FOREVER YOUNG FLOWER (FYP) has been reported to play an important role in regulating flower senescence/abscission. Here, we functionally analyzed five Arabidopsis FYP-like genes, two in the FYF subgroup (FYL1/AGL71 and FYL2/AGL72) and three in the SOC1 subgroup (SOC1/AGL20, AGL19, and AGL14/XAL2), and showed their involvement in the regulation of flower senescence and/or abscission. We demonstrated that in FYF subgroup, FYP has both functions in suppressing flower senescence and abscission, FYL1 only suppresses flower abscission and FYL2 has been converted as an activator to promote flower senescence. In SOC1 subgroup, AGL19/AGL14/SOC1 have only one function in suppressing flower senescence. We also found that FYF-like proteins can form heterotetrameric complexes with different combinations of A/E functional proteins (such as AGL6 and SEP1) and AGL15/18-like proteins to perform their functions. These findings greatly expand the current knowledge behind the multifunctional evolution of FYF-like genes and uncover their regulatory network in plants.
We have previously reported that Arabidopsis FOREVER YOUNG FLOWER (FYF) specifically regulates flower organ senescence and abscission by suppressing the downstream genes of ethylene signaling EDF1/2/3/4 and abscission-associated genes BOP1/2 and IDA. Conserved functions in regulating flower organ senescence and abscission have been reported for FYF orthologs identified in different species of orchids. Based on sequence homology and phylogenetic analysis, six putative closely related FYF-like genes could be subjected to two subgroups: (1) the FYF group composed of FYF, AGL71, and AGL72 and (2) the SOC1 group composed of AGL20 (SOC1), AGL19, and AGL14 (XAL2) in the Arabidopsis genome.

It has been reported that additional new genes could be generated in the genome through gene duplication, which was thought to play an important role in organisms during evolution. Phylogenetic analysis revealed that three duplication events from an FYF-like ancestor may have occurred (two within subgroups) to generate six Arabidopsis FYF-like genes. Functional analysis indicated that the majority of the duplicate FYF-like gene pairs (FYF/AGL71/AGL72/SOC1/AGL19/AGL14) may retain overlap of the original ancestral function, such as the regulation of flowering time, ovule development as seen for AGL14 (XAL2), which was described as subfunctionalization. Although different putative functions were uncovered for these Arabidopsis FYF-like genes, exploration of the function in regulating flower organ senescence and abscission was investigated for only the FYF gene and has never been reported for other five Arabidopsis FYF-like genes. It is therefore not clear whether the FYF gene evolved to have this unique function in regulating flower organ senescence and abscission from its ancestor or whether some of the other FYF-like genes also harbored this function during evolution. To uncover these questions, we comprehensively functionally characterized all putative Arabidopsis FYF-like genes for their involvement in regulating flower organ senescence and abscission. Furthermore, we used a FRET-based strategy to investigate the possible heterotetrameric protein complexes formed by the interactions of FYF-like and other MADS box proteins to further verify the regulatory networks for these duplicate FYF-like gene pairs in Arabidopsis.

Here, we show that all other five Arabidopsis FYF-like genes have a function in regulating flower senescence and/or abscission similar to FYF. In this work, we also found that FYF-like proteins can interact with different combinations of A/E functional proteins (such as AGL6 and SEP1) and AGL15/18-like proteins (such as AGL15/18) to form heterotetrameric complexes in regulating flower senescence and abscission.

Results

Characterization of two FYF closely related genes, FYL1 and FYL2. Two Arabidopsis italiana MADS box genes, known to be closely related to FYF (AT5G626165), named FYF-like 1, 2 (FYF1/AGL71, FYL2/AGL72), in the FYF group were analyzed. FYL1 (AT5G51870) encodes a protein containing 219 amino acids that showed 40.3% identity to FYF, with 86.2% (50/58) of the amino acids being identical in the MADS box domain (Supplementary Fig. 1). FYL2 (AT5G51860) encodes a protein containing 202 amino acids that showed 43.7 and 65% identity to FYF and FYL1, respectively, with 86.2% (50/58) and 94.8% (55/58) of the amino acids being identical in the MADS box domain (Supplementary Fig. 1), respectively. The amino acid identity and the phylogenetic tree relationship indicated that FYL1 was more closely related to FYL2 than to FYF (Supplementary Fig. 2).

The distinct expression patterns of FYL1 and FYL2. To investigate the expression patterns of the FYL1 and FYL2 genes, FYL1/2 expression was further analyzed in flowers at different developmental stages. The results indicated that higher FYL1/2 expression was observed during early flower development (before stage 9) than during late developmental stages (after stage 12) (Supplementary Fig. 3a, b), which was similar to the spatial and temporal expression pattern of FYF during flower development (Supplementary Fig. 3c). When FYL1::GUS and FYL2::GUS constructs were generated and transformed into Arabidopsis, GUS staining was exclusively detected in the abscission zone (AZ) of the sepal/petals of FYL1::GUS flowers (Fig. 1a–c) and shows a more extended pattern in the sepal/petals of FYL2::GUS flowers (Fig. 2a–d). Further RT-qPCR analysis indicated that FYL1 expression was highly detected whereas FYL2 expression was almost undetectable in the AZ (Supplementary Fig. 3d). This result is interesting since GUS staining was detected in both sepal/petals and in the abscission zones of FYF::GUS flowers, suggesting that FYL1 and FYL2 might have different subfunctions of FYF in regulating flower senescence and abscission.

