Plant Signaling & Behavior 7:9, 1151-1154; September 2012; © 2012 Landes Bioscience

Role of *SEPALLATA3* (*SEP3*) as a downstream gene of *miR156-SPL3-FT* circuitry in ambient temperature-responsive flowering

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Keywords: ambient temperature, flowering time, *miR156-SPL3-FT* circuitry, *SEP3*

Abbreviations: GA, Gibberellic acid; miRNAs, microRNAs; *SPL*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*; *FT*, *FLOWERING LOCUS T*; *SOC1*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*; *TSF*, *TWIN SISTER OF FT*; *FUL*, *FRUITFULL*; *SEP3*, *SEPALLATA3*; LD, Long-day; UTR, Untranslated region; DS, Developmental stage

SEPALLATA3 (*SEP3*) is important in determining flowering time as well as floral organ identity. Although much is known about the regulation of floral organ identity by *SEP3*, its role as a downstream gene of *FLOWERING LOCUS T* (*FT*) for the regulation of ambient temperature-responsive flowering is poorly understood. Here, we show that *SEP3* as a downstream gene of *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE3* (*SPL3*) and *FT* modulates the flowering time in response to different ambient temperatures. *SEP3* overexpression showed temperature-insensitive flowering at 23°C and 16°C. This suggests that altered *SEP3* activity affects ambient temperature-responsive flowering. However, a lesion in *SEP3* did not obviously affect ambient temperature-responsive flowering. *SEP3* expression was affected by altered *SPL3* and *FT* activities in the leaf and shoot apical regions at different temperatures. These results suggest that the *miR156-SPL3-FT* circuitry directly or indirectly regulates *SEP3* expression for the regulation of ambient temperature-responsive flowering in *Arabidopsis*.

Flowering, which is the transition from the vegetative phase to the reproductive phase, is very important for plant reproduction and survival in continuously changing environments.[1] This requires the precise perception and processing of various environmental factors and endogenous developmental cues. Thus, an extremely complicated network and a finely-tuned crosstalk control floral transition in plants. Molecular genetic analyses in *Arabidopsis thaliana* have revealed that flowering time is regulated by five major floral pathways: photoperiod, vernalization, autonomous, gibberellic acid (GA), and thermosensory.[2-4] Although some studies reported several components and mechanisms for the regulation of ambient temperature-responsive flowering in plants, the molecular mechanisms underlying the responses of plants to changes in ambient temperature are still limited.

The regulatory modules consisting of microRNAs (miRNAs) and their targets principally affect diverse growth and development in plants.[5,6] In Arabidopsis and other plant species, *miR156-SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) regulatory module is a well-known example of such modules. *miR156-SPL* regulatory modules, which are highly conserved in plant species,[7,8] play essential roles in the regulation of a variety of developmental processes.[9-15] A variety of genes, such as MADS box and MYB family genes, have been identified as downstream targets of this module. A recent report showed that the *miR156-SPL3* module controls ambient temperature-responsive flowering by regulating *FLOWERING LOCUS T* (*FT*) expression.[16]

A set of flowering time genes called floral integrators that include *FT*[17,18] and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*),[19,20] are pivotal in the integration of environmental and endogenous signals to induce flowering in Arabidopsis.[4,21] *FT* is a major output for ambient temperature-responsive flowering.[22,23] However, weakly temperature-sensitive flowering of *ft-10* mutants, an RNA null allele of *FT*, suggests that other flowering time genes, such as *SOC1* and *TWIN SISTER OF FT* (*TSF*),[24] redundantly function as outputs in the thermosensory pathway. Among the target genes of *FT* in the leaf,[25] we have shown that various loss- and gain-of-function alleles of *FRUITFULL* (*FUL*) normally respond to ambient temperature changes,[16] suggesting that *FUL* plays a limited role in the ambient temperature-responsive flowering.

