Overexpression of a novel MADS-box gene SlFYFL 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, SlFYFL, was isolated. SlFYFL 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 SlFYFL protein could interact with SlMADS-RIN, SlMADS1 and SlJOINTLESS, respectively. These results suggest that overexpression of SlFYFL 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 ethylene.

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 induction5. Meanwhile, the transcripts of E4 in fruit are suppressed through ethylene biosynthesis inhibition6. E8 is a tomato ripening-associated and fruit-specific expression gene. PG, transcriptionally activated during fruit ripening, is a major cell wall polyuronide degrading enzyme, catalyzes the depolymerization of pectins. Unraveling the regulation of these gene activities is important to understand the processes of ripening, senescence, abscission, and response to stress7.

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 zones8,9. MADS-box proteins have been found playing different and
important biological roles in tomato, such as the regulation of inflorescence and fruit ripening. TDR4 (FULI) and SLMBP7 (FUL2) both of which have high sequence similarity to Arabidopsis FRUITFULL. Studies have demonstrated that TDR4 acts in context of forming MADS-box transcription factor complexes with RIN15,16. The expression of FUL2 is up-regulated during ripening. 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 homoeotic conversion of stamens into petaloid organs.19. The antisense suppression of TAG1 results in ripening inhibition and pericarp thickness reduction.20. 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-RIN16. An APETALA2 transcription factor (SIAP2α), belonging to the AP2/ERF (Ethylene Response Element) family and encoding putative transcription factors, was identified through transcriptional profiling of fruit maturation. RNAi repression of SIAP2α results in fruits that over-produce ethylene, ripen early and modify carotenoid accumulation profiles by altering carotenoid pathway flux.21. These suggest that SIMADS1 and SIAP2α 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,20,21. 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.22,23. 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 yield.24. 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 fruit.25.

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.26,27. The mutant lateral suppressor (ls) suppresses 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.22,23. Transcriptional 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 development.22. 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. TAGP1 and cell wall hydrolase Cel2 are also required for abscission at the AZ.27. 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. This is a gene of which have high sequence similarity to Arabidopsis FYF/FOVER YOUNG FLOWER, AtAGL42, which acts as a repressor controlling floral organ senescence and abscission. In this study, SIFYFL 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 analysis. 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 lycopersicum Mill. cv. Alisa 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 sepal, the SIFYFL mRNA was highly accumulated in young sepal and increased to the highest level in mature sepal, then decreased slightly in senescence sepal (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:FIFYL 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:FIFYL 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 colonisation, 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 sepal have become senescent, while transgenic sepal 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 sepal 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 sepal.

35S:FIFYL 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:FIFYL 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 carotenoids. 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
in transgenic lines was down-regulated by 30–40% than wild type. Real-time PCR analysis results revealed that expression level of ***PSY1*** (Phytone synthease1), ***PDS*** (phytone desaturase) and ***ZDS*** (β-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 ***SlFYFL*** 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 ***SlFYFL*** in fruit ripening, a set of ethylene-related and ripening-related genes in wild 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 ***SlFYFL*** 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 ***SlFYFL*** might play a role in stress response.

**Ethylene production was reduced significantly in 35S:FYFL fruit.** To further investigate the relationship between ***SlFYFL*** 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, the storability of ***35S:FYFL*** fruits were improved.

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Figure 1 | ***SlFYFL*** 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), ***SlFYFL***(KF709444), JOINTLESS (AAO9811). (B) Multiple sequence alignment of FYFL and other MADS-Box proteins. ***SlFYFL*** 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.
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 plants. A smooth pedicel without obvious AZ cells was observed at the center of fruit stalk AZs in wild-type plant, while a smooth pedicel without obvious AZ cells was observed at the center of transgenic AZs (Fig. 4 A). To further explore the relationship between SIFYFL and AZs, we performed a yeast two-hybrid assay to determine whether SIFYFL interacts with key regulators of fruit ripening and ABZ formation. Our results showed that SIFYFL interacts with SlMADS-RIN, SlMADS1 and SlJOINTLESS, respectively.