FYL1 delayed flower senescence and abscission once ectopically expressed in transgenic Arabidopsis plants. To investigate function correlated to the expression pattern of FYL1, 35S::FYL1, 35S::FYL1::SRDX (containing a suppression motif), and 35S::FYL1::DR+VP16 (containing an activation VP16-AD) were transformed into Arabidopsis. The 35S::FYL1 plants showed a delay in both flower senescence and abscission (Fig. 1d, top and 1e), similar to what was observed in 35S::FYF plants. Furthermore, enhancement of the delay of flower senescence/abscission was observed in 35S::FYL1::SRDX transgenic plants (Fig. 1d, middle and 1f), and an opposite promotion of flower senescence/abscission was produced in 35S::FYL1::DR+VP16 transgenic plants (Fig. 1d, bottom left and 1g), suggesting that FYL1 should act as a repressor in suppressing flower senescence/abscission, similar to FYF. Once it has been converted into an activator, FYL1::DR+VP16, an opposite dominant negative mutant phenotype will be observed. We also found that the expression of the senescence-associated gene SAG12, downstream genes in ethylene signaling EDF1-3, and ERF1 and abscission-associated genes BOP1/2, IDA, and HAESA were all downregulated in 35S::FYL1 and 35S::FYL1::SRDX plants (Fig. 1h–j). In addition, 35S::FYL1 and 35S::FYL1::SRDX flowers were insensitive to ethylene treatment (Fig. 1k). Our data suggest that FYL1 could have a prominent role like FYF in controlling floral senescence/abscission once ectopically expressed in Arabidopsis flowers. However, FYL1 should only have a partial role in controlling floral abscission in real life since its expression was restricted to the abscission zone (AZ) of the sepal/petals of flowers (Fig. 1a–c and Supplementary Fig. 3d).

To further confirm the relationship between FYL1 and sepal/petal abscission, the FYL1 gene driven by the IDA promoter (IDA::FYL1) was transformed into Arabidopsis. A clear delay in the abscission of the perianth organs was observed in IDA::FYL1 flowers (Fig. 11–n). However, senescence of the perianth normally occurred from positions 4–5 in these flowers that were not abscised in the IDA::FYL1 transgenic plants (Fig. 11–n). This phenotype was very similar to what has been observed in ida mutants and Arabidopsis::FYF plants; thus, the result supported the role of FYL1 as a suppressor and its function together with FYF in suppressing IDA and sepal/petal abscission.

FYL2 promoted flower senescence and abscission when ectopically expressed in transgenic Arabidopsis plants. Similar to FYL1, 35S::FYL2, 35S::FYL2::SRDX, and 35S::FYL2::DR+VP16
Arabidopsis were generated. 35S:FYL2 plants surprisingly showed promotion of both flower senescence and abscission (Fig. 2e, first row and 2f). A similar promotion of flower senescence/abscission was observed in 35S:FYL2-DR+VP16 transgenic plants (Fig. 2e, third row and 2h), and an opposite delay of flower senescence/abscission was produced in 35S:FYL2+SRDX transgenic plants (Fig. 2e, second row and 2g), suggesting that FYL2 should act as an activator in promoting flower senescence/abscission, in contrast to FYF and FYL1. We also found that the expression of EDF1-4, ERF1, BOP1/2, IDA, and HAESA were all downregulated in 35S:FYL2+SRDX plants (Fig. 2i–k). By contrast, SAG12, EDF1-4, ERF1, IDA, and HAESA were all upregulated in 35S:FYL2 and 35S:FYL2-DR+VP16 transgenic plants (Supplementary Fig. 4). In addition, 35S:FYL2+SRDX flowers were
insensitive to ethylene treatment (Fig. 2l). This result suggested that FYL1 should function opposite to FYF and could have the same role as FYF once it is converted into a repressor as FYL1+SRDX, and a dominant negative mutant phenotype in suppressing floral senescence/abscission will be observed. However, FYL1 should only have an opposite role to FYF in controlling floral senescence in real life since its expression was specifically in the sepal/petal organs of flowers (Fig. 2a–d).

To further confirm the relationship between FYL1 and FYF in regulating sepal/petal senescence, 35S::FYF and 35S::FYL1 were doubly transformed into Arabidopsis, and plants ectopically expressing FYF and FYL1 were generated simultaneously. A clear wild-type-like phenotype by senescence and abscission of the perianth organs at approximately positions 3–4 was observed in the 35S::FYF/35S::FYL1 flowers (Fig. 2m, right), which was earlier than that in the 35S::FYF flowers (Fig. 2m, left) and later than that in the 35S::FYL1 flowers (Fig. 2e, first row). This 35S::FYF/35S::FYL1 intermediate phenotype between 35S::FYF and 35S::FYL1 clearly indicated an antagonistic relationship between FYF and FYL1. Thus, the results supported a role for FYL1 as an activator with a function antagonistic to part of the FYF function in controlling senescence of the sepal/petal.

**FYF can interact with AGL6 and SEP1 in regulating flower abscission/senescence.** To investigate which proteins could possibly interact with FYF to form a complex in regulating flower organ abscission and senescence, two potential candidates, AGL6 and SEP1, which have been reported to be able to interact with FYF through yeast two-hybrid screen, were identified. It is important to determine whether the spatial and temporal expression patterns of the AGL6 and SEP1 genes were correlated with FYF. Based on the AGL6:GUS assay, AGL6 expression could be detected in the basal parts and abscission zone of the floral organs during floral development (Supplementary Fig. 5a–c) and was detected to be more abundant in wild-type flowers before (BP) than after (AP) pollination (Supplementary Fig. 5d), which overlapped with the expression pattern of FYF. SEP1 has been reported to be expressed in all four whorls of flower organs, is more abundant during early morphological differentiation than in mature flowers, and was not reported in the abscission zone during floral development. These results suggested that FYF might interact with AGL6 to form a complex in regulating sepal/petal abscission. In addition, FYF can interact separately with SEP1 and AGL6 to form a complex in regulating sepal/petal senescence.

We further performed FRET analyses by using FYF-CFP and AGL6/SEP1-YFP to observe physical interactions of FYF and AGL6/SEP1 protein complexes in tobacco cells. The results confirmed that FYF/AGL6 formed heterodimers with high efficiency in the nucleus (Fig. 2n, column 1, Fig. 3a). The same result was observed for FYF/SEP1 (Fig. 2o, column 1). These results suggested that FYF is able to interact with AGL6 and SEP1 to form complexes in regulating flower senescence and/or abscission.