Here, we show that *SEPALLATA3* (*SEP3*), a downstream gene of *SPL3* and *FT*, regulates flowering time in response to different ambient temperatures. Increased *SEP3* activity resulted in the ambient temperature-insensitive flowering at 23°C and

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Submitted: 06/22/12; Accepted: 07/05/12
http://dx.doi.org/10.4161/psb/

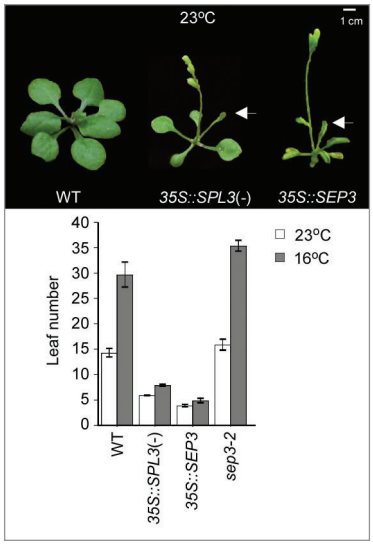

Figure 1. Flowering phenotypes of wild-type, *35S::SPL3*(-), *35S::SEP3*, and *sep3–2* plants. Photographs were taken when *35S::SPL3*(-) and *35S::SEP3* plants flowered at 23°C. *35S::SPL3*(-) and *35S::SEP3* plants are early flowering and small with curled leaves (denoted by arrows). Total leaf numbers of each plant grown at 23°C and 16°C under long-day (LD) conditions are presented. Error bars indicate the standard deviation (SD).

16°C. Also, *SEP3* expression was affected by altered *SPL3* and *FT* activities. Our results suggest that a model in which the *miR156-SPL3-FT* circuitry directly or indirectly regulates *SEP3* expression in the leaf and shoot apical regions to control ambient temperature-responsive flowering in *Arabidopsis.*

SEP3 is Involved in the Ambient Temperature-Responsive Flowering

Because overexpression of *SEP3* showed an early flowering and curled leaf phenotype, as seen in *35S::SPL3*(-) [a miR156-resistant version with the miR156 binding element mutated] and *35S::FT* plants[25] (**Fig. 1**), we investigated the phenotype of loss- and gain-of function mutants of *SEP3* (*sep3–2* and *35S::SEP3* plants, respectively) at 23°C and 16°C under long-day (LD) condition. *35S::SEP3* plants flowered with a similar number of leaves at both temperatures (3.9 and 4.9 leaves at 23°C and 16°C, respectively) (**Fig. 1**). This indicated that the flowering of *35S::SEP3* plants was almost insensitive to the changes in ambient temperature. This further suggested that altered *SEP3* activity can affect the ambient temperature-responsive flowering. Also, the curled leaf phenotype seen in *35S::SEP3* plants grown at 23°C was attenuated at the lower temperature (data not shown). This phenotypic attenuation at 16°C was also found in *35S::SPL3*(-) and *35S::FT* plants.[16,25] This suggests that low temperature influences leaf curling in these transgenic plants overexpressing *SEP3*, *SPL3*(-), or *FT*. In contrast to *35S::SEP3* plants, *sep3–2* mutants showed slightly late flowering at 23°C and 16°C (15.5 and 35.4 leaves, respectively). This indicated that *sep3–2* mutants normally responded to ambient temperature changes like wild-type plants.

SPL3 and *FT* Positively Regulate *SEP3* Expression in the Leaf and the Shoot Apical Regions

Because *35S::SEP3* plants showed temperature-insensitive flowering as similarly seen in *35S::SPL3*(-) plants (**Fig. 1**), we further investigated the *SEP3* expression in *35S::SPL3*(+) plants [*35S::SPL3*(+) plants had an intact miR156 binding element in its 3'-untranslated region (UTR)] and *35S::SPL3*(-) plants at defined growth stages (DS)1.02 and DS1.04.[26] We used a developmentally synchronized stage to compare *SEP3* expression levels due to the altered plastochron length of *SPL3*-overexpressing plants.[16] In *35S::SPL3*(-) plants at growth stage DS1.02, *SEP3* expression was significantly increased at 23°C and 16°C (**Fig. 2A**). However, the levels of *SEP3* expression were not altered in *35S::SPL3*(+) plants at both temperatures, compared with those in wild-type plants. This suggested that the miR156-sensitive *SPL3* version does not affect *SEP3* expression. Moreover, at DS1.04, the upregulation of *SEP3* was more apparent in *35S::SPL3*(-) plants. This indicated that increased *SPL3* activity upregulated *SEP3* expression at different temperatures.

