The *SPOROCYTELESS/NOZZLE* Gene Is Involved in Controlling Stamen Identity in Arabidopsis[1][W][OA]

Xiaodong Liu[2], Jian Huang, Sriram Parameswaran, Toshiro Ito, Brandon Seubert, Max Auer, Amy Rymaszewski, Gengxiang Jia[3], Heather A. Owen, and Dazhong Zhao*

Department of Biological Sciences, University of Wisconsin, Milwaukee, Wisconsin 53211 (X.L., J.H., B.S., M.A., A.R., G.J., H.A.O., D.Z.); and Temasek Life Sciences Laboratory, National University of Singapore, Singapore 117604 (S.P., T.I.)

The stamen, which consists of an anther and a filament, is the male reproductive organ in a flower. The specification of stamen identity in Arabidopsis (*Arabidopsis thaliana*) is controlled by a combination of the B genes *APETALA3* (*AP3*) and *PISTILLATA*, the C gene *AGAMOUS* (*AG*), and the E genes *SEPALLATA1* (*SEP1*) to *SEP4*. The "floral organ-building" gene *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*) plays a central role in regulating anther cell differentiation. However, much less is known about how "floral organ identity" and floral organ-building genes interact to control floral organ development. In this study, we report that ectopic expression of *SPL/NZZ* not only affects flower development in the wild-type background but also leads to the transformation of petal-like organs into stamen-like organs in flowers of *ap2-1*, a weak *ap2* mutant allele. Moreover, our loss-of-function analysis indicates that the *spl/nzz* mutant enhances the phenotype of the *ag* weak allele *ag-4*. Furthermore, ectopic expression and overexpression of *SPL/NZZ* altered expression of *AG*, *SEP3*, and *AP2* in rosette leaves and flowers, while ectopic expression of *SPL/NZZ* resulted in ectopic expression of *AG* and *SEP3* in the outer whorls of flowers. Our results indicate that the *SPL/NZZ* gene is engaged in controlling stamen identity via interacting with genes required for stamen identity in Arabidopsis.

The Arabidopsis (*Arabidopsis thaliana*) flower contains four types of organs that are arranged in four concentric whorls. Four sepals are found in the outermost whorl, four petals in the second whorl, six stamens in whorl 3, and two carpels in the innermost whorl. As in most angiosperms, flower development in Arabidopsis occurs in four steps: transition from vegetative growth to reproductive growth, establishment of floral meristem identity, specification of floral organ identity, and floral organ morphogenesis (Zhao et al., 2001a; Jack, 2004). Extensive molecular genetic studies have not only uncovered a large number of genes that are required for flower development but also elucidated regulatory relationships among key genes.

The ABC model describes three classes of homeotic genes, termed A, B, and C, which specify floral organ identity in four whorls (Haughn and Somerville, 1988; Bowman et al., 1991b; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Ma, 1994; Weigel and Meyerowitz, 1994). In Arabidopsis, the class A genes *APETALA1* (*AP1*) and *AP2* are active in whorls one and two, the class B genes *AP3* and *PISTILATA* are active in whorls two and three, and the class C gene *AGAMOUS* (*AG*) is active in whorls three and four. Class A genes alone are required for sepal identity. Class A and B genes together direct petal identity. Class B and C genes control stamen identity, while the class C gene solely specifies carpels. Moreover, class A and C genes function antagonistically (Drews et al., 1991; Mizukami and Ma, 1992). The activity of class A genes in whorls one and two prevents the function of the class C gene in the same whorls and vice versa in whorls three and four. A new addition to the ABC model is that the E genes (*SEPALLATA1* [*SEP1*]–*SEP4*) are involved in specifying organ identity in all four whorls via interacting with ABC regulators (Pelaz et al., 2000; Honma and Goto, 2001; Ditta et al., 2004; Melzer et al., 2009). The ABC model is applicable to diverse plant species in terms of regulation of floral organ identity. However, much less is known about genes that are necessary for building floral organs.

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[2] Present address: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

[3] Present address: Department of Genetics, University of Wisconsin, Madison, WI 53706–1574.

* Corresponding author; e-mail dzhao@uwm.edu.

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Stamens are male reproductive organs in a flower. Each stamen consists of an anther, where the male gametophyte develops, and a filament, which provides water and nutrients to the anther and anchors the anther to the flower. The anther is a four-lobed structure in Arabidopsis. Each mature anther lobe is composed of five types of well-organized cell layers: epidermis, endothecium, middle layer, tapetum, and microsporocyte (pollen mother cell; Goldberg et al., 1993; Sanders et al., 1999). Microsporocytes are reproductive cells that undergo meiosis and eventually develop into pollen grains. The remaining nonreproductive (somatic) cells are required for the normal development and release of pollen. In particular, the tapetum is essential for pollen development. After the specification of stamen identity, anther development involves a series of cell division, cell differentiation, and cell death, resulting in the formation of reproductive microsporocytes and somatic cell layers. Although microarray experiments uncovered many genes that are important for anther development (Zik and Irish, 2003; Hennig et al., 2004; Wellmer et al., 2004; Lu et al., 2006; Alves-Ferreira et al., 2007; Ma et al., 2007, 2008; Wijeratne et al., 2007), so far only a few genes have been identified to play direct roles in controlling differentiation of various anther cells.

Besides genes that encode signaling proteins (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Hord et al., 2006; Mizuno et al., 2007; Jia et al., 2008; Zhao, 2009), the *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*) gene plays a central role in controlling early anther cell differentiation (Schiefthaler et al., 1999; Yang et al., 1999). Anthers in the *spl/nzz* mutant do not produce microsporocytes or anther walls, suggesting that the *SPL/NZZ* gene is required for the differentiation of microsporocytes and anther walls, including the tapetum. The *SPL/NZZ* gene encodes a novel protein related to MADS box transcription factors. The direct activation of *SPL/NZZ* by AG is required for early anther development (Ito et al., 2004). Detected as early as the stage when stamen primordia are generated, the expression of *SPL/NZZ* coincides with the expression of *AG* during early anther development (Yanofsky et al., 1990; Bowman et al., 1991a; Drews et al., 1991; Ito et al., 2004). Therefore, we hypothesize that *SPL/NZZ* might be involved in controlling stamen identity.