**FYL1 can interact with AGL6 in regulating flower abscission.** To further investigate whether FYL1 could also interact with proteins similar to FYF in regulating flower organ abscission, FRET analyses were performed. When FYL1-CFP and AGL6/SEP1-YFP were used to observe the physical interactions of FYL1 and AGL6/SEP1, FYL1/AGL6 can form heterodimers with similar efficiency to FYF/AGL6 in the nucleus of tobacco cells (Fig. 2n, column 2, Fig. 3b). However, the efficiency for the formation of FYL1/SEP1 (Fig. 2o, column 2) was clearly lower than that for FYF/SEP1 (Fig. 2o, column 1). These results suggested that FYL1 is able to interact with AGL6 in a more stable manner than with SEP1 to form complexes. Since FYL1 was only expressed in the AZ of the sepal/petals and could only regulate flower organ abscission, it is reasonable to believe that FYL1 can only physically interact with AGL6 in the AZ to regulate abscission of the sepal/petals. Although they can interact, the FYL1/SEP1 complex should not exist during Arabidopsis flower development since these two genes have no overlapping expression pattern.

The ability of AGL6 to interact with FYF and FYL1 to form complexes reveals that its function should be related to FYF/FYL1. This assumption was further supported by the result that a similar delay in the flower senescence/abscission phenotype (Supplementary Fig. 5e–i) and downregulation of EDF1, BOP1/2, IDA, and HAESA (Supplementary Fig. 5j, k) were observed in 35S::AGL6+SRDX plants. This result revealed that FYF/FYL1 can interact with AGL6 to target similar downstream genes once expressed in the same places.

**FYF and FYL1 can interact with AGL6/AGL6 and AGL15 proteins to form stable heterotetrameric abscission complexes.** Based on the floral quartet model in which plant MADS-box proteins function as higher-order tetrameric complexes, we hypothesized that FYF/AGL6 heterodimer proteins would further form heterotetrameric complexes with other MADS box proteins in the AZ to regulate sepal/petal abscission. It is interesting to note that two MADS box genes, AGL15 and AGL18, have been reported to be expressed in flower organs and in the AZ of flowers (Supplementary Fig. 6a–d), and a delay in senescence/abscission of flowers has also been observed in 35S::AGL15 and 35S::AGL18 Arabidopsis plants (Supplementary Fig. 6i–j). The similar delay of flower senescence/abscission (Supplementary Fig. 6e–h) and downregulation of EDF1-4, ERF1, BOP1/2, IDA, and HAESA (Supplementary Fig. 6i–j) in 35S::AGL15+SRDX Arabidopsis...
supports the notion that AGL15/AGL18 function similarly to FYF/FYL1 as repressors in regulating flower organ abscission. To further explore whether AGL15/AGL18 could also interact with proteins similar to FYF/FYL1 in regulating flower organ abscission, FRET analyses were performed. The results indicated that AGL15/AGL6 are able to form heterodimers with similar efficiency to FYF/AGL6 in the nucleus of tobacco cells (Fig. 2n, column 6, Fig. 3c). In contrast, AGL18 showed a very weak interaction with AGL6 (Fig. 2n, column 7). This result revealed that AGL15 can form a complex with AGL6, whereas AGL18 might form different protein complexes to perform the redundant function in regulating flower organ abscission.

To test whether FYF (FYL1), AGL6, and AGL15 could form heterotetrameric abscission complexes, a strategy using in vivo
Fig. 2 Characterization of the FYL2 gene through transgenic plants and gene expression analysis in Arabidopsis. a GUS was stained in the sepals/petals of flowers of FYL2::GUS Arabidopsis. GUS staining gradually decreased in the mature flowers during the late stage of flower development. The numbers indicate the different developmental stages of Arabidopsis flowers. Bar =1 mm. Magnified view of stage 7 (b), 11 (c), and 13 (d) FYL2::GUS flowers. GUS was strongly and relatively weakly stained in stage 7 young and 11 mature flower buds and was barely detected in stage 13 mature flowers. s: sepal; p: petal; st: stamen. Bars = 0.5 mm. e Flowers along the inflorescences of 35S::FYF:35S::FYF/35S::FYF (first row), 35S::FYF::SRDX (second row), 35S::FYF::VP16 (third row) and wild-type (WT) (fourth row) plants. The numbers indicate the positions of the flowers. Bar = 2 mm. Magnified view of early senescent 35S::FYF (f) and 35S::FYF::VP16 (h) flowers and delayed senescent 35S::FYF::SRDX (g) flowers. s: sepal; p: petal; st: stamen. Bars = 0.5 mm. Detection of SAG12 (i), EDFI-4 and ERFI (j) and BOP2/2, IDA and HAESA (k) expression in 35S::FYF::SRDX Arabidopsis. Error bars show ± SD. n = 3 biologically independent samples. The expression of each gene in the transgenic plants is given relative to that of the wild-type plant, which was set at 1. The letter “a”, “b” and “c” indicates significant difference from the wild-type (WT) value (a: P < 0.05; b: P < 0.01, and c: P < 0.001). The two-sided Student’s t-test was used. Flowers along the inflorescence of 35S::FYF, 35S::FYF/35S::FYF, and wild-type (WT) plants. Magnified view of the flower organs that did not become senescent and did not undergo abscission (boxed) in the #7 35 S::FYF flower. s: sepal; p: petal. Bar = 2 mm. Analysis of the interaction of MADS proteins FYF, FYL1, FYL2, SOC1, AGL19, AGL15, and AGL18 fused with CFP to AGL6 (n) and SEPI (o) fused with YFP through the FRET technique. CyPet- and YPet-fused protein pair fluorescence signals were detected in the nucleus expressed in tobacco leaves. CFP and YFP channels were excited with a 440 nm laser, and these two channels were used to calculate the raw FRET signal. FRET values were divided by CFP signals to calculate the FRET efficiency. The average FRET efficiency values were quantified in multiple samples (n > 4). Image frame = 20 × 20 μm². N.D. indicates not determined. (Blue line: mean). Analysis of the effect of FYL2 on FYF-SEP1 interactions. The FRET efficiency for the formation of FYF-CPF/SEPI-YFP complexes was analyzed in tobacco cells by adding different amounts (0, 25, 75, and 100%) of unlabeled FYL2 proteins. The average FRET efficiency values were quantified in multiple samples (n > 4). Image frame = 20 × 20 μm². (Red line: mean).

