory factor *RIN* and abscission zone regulatory factors *JOINTLESS* and *MC*. Previous studies have shown *SIMADS1* was an ethylene biosynthesis inhibitory factor, thereby inhibiting fruit ripening; *RIN* positively regulated fruit ripening both by ethylene and non-ethylene mediated pathways. There were interactions in *FYFL* & *MADS1* and *FYFL* & *RIN*. *FYFL* may regulate the activity of *MADS1* and *RIN*, then control the ethylene and downstream genes, and achieve the purpose of regulating ripening. *JOINTLESS* and *MC* were needed in formation of abscission zone of tomato. There was interaction between *FYFL* & *JOINTLESS*, moreover, it was reported that there was interaction between *JOINTLESS* and *MC*. *FYFL* may regulate the activity of *JOINTLESS* and *MC*, then control the ethylene and downstream genes, subsequently regulate the development of abscission zone. Whether *JOINTLESS* and *MC* can directly control the downstream genes or not, it is still not clear.

encoding a GRAS family transcription factor⁴⁹, suppress the development of pedicel AZs²⁸. Tomato fruit ripening mutant *ripening-inhibitor (rin)* develops incomplete pedicel AZs structures that show a knuckle region on the pedicels similar to wild-type plants, but frequently they show insufficient fruit abscission¹². The *rin* mutation is a deletion that affects two tandemly arranged genes, *RIN* and *MC*. *RIN* controls fruit ripening and *MC* regulates sepal size, inflorescence determinacy and the development of tomato pedicel AZs^{11,12}. In our study, the expression of *JOINTLESS*, *Ls* and *MC* genes in the B + 7 and B + 14 fruit stalk AZs were all down-regulated by 30–82% in transgenic plants (Fig. 5 A, B and H). Transcriptome analyses revealed that *LeWUS*, *Bl* and *GOB* all expressed specifically in pedicel AZs, and these transcription factors might play an important role in abscission processes, such as the regulation of pedicel AZ development, the maintenance of the AZ cells in an undifferentiated state, or the acquisition of competence to respond to abscission signals^{50–52}. Mutation in *Bl* affects the jointless phenotype²⁸, supporting the possibility that *Bl* acts in AZ development. Thus, the expression of *LeWUS*, *Bl* and *GOB* genes in B + 7 and B + 14 fruit stalk AZs were examined and were all reduced by over 50% in transgenic plants (Fig. 5 E, F and G). Previous studies indicate that the expression of many genes encoding cell wall modification-related proteins, such as *Cel2* (endo-1,4-glucanase), polygalacturonase (PG) are drastically up-regulated at the onset of abscission⁵³. Our results showed that *Cel2* and *TAPG1* in the B + 7 and B + 14 fruit stalk AZs were



down-regulated in transgenic plants (Fig. 5 C, D). Meanwhile, ethylene is required to increase the expression of these cell wall modification genes with the onset of abscission⁵⁴. Expression levels of ethylene biosynthesis genes *ACO1*, *ACO3*, *ACS1A*, *ACS2* and *ACS6* were also reduced in AZs of transgenic plants (Fig. 5 I–M). These results indicate that *SIFYFL* overexpression may inhibit the expression of AZs related genes and the ethylene biosynthesis, thereby result in a developmental delay in AZs. This was proved in breakstrength test, breakstrength of B + 7 stage AZ in wild-type was approximately equal to that of B + 14 stage AZ in transgenic lines (Fig. 4 C). In addition, yeast two-hybrid assay results intimate that *SIFYFL* interacts with *SIJOINTLESS* in vivo (Fig. 6). It was reported that *MADS-MC* protein interacted physically with *JOINTLESS*, and regulated fruit abscission⁵⁵. So we also suspect that *FYFL*, *JOINTLESS* and *MC* might form a complex to control the development of abscission zone (Fig. 7). Moreover, yeast two-hybrid assay results also display that there exist interactions between *SIFYFL* and *SIMADS-RIN* and *SIMADS1* respectively, implying that *SIFYFL* might bind to *SIMADS-RIN* and *SIMADS1* to regulate their activity, subsequently inhibit the expression of cell wall modification-related and ethylene biosynthesis genes, ultimately affect the formation of abscission zone.