Here, we report our characterization of the *SPL/NZZ* function in specifying stamen identity. We show that ectopic expression of *SPL/NZZ* affects both leaf and flower development. Furthermore, the ectopic expression of *SPL/NZZ* leads to the transformation of petal-like organs into stamen-like organs in flowers of *ap2-1*, a weak *ap2* mutant allele. The *spl/nzz* mutant also enhances the phenotype of the *ag* weak allele *ag-4*. In addition, ectopic expression and overexpression of *SPL/NZZ* alter the expression of *AG*, *SEP3*, and *AP2* in rosette leaves and flowers. Our results indicate that the "floral organ-building" gene *SPL/NZZ* is not only essential for early anther cell differentiation but also is engaged in controlling stamen identity in Arabidopsis.

RESULTS

Ectopic Expression and Overexpression of *SPL/NZZ* Cause Abnormal Plant Development

The *SPL/NZZ* gene is critical for anther morphogenesis, since *spl/nzz* mutant plants fail to produce the main structures of the anther, including microsporocytes and anther walls (Schiefthaler et al., 1999; Yang et al., 1999). To further test the function of *SPL/NZZ* in anther development, we ectopically expressed *SPL/NZZ* under the control of the constitutively active cauliflower mosaic virus 35S promoter (*35S*). Eighty-seven percent of the 237 Pro_{35S}:*SPL/NZZ* transgenic plants examined were defective in both vegetative and reproductive growth. Pro_{35S}:*SPL/NZZ* plants were shorter than wild-type plants (Fig. 1A). In addition, fertility of most Pro_{35S}:*SPL/NZZ* plants was reduced. Interestingly, Pro_{35S}:*SPL/NZZ* plants produced curled rosette leaves, although the curling degree and leaf size were variable (Fig. 1, B–E). In the Pro_{35S}:*SPL/NZZ-GLUCOCORTICOID RECEPTOR* (*GR*) transgenic line, the *SPL/NZZ* activity can be induced by continuous treatment of the steroid hormone dexamethasome (DEX; Ito et al., 2004). The formation of curled rosette leaves in Pro_{35S}:*SPL/NZZ-GR* plants after DEX treatment indicated that curled rosette leaves were caused by the ectopic expression of *SPL/NZZ* (Fig. 1F). Additionally, an earlier study showed that the *spl-D* mutant, in which *SPL/NZZ* was overexpressed, exhibited

Figure 1. Pro_{35S}:*SPL/NZZ* plants are abnormal in development. A, Compared with the wild type (left), the Pro_{35S}:*SPL/NZZ* transgenic plant (right) had shorter stature and reduced fertility. B, Wild-type seedling. C to E, Pro_{35S}:*SPL/NZZ* seedlings showing variabilities in their up-curled rosette leaves. F, Pro_{35S}:*SPL/NZZ-GR* seedling exhibiting wrinkled and up-curled rosette leaves after treatment with DEX. G, Pro_{35S}:*AG* seedling displaying up-curled rosette leaves. Bars = 1 cm (A) and 0.5 cm (B–G).

similar phenotypes to Pro_{35S}:*SPL/NZZ* transgenic plants, including curled rosette leaves (Supplemental Fig. S1, A and C; Li et al., 2008). Furthermore, most curled rosette leaves in both Pro_{35S}:*SPL/NZZ* and *spl-D* plants were up-curled (Fig. 1, B–E; Supplemental Fig. S1, A–C) and resembled the rosette leaf phenotype of Pro_{35S}:*AG* plants (Fig. 1G). Our results indicate that ectopic expression and overexpression of *SPL/NZZ* caused abnormal vegetative and reproductive growth.

Ectopic Expression of *SPL/NZZ* Affects Flower Development

Pro_{35S}:*SPL/NZZ* plants were abnormal in flower development. Pro_{35S}:*SPL/NZZ* flowers were smaller than those of the wild type (Fig. 2, A and B). In addition, Pro_{35S}:*SPL/NZZ* plants frequently produced buds with unfurled sepals (Fig. 3A). The development of petals in Pro_{35S}:*SPL/NZZ* flowers was delayed, resulting in the late emergence of petals. More interestingly, Pro_{35S}:*SPL/NZZ* flowers sometimes produced small and narrow petals in whorl 2 (Figs. 2, B and C, and 3B). Occasionally, narrow petals were up-curled (Fig. 2F; Table I). Although no morphological defects of stamens and carpels were observed, Pro_{35S}:*SPL/NZZ* plants had reduced fertility. Anthers in many mature flowers could not reach the stigma due to elongation defects in filaments (Fig. 2, A and B). To further test the phenotype of Pro_{35S}:*SPL/NZZ* flowers, we analyzed the surface features of petals using scanning electron microscopy (SEM). Wild-type floral organs have distinctive epidermal cell morphologies. In the wild type, petal epidermal cells are uniform in size and conical in shape (Fig. 2D), while stamen epidermal cells exhibit irregular cell edges and have wavy ridges (Fig. 2E). In Pro_{35S}:*SPL/NZZ* flowers, epidermal cells of narrow petals were long and uneven in size, although some cells still had dome shapes (Fig. 2, C and G). Cells of some Pro_{35S}:*SPL/NZZ* petals were similar to those of wild-type stamens, indicated by irregular cell edges and wavy ridges (Fig. 2, F and H). Our results indicate that the ectopic expression of *SPL/NZZ* affects floral organ development. The stamen feature in some Pro_{35S}:*SPL/NZZ* petals suggests that the *SPL/NZZ* gene might be involved in specifying stamen identity by promoting C function in whorl 2.

Ectopic Expression of *SPL/NZZ* Enhances the *ap2-1* Flower Phenotype in Whorl 2

The ectopic expression of *SPL/NZZ* led to the subtle phenotype of floral organ transformation from petal to stamen in the second whorl, while *ap2-1* mutant flowers displayed a mild but similar phenotype in whorl 2. To test whether *SPL/NZZ* promotes the stamen identity, we transformed *ap2-1* mutant plants using the Pro_{35S}:*SPL/NZZ* construct. In *ap2-1* flowers, the whorl-one organs are transformed into leaf-like organs from sepals and sometimes have carpel-like structures (Fig. 4, A and D; Table I). In whorl 2, most petals are close to normal, although stamen-like structures were observed in some petals (Table I). In *ap2-1* Pro_{35S}:*SPL/NZZ*