FRET based on the distance change and distance symmetry of a stable tetrameric complex in tobacco leaf cells was performed. Since FYF is unlikely to interact with AGL15 (Fig. 3d) in the FRET analysis, we analyzed the possible abscission tetramer containing FYF/AGL6 and AGL15/AGL16 heterodimers. In the FYF-AGL6-AGL6-AGL15 complex (Fig. 3f), the FRET efficiency of the coexpression admixture of AGL6:CFP/AGL6:YFP/AGL15 (Fig. 3g) was similar to that of AGL6:CFP/AGL15:YFP/FYF (Fig. 3h) (31%/30%) (Fig. 3k), and the distribution range overlapped. The FRET efficiency of the coexpression admixture of AGL6:CFP/AGL6:YFP/AGL15 (Fig. 3i) was similar to that of FYF-CPF/AGL6:YFP/AGL6/AGL6 (Fig. 3j) (29%/24%) (Fig. 3k), and the distribution range overlapped. Similarly, FYL1 was unlikely to interact with AGL15 (Fig. 3e), and the FRET efficiency of the coexpression admixture of AGL6:CFP/FLYL1:YFP/AGL15 (Fig. 3l) was similar to that of AGL6:CFP/AGL15:YFP/FLYL1 (Fig. 3m) (32%/34%) (Fig. 3p), and the distribution range overlapped. Similarly, the FRET efficiency of the coexpression admixture of AGL6:CFP/AGL6:YFP/FLYL1/AGL15 (Fig. 3n) was similar to that of AGL15:CFP/FLYL1:YFP/AGL6/AGL6 (Fig. 3o) (36%/34%) (Fig. 3p), and the distribution range overlapped. These overlapped patterns for the distribution range observed for FYF-AGL6-AGL6-AGL15 and FYL1-AGL6-AGL6-AGL15 heterotetrameric complexes were similar to that for the most stable heterotetrameric complexes PI-AP3-AG-SEP3 described in our previous study. These results indicated that FYF-AGL6-AGL6-AGL15 and FYL1-AGL6-AGL6-AGL15 are likely stable heterotetrameric complexes in regulating flower organ abscission. In addition, FYF-AGL6-AGL6-AGL15 is also likely a stable heterotetrameric complex in regulating flower organ senescence.

FYL2 can interact with AGL6 and SEPI in regulating flower senescence. Similarly, the interaction of whether FYL2 could also interact with proteins similar to FYF in regulating flower organ senescence was also performed using FRET analyses. When FYL2-CPF and AGL6-YFP or SEPI-YFP were used to observe the physical interactions of FYL2 and AGL6 or SEPI, a lower efficiency of FYL2/AGL6 heterodimer formation than that of FYF/AGL6 (Fig. 2n, column 1) in the nucleus of tobacco cells was observed (Fig. 2n, column 3). The efficiency for the formation of FYL2/SEPI (Fig. 2o, column 3) was also lower than that for FYF/SEPI (Fig. 2o, column 1). These results suggested that similar to FYF, FYL2 can also interact with AGL6 and SEPI to form senescence complexes, although at a lower efficiency. Since FYL2 was only expressed in the flower organs of sepals/petals, which overlapped with part of AGL6 and SEPI expression, these data revealed that FYL2 can physically interact with AGL6 and SEPI during Arabidopsis flower development to regulate sepal/petal senescence.

Since we have already shown that FYL2 functions opposite to FYF in controlling sepal/petal senescence, FYL2 might compete to bind the interacting protein to form a functional complex. To examine this assumption, FRET efficiency for the formation of FYF-CPF/SEPI-YFP complexes was examined in tobacco cells by adding different amounts of unlabeled FYL2 proteins. The results indicated that the efficiency for FYF-CPF to interact with SEPI-YFP (Fig. 2p, column 1) was clearly decreased by the presence of 25–75% of the FYL2 proteins (Fig. 2p, columns 2 and 3). The ability of FYF-CPF to interact with SEPI-YFP was almost completely competed for by the presence of 100% FYL2 protein (Fig. 2p, column 4). Thus, FYL2 competes with FYF to interact with SEPI, performing opposite functions in controlling sepal/petal senescence.

**FYF-like genes AGL19/14 and SOC1 are complementary to FYF in regulating flower senescence.** We found a possible mechanism involving three FYF-like genes (FYF and FYL1/2) in regulating flower organ senescence and abscission. It is interesting to note that three other genes in the SOC1 subgroup, AGL19, AGL14 (XAL2), and AGL20 (SOC1), were also closely related to the FYF/FYL1/FYL2 genes (Supplementary Figs. 1, 2). Do these three genes also harbor similar functions to FYF/FYL1/FYL2 in regulating flower senescence/abscission? Interestingly, similar to that observed in 35S::FYF and 35S::FYF::SRDX Arabidopsis, a strong delay in flower senescence and abscission (Fig. 4a–c), insensitivity to ethylene treatment (Fig. 4d–i) and downregulation of EDFI-4, ERFI, BOP2, IDA, and HAESA (Fig. 4j, k) were observed in 35S::AGL19 and 35S::AGL19::SRDX Arabidopsis. In contrast to AGL19, only 35S::AGL14::SRDX and 35S::SOC1::SRDX caused a strong delay in flower senescence/abscission and a down-regulation of senescence/abscission-related genes (Supplementary Figs. 7a–d, 8a–c), whereas no or a reduced effect was seen in 35S::AGL14 and 35S::SOC1 plants. These results indicated that AGL19 and AGL14/SOC1 functioned as strong and weak repressors, respectively, and that part of their function
complemented FYF in suppressing flower organ senescence/abscission.