We further analyzed *SEP3* expression in the leaf and the shoot apical region of *35S::SPL3*(-), *ft-10,* and *35S::FT* plants, because *SEP3* is expressed in the leaf and the shoot apex,[27] and *SEP3* is a downstream gene of *FT* in the leaf.[25] In the leaf of 8-d-old *35S::FT* plants, *SEP3* expression was strongly upregulated at 23°C and 16°C (**Fig. 2B**), consistent with the results reported previously.[25] However, the downregulation of *SEP3* were more apparent in *ft-10* plants grown at 23°C than at 16°C. Furthermore, the upregulation of *SEP3* was observed in the leaf of 8-d-old *35S::SPL3*(-) plants. This suggested that *SEP3* is a downstream gene of *SPL3* and *FT* in the leaf. In the shoot apical region of *35S::SPL3*(-) plants, *SEP3* expression was highly increased at both temperatures (**Fig. 2B**). Collectively, these results suggested that *SEP3* is regulated by *SPL3* and *FT* in the leaf, whereas *SPL3* regulates *SEP3* in the shoot apical region.

Here, we have demonstrated that increased *SEP3* activity affects flowering time by the changes in ambient temperature. Also, we showed that *SEP3* acts downstream of *SPL3* and *FT* in the leaf and the shoot apical regions. Based on these results, we propose that *SEP3* acts as a downstream gene of *SPL3* and *FT* in the leaf and the shoot apical regions to modulate ambient temperature-responsive flowering (**Fig. 3**). In the leaf, changes in miR156 activity by different ambient temperatures affect transcriptional regulation of *FT* by directly binding of SPL3 protein to *FT* genomic region.[16] Because FT protein requires partner-dependent transcriptional regulation, the regulation of *SEP3* through the *FT-X* (unknown factor) module controls flowering time by the changes in different ambient temperatures. It is also possible that SPL3 protein could directly bind to *SEP3* genomic loci to regulate *SEP3* expression. Because the misexpressed version of *rSPL3* (miR156-resistant version) and *FT* in the shoot apex (*FD::rSPL3* and *FD::FT* plants) still showed ambient temperature-responsive flowering,[16] the *miR156-SPL3* module directly regulates *SEP3* expression for age-dependent flowering in the shoot apical region.[9] However, we cannot exclude the