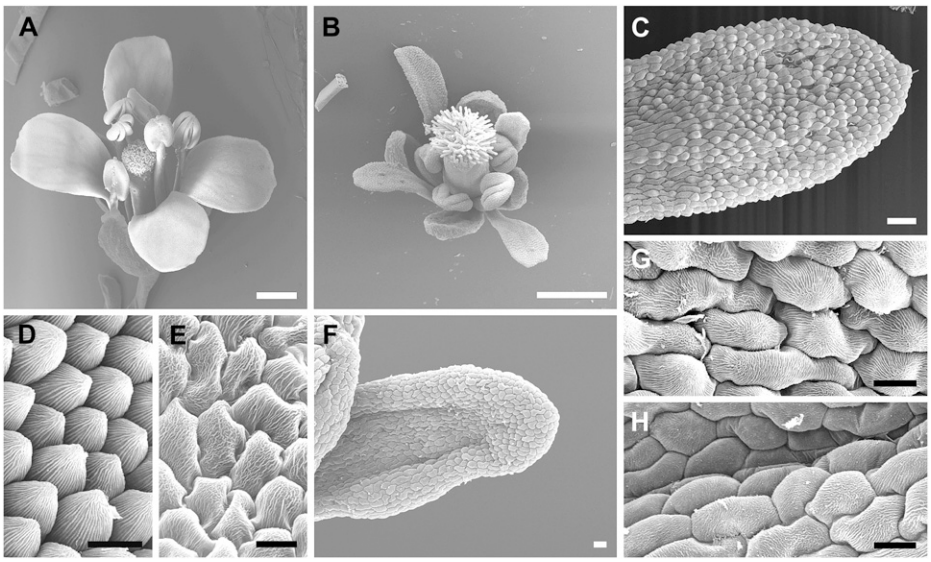

Figure 2. Scanning electron micrographs showing defects in flower development in Pro_{35S}:*SPL/NZZ* plants. A, A wild-type flower showing normal petals. B, A Pro_{35S}:*SPL/NZZ* flower showing narrow petals and shortened stamens. C, A narrow petal in whorl 2 of a Pro_{35S}:*SPL/NZZ* flower. D, Wild-type petal epidermal cells showing uniform size and conical shape. E, Wild-type anther epidermal cells exhibiting irregular cell edges and wavy ridges. F, A narrow and up-curled petal in whorl 2 of a Pro_{35S}:*SPL/NZZ* flower. G, High-magnification view of epidermal cells of the organ shown in C displaying elongated shape and uneven sizes. H, High-magnification view of epidermal cells of the organ shown in F exhibiting the resemblance to wild-type anther epidermal cells in E. Bars = 0.5 mm (A and B), 50 μm (C and F), and 10 μm (D, E, G, and H).

Figure 3. The petal phenotype caused by *Pro*$_{35S}$:*SPL/NZZ* may require a functional *AG*. A, A *Pro*$_{35S}$:*SPL/NZZ* young flower showing unclosed sepals and small petals. B, A *Pro*$_{35S}$:*SPL/NZZ* flower showing four narrow petals. C, An *ag-1* *Pro*$_{35S}$:*SPL/NZZ* young flower. D, An *ag-1* *Pro*$_{35S}$:*SPL/NZZ* flower showing normal petals in the second whorl. E, An *ag-1* flower. Bars = 0.5 mm.

flowers, a majority of petals in whorl 2 were narrow and up-curled (Fig. 4, B and E; Table I). In some *ap2-1 Pro*$_{35S}$:*SPL/NZZ* flowers, all of the petals were converted into stamens (Fig. 4C). Further SEM analyses revealed that stamen-like organs in *ap2-1 Pro*$_{35S}$:*SPL/NZZ* flowers produced cells that resembled anther and filament epidermal cells (Fig. 4, F–J). Moreover, we did not detect narrow petals in the second whorl of *ag-1 Pro*$_{35S}$:*SPL/NZZ* flowers (Fig. 3). Our results indicate that the ectopic expression of *SPL/NZZ* enhances the flower phenotype of *ap2-1* in whorl 2, which further supports the idea that *SPL/NZZ* is involved in promoting C function in whorl 2.

Loss-of-Function Analysis Indicates That *SPL/NZZ* Is Involved in Controlling Stamen Identity

Our gain-of-function analyses indicate that the *SPL/NZZ* gene promotes the specification of stamen identity. To further test whether *SPL/NZZ* controls stamen identity, we created the *spl ag-4* double mutant. Although *SPL/NZZ* (At4g27330) and *AG* (At4g18960) genes are located 3.3 Mb apart on chromosome 4, we found three *spl ag-4* double mutants from 287 F2 plants. Previous studies have shown that *spl/nzz* petals are normal, while mutant anthers do not produce microsporocytes and anther walls (Schiefthaler et al., 1999; Yang et al., 1999). Compared with the wild type (Fig. 5, A and H), mature *spl* anthers were somewhat flattened and did not produce pollen grains (Fig. 5, B and I). *ag-4*, a weak *ag* allele, produced normal petals in whorl 2 and stamens in whorl 3 (Fig. 5, C and K). However, although anthers in *spl ag-4* double mutant flowers were similar to those of *spl* (Fig. 5, B and D), SEM analyses revealed stamen-to-petal transformation of organs in the third whorl of *spl ag-4* flowers. Epidermal cells from *spl* stamens (Fig. 5J) and *ag-4* stamens in the third whorl (Sieburth et al., 1995; Chen and Meyerowitz, 1999) are similar to those of wild-type stamens (Fig. 2E). Conversely, in *spl ag-4* double mutant flowers, the third whorl organs (petaloid organs) exhibited cone-shaped cells (Fig. 5, L–O), which resembled wild-type petal epidermal cells (Fig. 5, E–G). Our results provided strong evidence to support the idea that the *SPL/NZZ* gene plays a role in promoting stamen identity by interacting with *AG*.

Ectopic Expression and Overexpression of *SPL/NZZ* Alter the Expression of Genes Required for Stamen Identity

Previous studies showed that class B, C, and E genes cooperate to control stamen identity. The expansion of C

Table I. *Comparison of floral organs*

Leaf like in whorl 1, sepals with leaf or stigma structures; stamen like in whorl 2, narrow, up-curled, or with stamen structures; stamen like in whorl 3, stamen with petal structures. The first 10 flowers from 10 plants were examined for each genotype.