Similar to the expression pattern of FYF/FYL2, higher AGL19/AGL14/SOC1 expression was observed during early flower development (before stage 9) than during late developmental stages (after stage 12) (Fig. 4l and Supplementary Figs. 7e, 8d), which further revealed possible similar and overlapping functions of FYF and AGL19/AGL14/SOC1. GUS staining was detected in sepal/petal organs and was absent in the AZ of AGL19::GUS (Fig. 4m, n) and SOC1::GUS flowers (Supplementary Fig. 8e–g), suggesting that AGL19/14 and SOC1 might have part of the functions of FYF in regulating flower senescence but not abscission. Although SOC1 and AGL19 might also be involved in regulating flower senescence, they have very low or complete
Fig. 3 The distance-measuring system validated that Arabidopsis AGL15-AGL6 forms stable abscission tetrameric complexes with FYF-AGL6 and FYL1-AGL6. The steady state of dimerization of the protein complexes FYF-AGL6 (a), FYL1-AGL6 (b), AGL15-AGL6 (c), FYF-AGL15 (d), and FYL1-AGL15 (e) is revealed in scatter diagrams showing pFRET and FRET efficiency. The black dots show the independent cell nuclei, and the yellow boxes indicate the steady-state FRET efficiency range for the protein complex. The mean value of FRET efficiency in the steady state is shown at the top of the schematic model, which is the baseline. The protein fused with CFP/YFP was attached by blue/yellow spots. f Schematic model of the protein interactions in the Arabidopsis abscission complexes (1) FYF-AGL6-AGL6-AGL15 and (2) FYL1-AGL6-AGL6-AGL15. Scatter diagram of the raw FRET (pFRET) and FRET efficiency values of the dimer pairs in adjacent lines AGL6-FYF (g) and AGL6-AGL15 (h) and diagonal lines AGL6-AGL6 (i) and FYF-AGL15 (j) in the abscission complex FYF-AGL6-AGL6-AGL15, with a different number of cell nuclei measured. The green dotted lines indicate the overlapping distribution range at the steady state. The yellow boxes (in g, h, j) indicate the baselines obtained for the dimer pairs. The protein fused with CFP/YFP was attached by blue/yellow spots. k Schematic model and FRET efficiency of four different pairs (two adjacent lines and two diagonal lines) of the protein interactions in the Arabidopsis stable abscission complex FYF-AGL6-AGL6-AGL15. The two adjacent lines (black) show similar FRET efficiencies (31%/30%), and the two diagonal lines (blue) show similar FRET efficiencies (29%/24%). Scatter diagram of the raw FRET (pFRET) and FRET efficiency values of the dimer pairs in adjacent lines AGL6-FYL1 (l) and AGL6-AGL15 (m) and diagonal lines AGL6-AGL6 (n) and FYL1-AGL15 (o) in the abscission complex FYL1-AGL6-AGL6-AGL15, with a different number of cell nuclei measured. The green dotted lines indicate the overlapping distribution range at the steady state. The yellow boxes (in l, m, o) indicate the baselines obtained for the dimer pairs. The protein fused with CFP/YFP was attached by blue/yellow spots. p Schematic model and FRET efficiency of four different pairs (two adjacent lines and two diagonal lines) of the protein interactions in the Arabidopsis stable abscission complex FYL1-AGL6-AGL6-AGL15. The two adjacent lines (black) show similar FRET efficiencies (32%/32%), and the two diagonal lines (blue) show similar FRET efficiencies (36%/34%).

Discussion

The Arabidopsis MADS box gene FYF can regulate flower organ senescence and abscission.2 Ectopic expression of FYF caused a delay of senescence and a deficiency of abscission in flowers of transgenic Arabidopsis and Eustoma grandiflorum.3 This study further showed that two tandem repeat FYF-like genes, FYL1, and FYL2, and three other FYF-like genes, AGL19/14 and SOC1, in Arabidopsis were also involved in the regulation of flower organ abscission and/or senescence, and their functions were complementary or antagonistic to FYF. FYL1 was found to act as a repressor to suppress abscission in sepal/petal organs with a complementary function to FYF (Fig. 5a). Unexpectedly, FYL2 could function as an activator and antagonize FYF in promoting the senescence of sepal/petal organs (Fig. 5a). The functions of FYL1/2 are correlated with their expression pattern since FYL1 was specifically expressed in the AZ of sepal/petals, whereas FYL2 expression was detected in the organs of sepal/petals. The amino acid identity and the phylogenetic tree relationship6 revealed that FYL1/2 were possibly the result of two duplication events. The first event generated an FYL1/2 ancestor from FYF, and the second event produced the two tandem repeats FYL1 and FYL2 from this FYL1/2 ancestor. The conserved role of FYF/FYL1/FYL2 during evolution in regulating flower senescence and/or abscission was further supported by their ability to interact with the same MADS box proteins AGL6 and SEP1. The original FYF gene must have contained both regulatory elements in its promoter or introns1,3,31–45, which are required for its expression in the organs and AZ of sepal/petals. In the AZ, FYF specifically interacts with AGL6 to suppress abscission of the sepal/petals (Fig. 5a, b). In the sepal/petal organs, FYF can interact with either AGL6 or SEP1 to suppress senescence (Fig. 5a, c). In the tandem repeat genes FYL1 and FYL2, the subfunctional alteration of the regulatory elements during evolution resulted in restriction of the expression of FYL1 in the AZ and FYL2 in sepal/petal organs. FYL1 should maintain the conserved ability of FYF to interact with AGL6 together to suppress the abscission of sepal/petals (Fig. 5a, b). Conversely, FYL2 only retained the conserved ability of FYF to interact with AGL6 and SEP1 in regulating sepal/petal senescence (Fig. 5a, c). However, FYL2 evolved into a role antagonistic to FYF and possibly helped to control FYF activity through a feedback loop to protect the flower buds from senescence and ensure the final senescence of the mature flowers (Fig. 5a, c).

In addition to FYL1/2, three putative FYF-like genes in the SOC1 subgroup, AGL19, AGL14, and SOC1, which are closely

no interactions with AGL6 (Fig. 2n, columns 4, 5) or SEP1 (Fig. 2o, columns 4, 5). This result indicated that the FYF/FYL1/FYL2 and AGL19/14/SOC1 groups might have evolved to have their own interacting partners in regulating flower senescence/abscission.