Methods

Plant materials and growth conditions. In this study, *Solanum lycopersicon* Mill. cv. Ailsa Craig (AC), a near-isogenic tomato line, was used as the wild type. The plants were planted in greenhouse and managed routinely. Transgenic cultures grew under standard greenhouse conditions. The ripening stages of tomato fruits were divided according to days after pollination (dpa) and fruit colour. In wild type, IMG (Immature green) fruits were defined as 20 dpa. MG (Mature green) fruits were defined as 32 dpa and were characterized as being green and shiny with no obvious colour change. B (Breaker) fruits were defined as the colour change from green to yellow. After breaker the fruit stages were divided B + 4 (4 days after B), B + 7 (7 days after B), B + 14, and so on. All plant samples were immediately frozen with liquid nitrogen and stored at -80°C until further use.

Isolation of *SIFYFL* and sequence analysis. Total RNA of tomato was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Then 1 μg total RNA was used to synthesis first strand cDNA through reverse transcription polymerase chain reaction (M-MLV reverse transcriptase, Takara) with Oligo d(T)₁₈ primer. 1–2 μl cDNA was used to clone the full length of *SIFYFL* gene with primers *SIFYFL-F* and *SIFYFL-R* (Table 1S) through high fidelity PCR (Prime STARTTM HS DNA polymerase, Takara). The amplified products were tailed by using DNA - Tailing kit (Takara) and linked with pMD18-T vector (Takara). Positive clones were picked out via *Escherichia coli* JM109 transformation and confirmed by sequencing (BGI, China). Multiple sequence alignments were performed by DNAMAN version 5.2.2. The phylogenetic tree was calculated by MEGA (Molecular Evolutionary Genetics Analysis) version 3.1⁵⁶.

Construction of 35S:*FYFL* vector and plant transformation. Above-mentioned *FYFL*-pMD18-T vector was used as the template and was amplified with primers *SIFYFL (F + X)* and *SIFYFL (R + S)* which have been tailed with *Xba* I and *Sac* I restriction site at the 5' end respectively. Then the amplified products were digested with *Xba* I and *Sac* I respectively, and linked into the plant binary vector pBI121 with *Sac* I and *Xba* I restriction sites. The transgene was under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The generated binary vectors were transferred into *Agrobacterium* LBA4404 and *Agrobacterium*-mediated transformation was performed following the protocols described by Chen et al⁵⁷. The transgenic plants were detected with primers NPTII-F (5' GAC AAT CGG CTG CTC TGA 3') and NPTII-R (5' AAC TCC AGC ATG AGA TCC 3'). The positive transgenic plants were selected and used for subsequent experiments.

Quantitative real-time PCR analysis. RNA extraction and cDNA synthesis were performed as the above described. The synthesized cDNAs were diluted 1 times in RNase/DNase-free water. Quantitative real-time PCR analysis was carried out using the CFX96TM Real-Time System (C1000TM Thermal Cycler, Bio-Rad). All reactions were performed using the SYBR[®] Premix Go Taq II kit (Promega, China) in a 10 μl total sample volume (5.0 μl 2 \times SYBR Premix Go Taq, 0.5 μl primers, 1.0 μl cDNA, 3.5 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. Three replications for each sample were used and standard curves were run simultaneously. Melt curve analysis of qPCR samples revealed that there was only one product for each gene primer reaction. The PCR products were sequenced to confirm the specific amplification. *SICAC*⁵⁸ gene was used as internal standard in tomato tissues. The primers *SIFYFL(RT)-F* and *SIFYFL(RT)-R* (Table S1) were used to determine the expression level of *SIFYFL* in wild type and transgenic lines.

Furthermore, the expression levels of fruit ripening and ethylene biosynthesis pathway genes *E4*, *E8*, *PG*, *PSY1*, *PDS*, *ZDS*, *RIN*, *ACO1*, *ACO3*, *ACS2* and *ERF1* were determined in fruits. Above-mentioned abscission zone related genes, *JOINTLESS*, *MC*, *WUS*, *GOB*, *Ls*, *Bl*, *Cel2*, *TAPG1* were detected in abscission zone. Ethylene biosynthesis genes *ACO1*, *ACO3*, *ACS1A*, *ACS2* and *ACS6* were detected in AZ, leaf and sepal. Primers were shown in Table 1S.

Measurement of carotenoid contents. 1.0 g sample was cut from pericarp in a 5 mm wide strip around the equator of B, B + 7 and B + 14 of wild type and 35S:*FYFL* lines, respectively. Then 10 ml of 60 : 40 (v/v) hexane-acetone was added respectively and total carotenoids 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*(OD450)*10 ml/1 g^{43,59}. Three independent experiments were performed for each sample.