Phenotype	Genotype				
	Wild Type	*spl*	*Pro*$_{35S}$:*SPL/NZZ*	*ap2-1*	*ap2-1Pro*$_{35S}$:*SPL/NZZ*
Whorl 1 sepal	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	0	0
Leaf like				4.00 ± 0.00	4.00 ± 0.00
Whorl 2 petal	4.00 ± 0.00	4.00 ± 0.00	3.47 ± 0.10	2.37 ± 0.11	0.65 ± 0.09
Stamen like	0	0	0.24 ± 0.06	0.62 ± 0.11	2.43 ± 0.14
Whorl 3 stamen	6.00 ± 0.00	5.44 ± 0.06	4.90 ± 0.12	5.25 ± 0.07	4.68 ± 0.08
Stamen like	0	0	0	0.21 ± 0.05	0.03 ± 0.01
Carpel	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00	2.00 ± 0.00

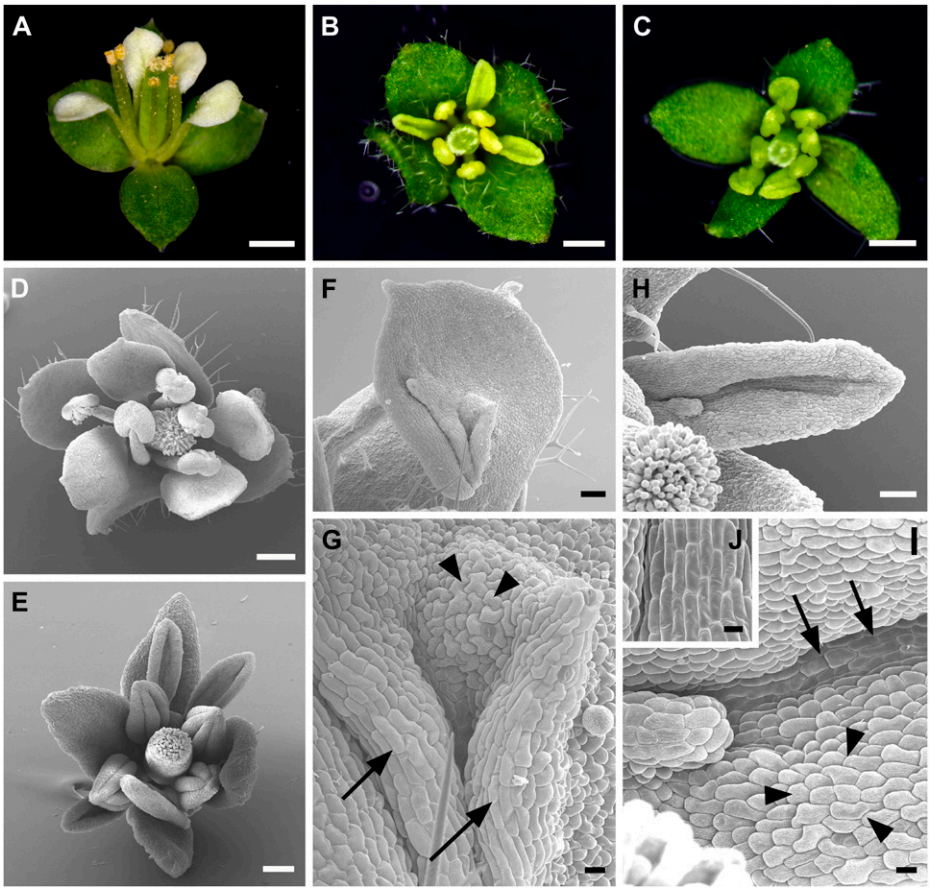

Figure 4. Ectopic expression of *SPL/NZZ* promoted stamen identity in the second floral whorl of the *ap2-1* mutant. A, An *ap2-1* flower showing leaf-like organs in whorl 1 and petals in whorl 2. B, An *ap2-1* Pro_{35S}:*SPL/NZZ* flower exhibiting narrow and up-curled petal-like organs in whorl 2. C, An *ap2-1* Pro_{35S}:*SPL/NZZ* flower showing all stamens in whorls two and three. D, SEM image showing a Pro_{35S}:*SPL/NZZ* flower. E, SEM image exhibiting an *ap2-1* Pro_{35S}:*SPL/NZZ* flower with up-curled stamen-like organs in whorl 2. F and G, SEM images showing a stamen-like organ (F) as well as filament-like (arrows) and anther-like (arrowheads) epidermal cells (G; high-magnification view of F) in an *ap2-1* Pro_{35S}:*SPL/NZZ* flower. H and I, SEM images displaying an up-curled stamen-like organ (H) as well as filament-like (arrows) and anther-like (arrowheads) epidermal cells (I; high-magnification view of H) in an *ap2-1* Pro_{35S}:*SPL/NZZ* flower. J, SEM image exhibiting wild-type filament cells. Bars = 0.5 mm (A–D), 100 μm (E), 50 μm (F and H), and 10 μm (G, I, and J).

gene function to whorls one and two leads to the formation of carpel and stamen-like structures in two outer whorls (Drews et al., 1991; Mizukami and Ma, 1992). In addition, ectopic expression of *AG* and *SEP3* resulted in the formation of curled rosette leaves (Fig. 1G; Mizukami and Ma, 1992; Goodrich et al., 1997; Castillejo et al., 2005). Plants impaired in function of the *CURLY LEAF* (*CLF*) gene, which is required for maintaining the repression of *AG* epigenetically, also produced curled rosette leaves (Goodrich et al., 1997). Our results demonstrated that the ectopic expression of *SPL/NZZ* promotes stamen identity in the second whorl and causes the formation of curled rosette leaves. Therefore, to test how *SPL/NZZ* controls stamen identity, we carried out regular semiquantitative and quantitative real-time reverse transcription (RT)-PCR experiments to examine the expression of *AP1*, *AP2*, *AP3*, *AG*, *SEP3*, and *CLF* in Pro_{35S}:*SPL/NZZ* and *spl-D* plants.

As expected, the expression of *SPL/NZZ* was greatly increased in both Pro_{35S}:*SPL/NZZ* and *spl-D* rosette leaves (Fig. 6A). The expression of *AG* and *SEP3* was also significantly increased, while there was no detectable change in the expression of *CLF*. We further examined gene expression in flowers. Compared with wild-type flowers, RT-PCR and semiquantitative RT-PCR results demonstrated that expression of *SPL/NZZ*, *AG*, and *SEP3* was increased in both Pro_{35S}:*SPL/NZZ* and *spl-D* flowers (Fig. 6B; Supplemental Fig. S2). Conversely, the expression of *AP2* was decreased. The expression of *AP1* was decreased only in Pro_{35S}:*SPL/NZZ* flowers, while the expression of *AP3* was unaffected (Fig. 6B; Supplemental Fig. S2). Our quantitative real-time RT-PCR results confirmed that expression of *AG* and *SEP3* was significantly increased, while the expression of *AP2* was decreased, in rosette leaves and flowers of both Pro_{35S}:*SPL/NZZ* and *spl-D* plants (Fig. 6, C–E).