FYF activates FYL1 and AGL19/14/SOC1 expression to enhance the regulation of flower abscission and senescence. Since FYF has the same function as FYL1 and AGL19/14/SOC1 to regulate abscission and senescence of the sepal/petal, respectively, we were also interested in determining how they work together. When the expression pattern of endogenous FYL1 and AGL19/14/SOC1 was analyzed in 35S::FYF flowers, we found that the expression of all three genes was clearly upregulated (Fig. 4o). Our results revealed that FYL1 was activated by FYF in the AZ during flower development and could enhance the function of the FYF gene in suppressing sepal/petal abscission, whereas AGL19/14/SOC1 were activated by FYF in sepal/petals during flower development, which could enhance the function of the FYF gene in suppressing sepal/petal senescence. Interestingly, we found that FYF, FYL1, AGL14, and SOC1 expression was also upregulated in 35S::AGL19 flowers (Fig. 4p). This result suggested that FYF and other FYF-like genes with the strong repressor role, such as AGL19, could reciprocally activate each other to enhance the suppression of sepal/petal senescence.

FYF activates FYL2 expression to regulate flower senescence possibly through a feedback loop. Exploring how FYF competes with FYL2 to oppositely regulate sepal/petal senescence is interesting. When the expression pattern of endogenous FYL2 was analyzed in 35S::FYF flowers, we found that FYL2 expression was upregulated (Fig. 4o). Our results revealed that FYL2 was activated by FYF during flower development and that FYL2 could possibly form a feedback loop to contend with endogenous FYF function and to more appropriately control flower senescence. This assumption was further supported by the downregulation of FYL2 expression in fyf/agl15 double mutants (Supplementary Fig. 9a). We also found that FYL2 expression was upregulated in 35S::AGL19 flowers (Fig. 4p). This result suggested that FYF and AGL19 might control sepal/petal senescence by regulating FYL2 in a similar way.
related to FYF/FYL1/FYL2 genes based on the phylogenetic tree relationship\textsuperscript{6,16}, were also characterized. Based on the results of the functional analysis and the expression patterns of these three genes, we found that AGL19, AGL14, and SOCI were all involved in the regulation of senescence but not abscission of flower organs (Fig. 5a, c). AGL19 might play a stronger repressive role than AGL14/SOCl, which functions similarly to FYF in suppressing flower senescence (Fig. 5a). Although AGL19/14 and SOCl were found to be involved in regulating flower senescence, similar to FYF/FYL2, they seemed to perform their function in a different way in terms of finding interacting partners to form functional complexes. For example, FYF/FYL1/FYL2 can interact sufficiently with AGL6/SEP1, whereas AGL19/14/SOCl can not interact with AGL6/SEP1. This finding indicated that the AGL19/14/
SOCI subgroup might have their own interacting partners in regulating flower senescence that differ from those of FYF/FYL1/FYL2 during evolution.

One interesting finding is that these FYF-like genes could regulate the expression of each other. For example, FYF could positively regulate the expression of FYL1 and AGL19/14/SOC1 to enhance suppression of the abscission and senescence of flower organs, respectively, whereas AGL19 could positively regulate the expression of FYF and AGL14/SOC1 to enhance the suppression of flower organ senescence. This positive reciprocal regulatory network among the FYF-like genes should provide a mechanism to ensure the suppression of senescence/abscission during the early stage of flower organ development (Fig. 5b, c). In addition, we also found a possible feedback loop regulatory mechanism between FYF and its opposite functional activator FYL2. FYF could activate the expression of FYL2, which possibly sequentially antagonized the activity of FYF. In this case, FYF activity will be counterbalanced at an appropriate level by FYL2, which is high in early and low in late flower development and ensures that sepal/petal senescence will occur after flower maturation and will not occur in the flower bud stage (Fig. 5c).

In addition to the FYF-like genes studied, we also found that one other MADS box protein, AGL15, which has also been characterized to be able to regulate flower organ senescence/abscission (Fig. 5a)\(^{38,39}\), was able to interact with AGL6 to form stable heterotetrameric abscission complexes (FYF-AGL6-AGL6-AGL15 and FYL1-AGL6-AGL6-AGL15) and a senescence complex (FYF-AGL6-AGL6-AGL15) with FYF/FYL1 (Fig. 5b, c). Interestingly, AGL18, a closely related gene to AGL15, has both functional proteins (AGL6, SEP1, and unidentified X) and AGL15/18-like proteins (AGL15/18 and unidentified Y) to perform their functions (Supplementary Fig. 2 and Fig. 5d). In the AZ, FYF/AGL6/AGL6/AGL15 and FYL1/AGL6/AGL6/AGL15 were two identified heterotetrameric abscission complexes that suppressed flower abscission (Fig. 5b). In sepal/petals, we identified FYF/AGL6/AGL6/AGL15 together with FYF/SEP1/Y, AGL19/X/Y, AGL14/X/Y, and SOC1/X/Y heterotetrameric senescence complexes in suppressing flower senescence (Fig. 5c).

Our findings reveal the potential immense complexity of the different combinations of FYF-like, A/E functional, and AGL15/18-like proteins in forming heterotetrameric abscission/senescence complexes (Fig. 5d). This complicated gene redundancy might explain why it is difficult to identify the senescence/abscission mutant phenotype in a single gene mutation for these genes. In an attempt to mutate FYF (key gene in FYF-like) and AGL15 (key gene in AGL15/18-like) simultaneously, T-DNA mutants for each gene were crossed to generate fyf/agl15 double mutants. Very interestingly, early senescence and abscission of the flowers was observed in these fyf/agl15 double mutants (Supplementary Fig. 9). This result strongly supported our assumption that abscission/senescence heterotetrameric complexes are at least composed of different combinations of FYF-like and AGL15/18-like proteins. Simultaneous mutations in FYF and AGL15 proteins will disrupt the functions of various combinations of the complexes and result in early senescence/abscission mutant phenotypes. In conclusion, our findings not only greatly expand the current knowledge concerning the multifunctional evolution of FYF-like genes in regulating flower senescence/abscission but also provide an excellent example for the study of diverse functionalizations of duplicate gene pairs in plants.

**Methods**

**Plant materials and growth conditions.** The T-DNA insertion mutants of FYF (fyf, SALK_047915) and AGL15 (agl15, SALK_076234C) mutants Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, USA. Seeds for Arabidopsis were germinated and grown as described previously\(^{2,26}\). Arabidopsis seeds were sterilized and placed on agar plates containing 1/2 X Murashige & Skoog medium\(^{26}\) at 4 °C for 2 days. Before being transplanted to soil, the seedlings were grown in growth chambers under long-day conditions (16 h light/8 h dark) at 22 °C for 10 days. The light intensity of the growth chambers was 150 μmol m\(^{-2}\) s\(^{-1}\).