**Yeast two-hybrid assay demonstrated SIFYFL interacted with SIMADS-RIN, SIMADS1 and SJ0INTLESS, 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 SJ0INTLESS were preferentially selected for yeast two-hybrid assay. The open reading frame of SIFYFL was amplified and cloned into pGBK7 as the bait. Self-activation of FYFL-pGBK7 was tested, and the result was minus. The open reading frame of SIMADS-RIN, SIMADS1 and SJ0INTLESS 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-a-gal indicator plate, suggesting that there exist interactions between SIFYFL and SlMADS-RIN, SlMADS1 and SlJOINTLESS.
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.
interaction between SlFYFL and SlMADS-RIN, SlJOINTLESS and SlMADS1 in vivo respectively.

**Discussion**

Overexpression of SlFYFL 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 B14 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 SlFYFL may decrease the ethylene biosynthesis, thereby delay the senescence of tomato plant. SlFYFL impacts sepal development. To date, five classes of MADS-box genes (A, B, C, D and E) determine the identities of floral organ34,35. In the ABCDE model, sepal structures are specified by genes of A-class. So far, a number of MADS-box genes including SlMADS-MC and TAGL1 were reported to influence sepal development. TAGL1 overexpression induces swelling and ripening of sepals36,37. The mutant rin displays enlarged sepals because of missing of SlMADS-MC35. In addition, SlMADS-MC has been reported to be the homolog of AP1, which is a A-class gene of Arabidopsis11. In our study, the 35S:FYFL lines represented longer sepals (Fig. S3 A–C), and SlMADS-MC was down-regulated in sepals of 35S:FYFL lines (Fig. S3 D). These results indicated that SlFYFL might be a member of A-class gene and regulate the development of sepal. SlFYFL 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 defined38. 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\textsuperscript{58}. Transcriptional regulation of ACS is one of the major control points of ethylene biosynthesis\textsuperscript{40}. ACS2 is an important factor to transit System 1 to System 2\textsuperscript{2}. Antisense suppression of tomato ACS2 prevents ripening in a manner recoverable with exogenous ethylene\textsuperscript{34,44}. SIACT1 and SIACT6 are involved in system 1 and present in tomato fruits before the onset of ripening\textsuperscript{50}. In addition, SIACT1 and SIACT3 have been reported to contribute to triggering fruit ripening. SIACT3 is induced but transitory at the breaker stage while SIACT1 expression is sustained during ripening\textsuperscript{52}. 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. Ethylene and E8 are well known to be important ethylene response factors impacting fruit ripening\textsuperscript{52}. 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 ripening\textsuperscript{3}, 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 proteins\textsuperscript{44}. 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\textsuperscript{44,55}. ACO1 is indirectly influenced by SIMADS-RIN through a homeobox gene HBI\textsuperscript{47,54}. E8 is identified as a novel direct target of SIMADS-RIN, which can be rapidly induced following ethylene induction and during normal fruit ripening\textsuperscript{46,47}. 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\textsuperscript{50,56}. 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 ripening\textsuperscript{48}. 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 pedicels\textsuperscript{28}, which phenotype facilitates large-scale harvesting of tomato fruit by saving time removing the calices\textsuperscript{27}. Another mutant lateral suppressor (ls), encoding a GRAS family transcription factor\textsuperscript{29}, suppress the development of pedicel AZs\textsuperscript{28}. 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 abscission\textsuperscript{12}. 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\textsuperscript{11,12}. In our study, the expression of JOINTLESS, Ls and MC genes in 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\textsuperscript{50–52}. Mutation in Bl affects the jointless phenotype\textsuperscript{28}, 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 abscission\textsuperscript{52}. Our results showed that Cel2 and TAPG1 in the B + 7 and B + 14 fruit stalk AZs were

![Figure 7 | Patterns exist in tomato fruit ripening and abscission zone formation network.](image-url)
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 abscission. Expression levels of ethylene biosynthetic genes ACO1, ACO3, ACS1A, ACS2 and ASC6 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 break strength test, break strength 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 addition, yeast two-hybrid assay results indicate that SlFYFL interacts with 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 lycopersicum 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 that the 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 synthesize first strand cDNA through reverse transcription polymerase chain reaction (M-MLV reverse transcriptase, Takara) with oligo d(T)15 primer. 1.2 μM 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 START™ HS DNA polymerase, Takara). The amplified products were tailied 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 3.1.