To examine whether ectopic expression *SPL/NZZ* affects the expression of *AG*, *SEP3*, and *AP2* in sepals and petals, young buds from both wild-type and Pro_{35S}:*SPL/NZZ* plants were dissected and then sepals and petals were collected with the dissection microscope. Both RT-PCR and quantitative real-time RT-PCR results showed that *AG* and *SEP3* were ectopically expressed in sepals and petals of Pro_{35S}:*SPL/NZZ* flowers (Fig. 6, B–D). Additionally, the expression of *AP2* was decreased in sepals and petals (Fig. 6, B and E). In summary, our results indicate that both ectopic expression and overexpression of *SPL/NZZ* increase the expression of *AG* and *SEP3* in rosette leaves and flowers. Furthermore, the ectopic expression of *SPL/NZZ* causes ectopic expression of *AG* and *SEP3* in flower whorls one and two, which may affect the A gene function.

To test whether SPL/NZZ directly activates the expression of *AG* or *SEP3*, the expression of *AG* and

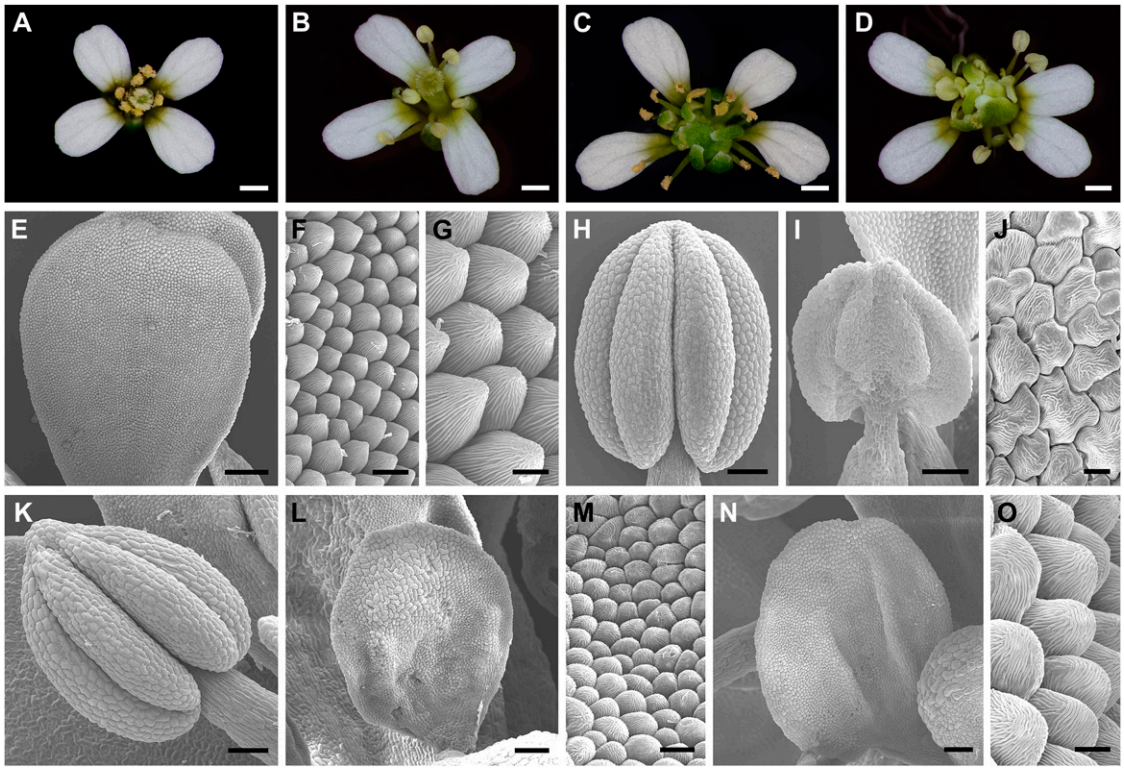

Figure 5. Loss-of-function analysis suggested that *SPL/NZZ* was involved in regulating stamen identity. A, A wild-type flower showing petals in whorl 2 and stamens in whorl 3. B, An *spl* flower exhibiting normal petals in whorl 2 and sterile stamens (no pollen released) in whorl 3. C, An *ag-4* flower showing normal petals in whorl 2 and normal stamens in whorl 3. D, An *spl ag-4* double mutant flower displaying normal petals in whorl 2 and stamens resembling those of *spl* inward whorl 2. E, SEM image exhibiting a wild-type petal. F and G, Low-magnification (F) and high-magnification (G) views of wild-type petal epidermal cells showing uniform size and cone shape. H, A wild-type anther. I, An *spl* anther showing flattened shape and undeveloped lobes. J, High-magnification view of epidermal cells of the *spl* anther, which are the same as wild-type anther epidermal cells (Fig. 2D). K, An *ag-4* anther is similar to the wild-type anther. L, An *spl ag-4* double mutant petal-like (petaloid) organ from whorl 3 showing no developed anther lobes. M, High-magnification view of epidermal cells of the organ shown in L showing the resemblance to wild-type petal epidermal cells in F. N, An *spl ag-4* double mutant petal-like organ from whorl 3. O, High-magnification view of epidermal cells of the organ shown in N, which is similar to wild-type petal epidermal cells in G. Bars = 0.5 mm (A–D), 200 μm (E), 50 μm (L and N), 20 μm (H, I, and K), 10 μm (F and M), and 5 μm (G, J, and O).

SEP3 was examined in *nzz-2* Pro_{35S}*:SPL/NZZ-GR* plants after DEX as well as both DEX and cycloheximide (Cyc) treatments. The expression of *AG* was induced by DEX alone, while when Cyc was present, the expression of *AG* did not change (data not shown). The expression of *SEP3* was rapidly induced in *nzz-2* Pro_{35S}*:SPL/NZZ-GR* inflorescences in the presence of DEX as well as both DEX and Cyc after 4 h (Fig. 6F).