**Cloning of the cDNA for FYF, FYL2, AGL6, AGL19, AGL14, SOC1, and AGL15 from Arabidopsis.** For 35S:MADS constructs, the cDNAs for FYF, FYL2, AGL6, AGL19, AGL14, SOC1, and AGL15 were obtained by PCR amplification using gene-specific 5′ and 3′ primers. The primers contained the XbaI and KpnI recognition sites to facilitate the cloning of the cDNAs. The XbaI-KpnI fragment containing the cDNA was cloned into the binary vector pEpyron-22K\(^{1}\) under the control of the

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**Fig. 4 Characterization of the AGL19 gene through transgenic plants and gene expression analysis in Arabidopsis.** a. Flowers along the inflorescence of 35S::AGL19 plants. The numbers indicate the positions of the flowers. Bars = 2 mm. b. Magnified view of the flower organs of a 35S::AGL19 flower that were not senescent/abscised from (a). s: sepal, p: petal, st: stamen. Bars = 0.5 mm. c. Flowers along the inflorescence of 35S::AGL19+SRDX plants. The numbers indicate the positions of the flowers. Bars = 2 mm. Flowers along the inflorescence of wild-type (d, e), 35S::AGL19 (f, g), and 35S::AGL19+SRDX (h, i) plants after exposure to ethylene. Wild-type flowers were senescent (arrowed in d, e), whereas 35S::AGL19 and 35S::AGL19+SRDX flowers were not senescent/abscised. Bars = 2 mm. Detection of EDFI-4 and EFPI (f) and BOP/2, IDA and HAE5A (k) expression in 35S::AGL19 and 35S::AGL19+SRDX Arabidopsis. Error bars show ± SD. n = 3 biologically independent samples. The expression of each gene in the transgenic plants is given relative to that of the wild-type plant, which was set at 1. The letter “a”, “b” and “c” indicates significant difference from the wild-type (WT) value (a: P < 0.05, b: P < 0.01, and c: P < 0.001). The two-sided Student’s t-test was used. The finding not only greatly expand the current knowledge concerning the multifunctional evolution of FYF-like genes in regulating flower senescence/abscission but also provide an excellent example for the study of diverse functionalizations of duplicate gene pairs in plants.
Table 1. For the IDA construct, the 5'UTR and 3'UTR were cloned into the pEpyon-2aK plasmid upstream of the SRDX (LDLDLELRLGFA') sequence, under the control of the CaMV 35S promoter as described previously. Sequences for the primers are listed in Supplementary Table 1.

Cloning of the promoter DNA fragment from Arabidopsis. For the FYL1::GUS and FYL2::GUS constructs, the promoter regions which included the 5'UTR and first intron for FYL1 (2.65 kb) and FYL2 (2.43 kb) were obtained by PCR amplification using specific primer pairs from the genomic DNA followed by cloning into the pGEM-T easy vector (Promega, Madison, WI, USA). These promoter fragments were then subcloned into the linker region before the β-Glucuronidase (GUS) coding region in the binary vector pEpyon-01K. For the A/E functional genes AGL6/AGL19/AGL14/SOC1 constructs, the promoter regions which included the 5'UTR and first intron for AGL6 (4.96 kb), AGL15 (0.94 kb), AGL19 (4.59 kb) and SOC1 (5.68 kb) were obtained by PCR amplification and these promoter fragments were subcloned into the linker region before the β-Glucuronidase (GUS) coding region in the binary vector pEpyon-01K. Sequences for the primers are listed in Supplementary Table 1. For the IDA::FYLI construct, the 5'UTR and 3'UTR were cloned into the pEpyon-2aK plasmid upstream of the SRDX (LDLDLELRLGFA') sequence, under the control of the CaMV 35S promoter as described previously. Sequences for the primers are listed in Supplementary Table 1.

Construction of the MADS + SRDX constructs. For the 35S::FYF1::SRDX, 35S::FYL1::SRDX, 35S::AGL6::SRDX, 35S::AGL15::SRDX, 35S::AGL19::SRDX, 35S::AGL14::SRDX, 35S::SOC1::SRDX constructs, the cDNAs for FYF1/FYL1/AGL19/AGL14/SOC1 were obtained by PCR amplification and cloned into the pEpyon-2aK plasmid upstream of the SRDX (LDLDLELRLGFA') sequence, under the control of the CaMV 35S promoter as described previously. Sequences for the primers are listed in Supplementary Table 1.

Construction of the MADS + VP16 constructs. For the 35S::FYF1::VP16, and 35S::FYL2::VP16 constructs, the cDNAs for FYF1/FYL2 were obtained by PCR amplification and cloned into the pEpyon-2aK plasmid upstream of the VP16-AD fragment sequence, under the control of the CaMV 35S promoter as described previously. Sequences for the primers are listed in Supplementary Table 1.

CaMV 35S promoter and used for plant transformation. Sequences for the primers are listed in Supplementary Table 1.