Construction of 3SS:SIFYFL 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 + 5) which have been tailied with XbaI and SacI restriction site at the 5′ end respectively. Then the amplified products were digested with XbaI and SacI respectively, and linked into the binary vector binary pBI121 with SacI and XbaI 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 al.30. The transgenic plants were detected with primers NPTII-F (5′ GAC AAT CCG GTC TTC TGA 3′) and NPTII-R (5′ AAC TCC AGC AGT 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 CFX96™ Real-Time System (C1000™ Thermal Cycler, Bio-Rad). All reactions were performed using the SYBR® Premix Ex Taq™ (Takara) with Go Taq™ (Promega, China) at 10 μl total sample volume (5.0 μl 2× SYBR Premix Ex Taq, 0.5 μl primers, 1.0 μl cDNA, 3.5 μl dH₂O). 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. Fruit tissue analysis of PCR samples revealed that there was only one product for each gene primer reaction. The PCR products were sequenced to confirm the specific amplification. SICA2™ 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, P531, PDS, ZDS, RIN, ACO1, ACO3, ACS2 and ERF1 were determined in fruits. Above-mentioned abscission zone related genes, JOINTLESS, MC, WUS, GOB, Ls, Bl, Cc2, TAPG1 were detected in abscission zone. Ethylene biosynthesis genes ACO1, ACO3, ACO5, 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 3SS:SFFYFL 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 equation: total carotenoid mg mL⁻¹ OD450(10 mL)1 g⁻¹. Three independent experiments were performed for each sample.

Ethylene measurements. Fruits at B + 1, B + 3, B + 7 and B + 14 stages were harvested in open field (Fig. 7A). Fruits placed in open field for 21 d were treated with 5 μL/L of 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 pedicels materials at B + 7 and B + 14 stages of transgenic plants and wild type. Repeat twice for each observation.

Break strength 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 break strength meter. Then the break strength of fruit stalk abscission zone was measured using the BOSE ELITE MICROFORCE 3500 break strength 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 assay 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 EcoRI and Psi I and cloned into the EcoRI and Psi I sites of the pGBKTK7 bait vector to obtain the vector FYFL-pGBKTK7. Then FYFL-pGBKTK7 vector was transformed into Y2HGold. The Y2HGold with bait was plated on SD medium lacking Trp (SDO) and SD medium lacking Leu, His, Ade (TDO) to test self-activation of FYFL-pGBKTK7. In parallel, the open reading frame of SIRIN, SIJOINTLESS and SIMADS1 were also amplified by primers (SIRIN(Y)-F, SIRRNY)-R), (SII(Y)-F, SII(Y)-R) and (SIMADS1(Y)-F, SIMADS1(Y)-R) (Table 1S). The products were cloned into the pGAD17 vector, and introduced into Y187. Subsequently, Y2HGold with bait ml Y187 with prey were cultured together and the culture 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.

Detached leaf senescence experiment. Mature leaves were cut from 2 month 3SS:SFFYFL and wild-type plants and placed on three layers of wet filter paper in 14 cm Petri dishes, then wrapped in aluminum foil, incubated in the dark at 25°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 sepal 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°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. Chlorophyll content were calculated using the formulas according to the method of Arnon:31 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 sepa
s were longer than the wild type. We measured the length of septals at the fully open flower
stage, at least 10 flowers per plant were measured (Fig 35 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.

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