To further examine whether *AG* is required for the formation of curled rosette leaves, the Pro_{35S}*:SPL/NZZ* construct was transformed into *ag-1* heterozygous plants and the *spl-D* mutant was crossed to *ag-1* heterozygous plants. The rosette leaves in both *ag-1* Pro_{35S}*:SPL/NZZ* and *ag-1 spl-D* plants also exhibited the curled-leaf phenotype (Supplemental Fig. S1). However, plants with the most severe curled-leaf phenotype were not observed. Therefore, the formation of curled leaves was not fully dependent on a functional *AG*. Our results suggest that the induction of *AG* expression might be caused by the direct activation of *SEP3* by SPL/NZZ in Pro_{35S}*:SPL/NZZ* and *spl-D* plants.

DISCUSSION

Anther development entails a series of processes, including specification of stamen identity, establishment of anther adaxial and abaxial polarity, and differentiation of reproductive microsporocytes and somatic cell layers. To date, interactions between "stamen identity" and "stamen-building" genes have not been extensively studied in the context of development, although excellent advances have been made in understanding stamen identity, early anther cell differentiation, male meiosis, pollen development, and anther dehiscence (Goldberg et al., 1993; Walbot and Evans, 2003; Jack, 2004; McCormick, 2004; Scott et al., 2004; Ma, 2005; Feng and Dickinson, 2007; Borg et al., 2009; Wilson and Zhang, 2009; Zhao, 2009). Stamen

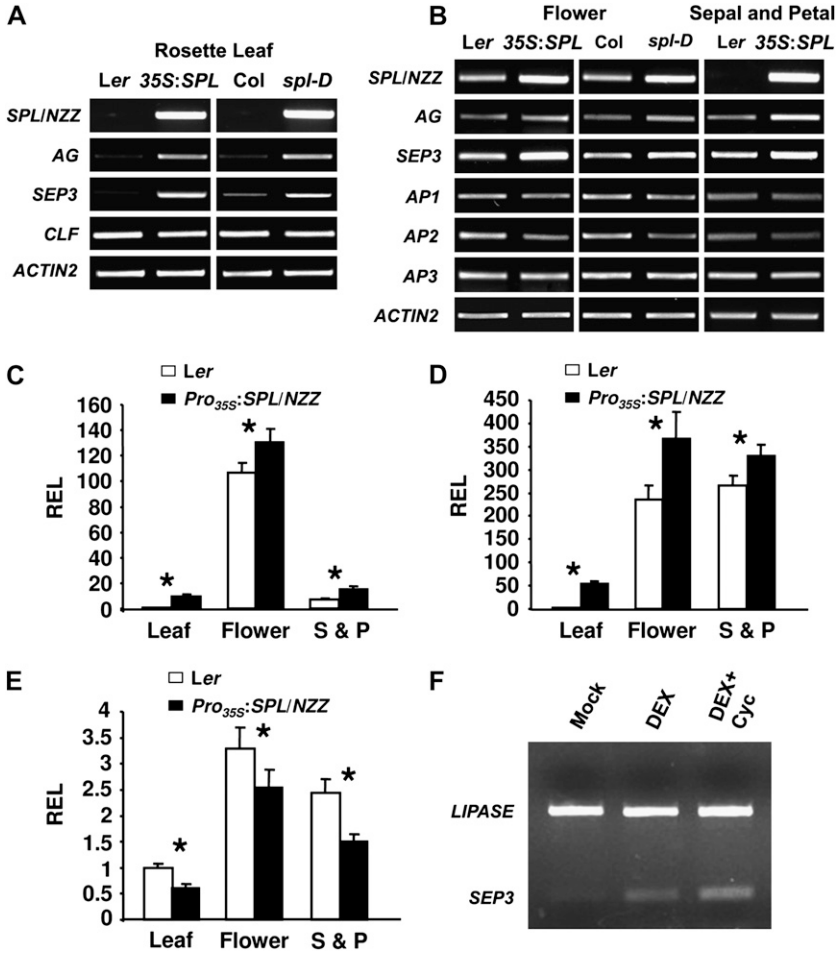

Figure 6. Ectopic expression and overexpression of *SPL/NZZ* altered the expression of genes required for stamen identity in rosette leaves and flowers. A, RT-PCR results showing greatly increased expression of *SPL/NZZ*, *AG*, and *SEP3* in both Pro_{35S}:*SPL/NZZ* and *spl-D* rosette leaves. There was no detectable change of *CLF* expression. B, RT-PCR results showing that expression of *SPL/NZZ*, *AG*, and *SEP3* was increased in Pro_{35S}:*SPL/NZZ* and *spl-D* flowers as well as Pro_{35S}:*SPL/NZZ* sepals and petals. The expression of *AP2* was slightly decreased, while the expression of *AP1* and *AP3* seemed unchanged. C, Quantitative real-time RT-PCR results showing the increased expression of *AG* in rosette leaves, flowers, sepals, and petals of Pro_{35S}:*SPL/NZZ* plants. D, Quantitative real-time RT-PCR results showing the increased expression of *SEP3* in rosette leaves, flowers, sepals, and petals of Pro_{35S}:*SPL/NZZ* plants. E, Quantitative real-time RT-PCR results illustrating the decreased expression of *AP2* in rosette leaves, flowers, sepals, and petals of Pro_{35S}:*SPL/NZZ* plants. F, Semiquantitative RT-PCR results showing the increased expression of *SEP3* in Pro_{35S}:*SPL/NZZ-GR ag-1* inflorescences after both DEX and DEX + Cyc treatments for 4 h. The *LIPASE* gene was used as a control. The transcripts in wild-type rosette leaves in C to E were used as a standard for normalization. Asterisks indicate that the difference is significant ($P < 0.01$ or $P < 0.05$). REL, Relative expression level; S & P, sepal and petal; *35S:SPL*, Pro_{35S}:*SPL/NZZ*.

identity is controlled by B, C, and E genes. The SPL/NZZ transcription factor plays a central role in regulating early anther cell differentiation, since the *spl/nzz* mutant anther is defective in producing the majority of anther cells, including endothecium, middle layer, tapetum, and microsporocyte (Schiefthaler et al., 1999; Yang et al., 1999). Different from *SPL/NZZ*, genes encoding Leu-rich repeat receptor-like protein kinases directly regulate the differentiation of fewer anther cell types in Arabidopsis (Zhao, 2009). EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS (EMS1/EXS) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1/2 determine the tapetum (Canales et al., 2002; Zhao et al., 2002; Albrecht et al., 2005; Colcombet et al., 2005). BARELY ANY MERISTEM1/2 is required for the differentiation of anther somatic cell layers (Hord et al., 2006). In addition, RECEPTOR-LIKE PROTEIN KINASE2 specifies middle layer cells (Mizuno et al., 2007). TAPETUM DETERMINANT1 defines the tapetum by acting as a potential ligand of EMS1/EXS (Yang et al., 2003; Jia et al., 2008). Therefore, SPL/NZZ may control anther cell differentiation by regulating a subset of downstream genes necessary for anther establishment. On the other hand, in this study, our gain-of-function and loss-of-function analyses demonstrated that *SPL/NZZ* is involved in controlling stamen identity. Furthermore, we showed that the ectopic expression and overexpression of *SPL/NZZ* altered the expression of several key genes that are required for stamen identity. Besides the role played in early anther cell differentiation, our results suggest that the floral organ-building gene *SPL/NZZ* might regulate stamen identity by interacting with floral organ identity genes such as *AG*, *SEP3*, and *AP2*.