**Fig. 5** The functional evolution and regulatory network of the FYF-like genes in regulating flower senescence/abscission. a In Arabidopsis, six FYF-like genes in two subgroups (FYF/FYL1/FYL2 and AGL19/AGL14/SOC1) were all involved in regulating flower senescence and/or abscission. In the FYF subgroup, FYF acts as a repressor (R) in suppressing both flower senescence (indicated by a blue box) and abscission (indicated by a pink box), and FYL1 acts as a repressor (R) and only suppresses flower abscission (indicated by a pink box). FYL2 functions as an activator (A) in promoting flower senescence (indicated by a gray box). In the SOCI subgroup, AGL19, AGL14, and SOCI function as repressors (R) and have only one function in suppressing flower senescence (indicated by a light blue box), with the effect of AGL19 being stronger than that of AGL14/SOCI. In addition, the A/E functional genes AGL6/AGL15 (AGL15/18-like gene) can regulate senescence (indicated by a blue box) by interacting with FYF/FYL2, whereas AGL6 and AGL15 can also regulate abscission (indicated by a pink box) by interacting with FYF/FYL1. The size of the letter R in the box correlated with the strength of the repressor for the MADS box proteins. b In the AZ of the perianth, FYF and FYL1 complement each other by forming two identical heterotetrameric senescence complexes, FYF/AGL6/AGL15 and FYL1/AGL6/AGL15, suppressing flower abscission through the downregulation (−) of BOP1/2 and IDA/HAESA expression. c In sepals/petals, FYE, AGL19, AGL14, and SOCI functioned antagonistically to FYL2 in suppressing flower senescence. An identified FYF/AGL6/AGL15/AGL19 (AGL15/18-like proteins) together with FYF/SEPI/Y, AGL19/X/Y, AGL14/X/Y, and SOCI/Y/Y heterotetrameric senescence complexes suppressed sepal/petal senescence through the downregulation (−) of ethylene downstream gene expression. In contrast, FYL2/AGL6/Y and FYL2/SEPI/Y heterotetrameric complexes promoted sepal/petal senescence by the activation (→) of ethylene downstream gene expression, possibly through a negative feedback loop to FYF/X/Y, AGL19/X/Y, AGL14/X/Y, and SOCI/X/Y. d In these cases from (c), the heterotetrameric complexes are composed of FYF-like, X (in red) and Y (in blue) proteins. FYF-like can be either one of the FYF/FYL1/FYL2/AGL19/AGL14/SOC1, X can be AGL6, SEPI, or any unidentified A/E proteins, whereas Y can be AGL15, AGL18, or any unidentified AGL15/18-like proteins.
Plant transformation and transgenic plant analysis. A floral dip method as described elsewhere was used to introduce constructs made in this study in the Agrobacterium tumefaciens strain GV3101 into Arabidopsis plants. PCR and RT-PCR analyses were used to verify the transformants that survived in medium containing kanamycin (50 µg/ml). To generate 35S::FYF/35S::FY2 Arabidopsis, constructs of 35S::FY2 which contained hygromycin resistant gene co-transformed with 35S::FY (kanamycin resistant) into Arabidopsis plants. Transformants that survived in medium containing both kanamycin (50 µg/ml) and hygromycin (5 µg/ml) were selected for further analysis. To generate fyf/agl15 double mutant Arabidopsis, homozygous fyf were crossed with the agl15 T-DNA mutants in the Columbia background and F2 plants were used to further generate the F2 generation. One quarter of the F2 plants were fyf/agl15 and were further verified and selected for further analysis.

Real-time PCR analysis. For real-time quantitative RT-PCR, the reaction was performed on a MJ Opticon system (MJ Research, Waltham, MA) using SYBR® Green Real-time PCR Master Mix (TOYOBO Co., LTD.). The amplification condition was 95°C for 10 min, followed by 40 cycles of amplification (95°C for 15 s, 58°C for 15 s, 72°C for 30 s and then plate reading) and melted (50–95°C with plate readings every 1°C) as described previously. Sequences for the primers used for real-time quantitative RT-PCR for FYF, FYL1, FYL2, AGL15, AGL19, AGL14, SOCI, EDF1, EDF2, EDF3, ERFI, SAG12, BOP1, BOP2, IDA, and HAESA, were listed in Supplementary Table 1. The housekeeping gene UBQ10 was used as normalization control with the following primers: RT-UBQ10-1 and RT-UBQ10-2. Data were analyzed using Gene Expression Macro software (version 1.1, Bio-Rad).

Ethylene responses. As described previously, wild-type and transgenic Arabidopsis plants were sealed in plastic chambers and gassed with air or air containing 10 ppm ethylene for 3 days in a 16 h light/8 h dark cycle and phenotype analyzed.

FRET analysis. The procedure used to prepare FRET-associated fusion constructs was described in previous studies. To fuse FYF/FYL1/FYL2/AGL6/AGL15/AGL18/AGL19/SOC1 with CFP or YFP, the cDNAs for the fusion proteins were expressed in tobacco cells and a confocal microscope was used to detect fluorescence signals in the nucleus. To perform the subcellular localization assay, Agrobacterium-infiltrated N. benthamiana leaves were vacuum infiltrated in 10 mM MgCl2 at room temperature until immersed. As previously described, an Olympus FV1000 confocal microscope (Olympus FV1000, Tokyo, Japan) and the FV-AW 3.0 software were used to visualize fluorophores and to calculate the raw FRET and FRET efficiency values. To evaluate the variation in protein interaction distances among different protein complexes (n > 4), the mean value of FRET efficiency in the nucleus was calculated.

Statistics and reproducibility. Data in the analysis of gene expression in various transgenic plants were analyzed using the two-sided Student’s t-test and represented as the mean ± SD. In these cases, n = 3 biologically independent samples. The letter “a”, “b” and “c” indicates significant difference from the wild-type (WT) value (a: P < 0.05; b: P < 0.01, and c: P < 0.001).

Received: 11 January 2022; Accepted: 24 June 2022; Published online: 05 July 2022

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Data availability

The data supporting the findings of this work are available within the paper and the Supplementary Information files. The data sets generated and analyzed during this study are available from the corresponding author upon request.
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Acknowledgements
This work was supported by grants to C.-H.Y. from the Ministry of Science and Technology, Taiwan, ROC, grant number: MOST 107-2313-B-005-018-MY3 and MOST 109-2312-B-005-001. This work was also financially supported (in part) by the Advanced Plant Biotechnology Center from The Featured Areas Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan.

Author contributions
C.-H.Y. developed the overall strategy, designed experiments, and coordinated the project. W.-H.C., P.-T.L., C.-W.T., and M.-C.H. generated transgenic Arabidopsis plants, performed phenotypic and gene expression analyses. H.-F.H. and Y.-C.L. performed gene expression analyses. W.-H.H., C.-W.T., P.-T.L., and W.-T.M. performed FRET analyses. C.-H.Y. prepared and revised the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s42003-022-03629-w.

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Peer review information Communications Biology thanks Jazmin Abraham and the other anonymous reviewer(s) for their contribution to the peer review of this work.

Primary Handling Editors: José Estevez and Luke R. Grinham.

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