Ethylene measurements. Fruits at B, B + 1, B + 3, B + 7 and B + 14 stages were harvested and placed in open 100 mL jars for 2 h to minimize the effect of wound ethylene caused by picking. Jars were then sealed and incubated at room temperature for 2 h, and 0.5 mL of headspace gas was injected into a Focus GS gas chromatograph (Thermo-Electron) equipped with a flame ionization detector. Samples were compared with reagent grade ethylene standards of known concentration and normalized for fruit weight.

Observation of abscission zone in fruit pedicels. In our experiments, we found that formation of fruit stalk abscission zone in transgenic plants were different from wild type (Fig. 4 A). Therefore, we carried out observation and analysis. The optical microscope (Olympus-BMF) was used to observe fresh abscission zone in fruit pedicel materials at B + 7 and B + 14 stages of transgenic plants and wild type. Repeat twice for each observation.

Breakstrength analysis of abscission zone in fruit pedicels. Every single fruit stalk of wild type and transgenic lines was clipped with a small clamp which was attached to a breakstrength meter. Then the breakstrength of fruit stalk abscission zone was measured using the BOSE ELECTROFORCE 3300 breakstrength meter. The measured value represents the force necessary to break the fruit stalk from the abscission zone. Fruit stalk materials were taken from B + 7 and B + 14 stages fruits, and three measurements were performed at each stage for each plant.

Postharvest storage test. Fruits of wild type and transgenic lines were harvested at B + 7 stage, and placed on filter paper in standard greenhouse conditions. Phenotype was observed once every two days.

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 *SIFYFL* was amplified by PCR with the primer pairs *SIFYFL(Y)-F* and *SIFYFL(Y)-R* (Table 1S). The PCR products were digested using *Eco*RI and *Pst* I and cloned into the *Eco*RI and *Pst* I sites of the pGBKT7 bait vector to obtain the vector *FYFL-pGBKT7*. Then *FYFL-pGBKT7* vector was transferred 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 *FYFL-pGBKT7*. In parallel, the open reading frame of *SIRIN*, *SIJOINTLESS* and *SIMADS1* were also amplified by primers (*SIRIN(Y)-F*, *SIRIN(Y)-R*), (*SIJ(Y)-F*, *SIJ(Y)-R*) and (*SIMADS1(Y)-F*, *SIMADS1(Y)-R*) (Table 1S). The products were cloned into the pGADT7 vector, and introduced into Y187. Subsequently, Y2HGOLD with bait and Y187 with prey were cultured together in 2 \times YPDA medium for 24 h. 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 *SIFYFL* can interact with *SIRIN*, *SIJOINTLESS* and *SIMADS1* or not, respectively. Plates were incubated for 3 to 7 days at 30 $^{\circ}\text{C}$. 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.

Detached leaf senescence experiment. Mature leaves were cut from 2 month 35S:*FYFL* and wild-type plants and placed on three layers of wet filter paper in 14 cm Petri dishes, then wrapped in aluminium foil, incubated in the dark at 25 $^{\circ}\text{C}$ for 5 d. Some dishes were placed in a glass desiccator with 20 ml⁻¹ ethylene. The others were incubated under air condition.

Extraction and quantitation of leaf and sepal chlorophyll. Weighted 1 g fresh sepals of B + 7 and B + 14 fruits of wild-type and transgenic lines, pounded to pieces with liquid nitrogen, extracted with 10 ml mixed solution acetone and ethanol (2 : 1, V/V) for 48 h in dark, centrifuged 5000 rpm for 10 min at 4 $^{\circ}\text{C}$. The absorbance of the supernatant was measured at 645 and 663 nm in a PerkinElmer Lambda 900 UV/VIS/NIR spectrophotometer using above-mentioned mixed solution as a blank. Total chlorophyll content were calculated using the formulas according to the method of Arnon⁶⁰: Chl (mg/g) = 20.29A645 + 8.02A663. The chlorophyll of each sample was extracted and measured in triplicate. Chlorophyll contents of mature leaves, treated by ethylene/air were measured using the same method.



Statistics of sepal length of flower. In our study, we found that transgenic sepals were longer than the wild type. We measured the length of sepals at the fully open flower stage, at least 10 flowers per plant were measured (Fig. 3S C).

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Author contributions

G.C. and Z.H. designed and managed the research work and improved the manuscript. Q.X., Z.Z., T.D., Z.Z. and B.C. performed the experiments. Q.X. and T.D. wrote the manuscript.

Additional information

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