SPL/NZZ may be involved in controlling stamen identity through interacting with *AG*. The *AG* gene, which encodes a MADS box protein, is essential for specifying stamens and carpels as well as repressing stem cell proliferation in the center of the flower (Yanofsky et al., 1990; Lenhard et al., 2001; Lohmann et al., 2001). The AG transcription factor controls early stamen development by directly activating the expression of *SPL/NZZ* (Ito et al., 2004). In addition, AG is necessary for regulating anther dehiscence, a late event in stamen development, by directly activating the expression of *DEFECTIVE IN ANTHER DEHISCENCE1*, which encodes a catalytic enzyme involved in the biosynthesis of jasmonic acid (Ito et al., 2007). Therefore, *AG* not only controls early stamen development,

including stamen identity and early anther cell differentiation, but also regulates late stamen development, such as anther dehiscence. Previous studies have shown that *AG* is ectopically expressed in two outer whorls in *ap2* mutant flowers, while plants ectopically expressing *AG* form *ap2*-like flowers, which have stamen-like structures in the second whorl (Drews et al., 1991; Mizukami and Ma, 1992). Our results showed that both ectopic expression and overexpression of *SPL/NZZ* result in the increased expression of *AG* in rosette leaves and flowers. In particular, the ectopic expression of *SPL/NZZ* led to ectopic expression of *AG* in sepals and petals. Therefore, the ectopic expression of *AG* may cause the flower phenotypes in Pro_{35S}:*SPL/NZZ* plants and enhance the stamen phenotype in the second whorl of *ap2-1* Pro_{35S}:*SPL/NZZ* flowers. Although Pro_{35S}:*SPL/NZZ* flowers produced abnormal sepals (Fig. 3A), we have not detected carpel-like structures in whorl 1 organs of Pro_{35S}:*SPL/NZZ* and *ap2-1* Pro_{35S}:*SPL/NZZ* flowers. Hence, *SPL/NZZ* seemed to have no carpel identity function. It is possible that the expression level of *AG* might not reach the threshold for promoting the formation of carpel-like structures in sepal-like organs of both Pro_{35S}:*SPL/NZZ* and *ap2-1* Pro_{35S}:*SPL/NZZ* flowers. The other reason could be that *AG* was not ectopically expressed in whorl 1. In *ag-1* flowers, ectopic expression of *SPL/NZZ* is able to induce microsporogenesis in whorl 3 petal-like structures, which, however, lack normal anther structure and filament morphology (Ito et al., 2004). In this study, we did not detect narrow petals in the second whorl of *ag-1* Pro_{35S}:*SPL/NZZ* flowers. Although the third whorl stamens in *spl ag-4* double mutant flowers showed petal identity, the null *SPL/NZZ* mutants appeared normal in stamen identity. Thus, SPL/NZZ should not play an equal role to AG in specifying stamen identity. The expression of *SPL/NZZ* coincides with the expression of *AG* during early anther development, when anther cell division and differentiation actively occur (Yanofsky et al., 1990; Bowman et al., 1991a; Drews et al., 1991; Ito et al., 2004; Xing and Zachgo, 2008). After the anther structure is established, *AG* is expressed in anther connective tissues and anther walls (Bowman et al., 1991a; Ito et al., 2004). Therefore, SPL/NZZ may contribute to maintaining the expression of *AG* during anther development.

The spatial and temporal specificity of *AG* expression is achieved by both positive and negative regulators. The transcription factor LEAFY (LFY) is a key positive regulator of *AG*, which binds to cis-elements in an unusually large intron of *AG* to activate its expression (Schultz and Haughn, 1991; Weigel et al., 1992; Parcy et al., 1998; Busch et al., 1999). In addition, the homeodomain transcription factor WUSCHEL activates the expression of *AG* in the center of developing flowers via binding to the same regulatory region as LFY (Mayer et al., 1998; Lenhard et al., 2001; Lohmann et al., 2001). Also, several other positive regulators of *AG* have been identified with enhancer screenings. The *ag-4* enhancers *HUA1* and *HUA2*, as well as *HUA ENHANCER4*, are involved in facilitating the processing of *AG* pre-mRNA, particularly splicing the large intron (Chen and Meyerowitz, 1999; Li et al., 2001; Cheng et al., 2003). The expression of *AG* is decreased after stage 6 in *hen2-1 hua1-1 hua2-1* flowers, suggesting that HEN2 is important for maintaining the expression of *AG* (Western et al., 2002). Recent studies found that the bZIP transcription factor PERIANTHIA directly activates the expression of *AG* (Das et al., 2009; Maier et al., 2009).

AG expression is also negatively regulated by repressors. AP2 represses the expression of *AG* in outer whorls, possibly by binding to the *AG* large intron (Drews et al., 1991; Mizukami and Ma, 1992; Jofuku et al., 1994; Sieburth and Meyerowitz, 1997; Bomblies et al., 1999). Moreover, microRNA172 acts as a negative regulator of *AP2* (Aukerman and Sakai, 2003; Chen, 2004; Zhao et al., 2007). LUG and SEU serve as corepressors to form a transcriptional repressor complex of *AG* together with DNA-binding transcription factors, including BELLRINGER, AP1, SEP3, and AGAMOUS-LIKE 24 (Liu and Meyerowitz, 1995; Conner and Liu, 2000; Bao et al., 2004; Sridhar et al., 2004, 2006; Franks et al., 2006; Gregis et al., 2006). Furthermore, polycomb group proteins CLF and EMBRYONIC FLOWER2, as well as the plant-specific protein EMF1, repress the expression of *AG* epigenetically (Goodrich et al., 1997; Sieburth and Meyerowitz, 1997; Aubert et al., 2001; Yoshida et al., 2001; Calonje et al., 2008).