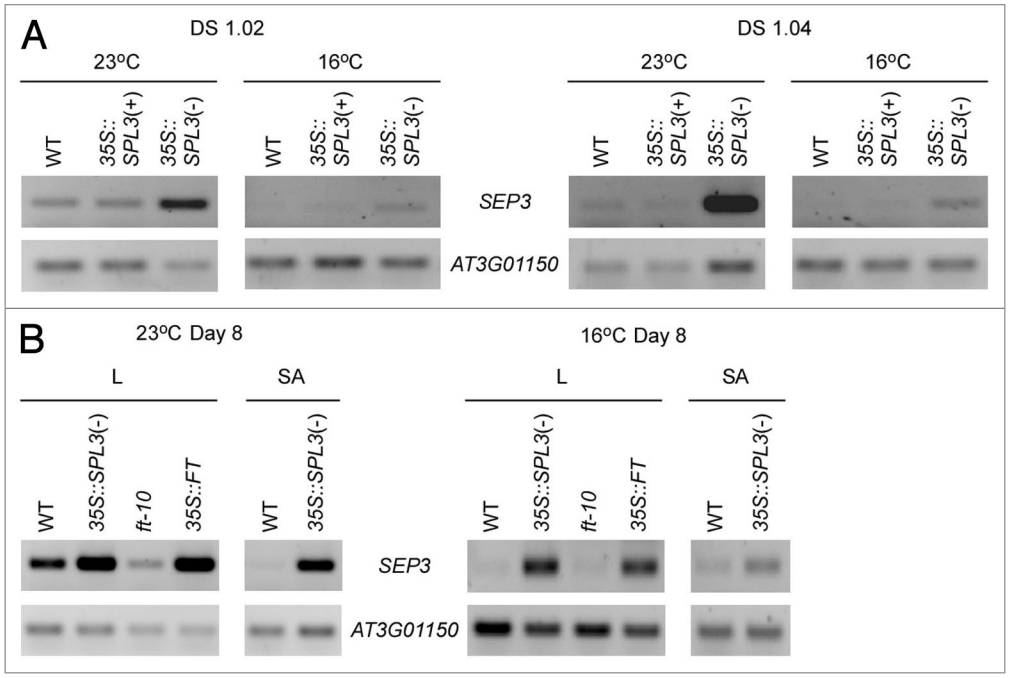

Figure 2. *SEP3* expression in *35S::SPL3*(+), *35S::SPL3*(-), *35S::FT*, and *ft-10* plants. (A) RT-PCR analyses of *SEP3* expression at defined growth stages (DS)1.02 and DS1.04[26] of wild-type (WT), *35S::SPL3*(+), and *35S::SPL3*(-) plants grown at 23°C and 16°C under LD conditions. (B) RT-PCR analyses of *SEP3* expression in the leaves (L) and in the shoot apical regions (SA) of 8-d-old seedlings in WT, *35S::SPL3*(-), *ft-10*, and *35S::FT* plants grown at 23°C and 16°C under LD conditions. AT3G01150 was used as an internal control.[29]

possibility that the regulation of *SPL3* via *FT-FD* module affects *SEP3* activity to modulate photoperiodic flowering in the shoot apical region, because FD protein binds to the G-box motifs in the *SPL* genomic region.[16,28] Thus, further investigation is required to elucidate the mechanism of interaction between the *FT-X* module and *SEP3* in the leaf and between *SPL3* and *SEP3* in the shoot apical region. Furthermore, because loss-of-function mutants of *SEP3* showed ambient temperature-responsive flowering, the temperature response of *ft sep3* double mutants will shed light on the question whether *SEP3* redundantly acts as an output gene with *FT* in the control of flowering time by the changes in different ambient temperatures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Creative Research Initiatives of the National Research Foundation for the Ministry of Education, Science and Technology (R16–2008–106–01000–0 to J.H.A.) and by the Brain Korea 21 program (to J.H.L).

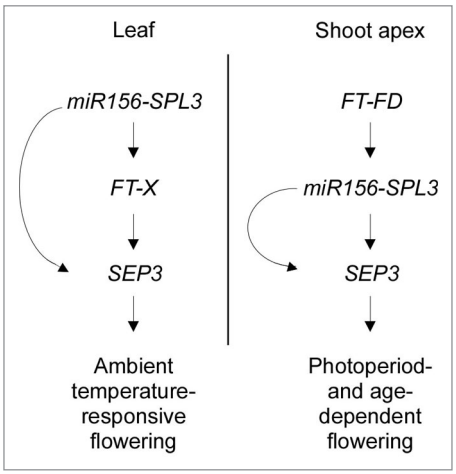

Figure 3. A proposed molecular interaction between the *miR156-SPL3-FT* circuitry and *SEP3* for ambient temperature-responsive flowering. In the leaves, changes in ambient temperature affect the *miR156-SPL3* module, which regulates the *FT* expression by directly SPL3 binding to the *FT* genomic region.[16] Because *FT* functions through partner-dependent transcriptional activation of unknown factors, the *FT-X* module controls *SEP3* expression to regulate ambient temperature-responsive flowering. However, we cannot dismiss the possibility that SPL3 protein directly binds to *SEP3* loci. In the shoot apical region, the *miR156-SPL3* module regulates *SEP3* expression to modulate age-dependent flowering.[9] Furthermore, the *FT-FD* module positively regulates *SPL3* expression, which affects *SEP3* expression for the photoperiodic flowering.[28] Arrows represent promotion effects.

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