Our results suggest that SPL/NZZ induces the expression of *AG* via *SEP3*. Both ectopic expression and overexpression of *SPL/NZZ* caused the increased expression of *AG*. Furthermore, ectopic expression of *SPL/NZZ* could directly induce the expression of *SEP3*. Previous studies showed that the ectopic expression of *SEP3* is sufficient to ectopically activate *AG* (Castillejo et al., 2005). Depending on regulatory conditions, SEP3 may play a positive or negative role in regulating the expression of *AG* (Sridhar et al., 2006). Very recently, studies using the ChIP-Seq technique revealed that the SEP3 binding site is located in the second intron of *AG* (Kaufmann et al., 2009). AG controls early stamen development by directly activating the expression of *SPL/NZZ* (Ito et al., 2004). Our results suggest that the positive feed-forward regulation between *AG* and *SPL/NZZ* plays an important role in stamen identity specification and stamen morphogenesis.

It is worthwhile to point out that ectopic expression and overexpression of *SPL/NZZ* led to the decreased expression of *AP2*. AG prevents the expression of *AP1* in whorls three and four of wild-type flowers (Gustafson-Brown et al., 1994). However, no report has shown that AG represses *AP2* (Jofuku et al., 1994; Chen, 2004). In *spl/nzz* anthers, the expression of *AP2* is increased 5-fold, suggesting that SPL/NZZ may repress the expression of *AP2* in the third whorl (Wijeratne et al., 2007). Therefore, it might be possible that SPL/NZZ plays a direct role in negatively regulating the

expression of *AP2*. Further studies of interactions among *SPL/NZZ*, *AG*, *SEP3*, and *AP2* as well as identifying the target genes of SPL/NZZ should lead to a better understanding of anther development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) plants are in the Landsberg *erecta* (L*er*) background, except for the *spl-D* mutant, which is in the Columbia (Col-0) ecotype. To construct the *spl ag-4* double mutant, pollen from the *ag-4* mutant was used to pollinate *spl* heterozygous plants. In the F2 generation, plants exhibiting the *ag-4* flower phenotype were genotyped for the *spl* mutant by PCR (Supplemental Table S1; Yang et al., 1999; Zhao et al., 2002). The Pro_{35S}:*SPL/NZZ-GR* line was generated in the Meyerowitz Laboratory (Ito et al., 2004). Plants were grown on Metro-Mix 360 soil at 22°C under a 16-h-light/8-h-dark cycle.

Vector Construction and Plant Transformation

The cDNA of *SPL/NZZ* was amplified by Phusion High-fidelity DNA polymerase (Supplemental Table S1; New England Biolabs) and then was cloned into the pENTR TOPO vector (Invitrogen). After verification by sequencing, the cDNA fragment was introduced into the Gateway binary vector (a gift from Dr. T. Nakagawa, Shimane University) by an LR recombination reaction using Gateway LR Clonase II enzyme mix (Invitrogen). The resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101. Wild-type, *ap2-1* mutant, and *ag-1* heterozygous plants were transformed. T0 seeds were screened for transformants on half-strength Murashige and Skoog agar plates containing 50 mg L^{-1} kanamycin and 50 mg L^{-1} hygromycin (Clough and Bent, 1998). The Pro_{35S}:*AG* construct was kindly provided by Dr. Hong Ma (Penn State University).

DEX Treatment

The Pro_{35S}:*SPL/NZZ-GR* T3 seedlings were screened on half-strength Murashige and Skoog plates containing 10 μg mL^{-1} phosphinothricin. The phosphinothricin-resistant seedlings were transferred to soil. Seedlings were then treated with a DEX solution containing 10 μM DEX and 0.015% Silwet L-77 and a mock solution (0.015% Silwet L-77 and the same concentration of ethanol used for dissolving DEX; Wagner et al., 1999; Ito et al., 2004). Rosette leaves were collected for total RNA extraction after 6 h, 12 h, 3 d, and 6 d. Inflorescences of *nzz-2* Pro_{35S}:*SPL/NZZ-GR* and *ag-1* Pro_{35S}:*SPL/NZZ-GR* plants were treated similarly for 4 h.

Phenotype Analyses and Microscopy

Micrographs were taken with an Olympus DP70 digital camera through a stereomicroscope (Olympus SZX-RFL). Samples for SEM were fixed, dried, dissected, and coated as described previously (Bowman et al., 1989; Zhao et al., 2001b). Specimens were then examined using a Hitachi S-570 scanning electron microscope.

RT-PCR and Quantitative Real-Time RT-PCR

Two-week-old rosette leaves and young buds from L*er*, Col-0, Pro_{35S}:*SPL/NZZ*, *spl-D*, and *ag-1* Pro_{35S}:*SPL/NZZ-GR* plants were collected. To examine gene expression in sepals and petals, about 500 L*er* and Pro_{35S}:*SPL/NZZ* young buds were dissected, and then sepals and petals were collected using a dissection microscope (Olympus SZ51). Total RNAs were extracted from different plant tissues using the RNeasy Plant Mini Kit (Qiagen). RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer. RT reactions were carried out using the QuantiTect Reverse Transcription Kit (Qiagen).

Primers for regular RT-PCR are listed in Supplemental Table S1. PCR cycles varied with expression levels for examined genes: for *ACTIN2*, 21 cycles in rosette leaves, flowers, sepals, and petals; for *SPL/NZZ*, 28 cycles in rosette leaves, flowers, sepals, and petals; for *AG*, 33 cycles in leaves, sepals, and petals and 29 cycles in flowers; for *CLF*, 33 cycles in leaves; for *AP1*, 28 cycles in flowers and 26 cycles in sepals and petals; for *AP2*, 28 cycles in flowers, sepals, and petals; for *AP3*, 26 cycles in flowers and 30 cycles in sepals and petals; for *SEP3*, 34 cycles in leaves and 28 cycles in flowers, sepals, and petals.

Quantitative real-time PCR was performed with a DNA Engine Opticon 2 system (Bio-Rad) using Fast SYBR Green PCR Master Mix (Applied Biosystems). The *ACTIN2* gene was used as a control. The quantitative RT-PCR results were analyzed as described previously (Pfaffl et al., 2002). Three independent experiments were repeated. Each value indicates the average and SE.

Supplemental Data

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

Supplemental Figure S1. Leaf phenotypes.

Supplemental Figure S2. Semiquantitative RT-PCR.

Supplemental Table S1. Primer list.

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