Flowering Time Modulation by a Vacuolar SNARE via FLOWERING LOCUS C in Arabidopsis thaliana

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Abstract

The transition of plant growth from vegetative to reproductive phases is one of the most important and dramatic events during the plant life cycle. In Arabidopsis thaliana, flowering promotion involves at least four genetically defined regulatory pathways, including the photoperiod-dependent, vernalization-dependent, gibberellin-dependent, and autonomous promotion pathways. Among these regulatory pathways, the vernalization-dependent and autonomous pathways are integrated by the expression of FLOWERING LOCUS C (FLC), a negative regulator of flowering; however, the upstream regulation of this locus has not been fully understood. The SYP22 gene encodes a vacuolar SNARE protein that acts in vacuolar and endocytic trafficking pathways. Loss of SYP22 function was reported to lead to late flowering in A. thaliana plants, but the mechanism has remained completely unknown. In this study, we demonstrated that the late flowering phenotype of syp22 was due to elevated expression of FLC caused by impairment of the autonomous pathway. In addition, we investigated the DOC1/BIG pathway, which is also suggested to regulate vacuolar/endosomal trafficking. We found that elevated levels of FLC transcripts accumulated in the doc1-1 mutant, and that syp22 phenotypes were exaggerated with a double syp22 doc1-1 mutation. We further demonstrated that the elevated expression of FLC was suppressed by ara6-1, a mutation in the gene encoding plant-unique Rab GTPase involved in endosomal trafficking. Our results indicated that vacuolar and/or endocytic trafficking is involved in the FLC regulation of flowering time in A. thaliana.

Introduction

Membrane trafficking is a major regulatory system for protein transport in eukaryotic cells. Evolutionary conserved regulatory molecules play substantial roles in regulating the budding of transport vesicles from organelles and the fusion to target membranes. For example, SAR/ARF GTPases regulate budding processes, and RAB GTPases and soluble N-ethyl-maleimide sensitive factor attachment protein receptors (SNAREs) are key regulators of membrane tethering and fusion in any medium, provided the original author and source are credited.

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mechanism that linked flowering regulation to vacuolar/endocytic trafficking. In this study, we examined the mechanism underlying the late flowering phenotype of syp22 to unveil this hidden link.

Upon floral induction, plants in the vegetative stage start producing floral meristems around the shoot apical meristem (SAM). In A. thaliana, the FT gene encodes an important floral inducer that is synthesized in leaves and transported to the SAM to induce expression of other floral inducers, including the SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1).

Expression of FT and SOC1 in A. thaliana is known to be regulated by at least four distinguishable genetic pathways: the photoperiodic-dependent, gibberellin-dependent, vernalization-dependent, and autonomous promotion pathways [12,13]. The photoperiodic pathway modulates expression of FT in leaves in response to changes in the photoperiod [14]. The gibberellin pathway regulates flowering by controlling expression of SOC1 in the SAM [15]. The vernalization and autonomous pathways cooperatively modulate expression of FLC, which represses the expression of FT and SOC1 by directly interacting with the promoter regions of these genes [16]. Thus, FLC is a key integrator of the vernalization-dependent and autonomous promotion pathways.

To date, many putative nuclear factors have been identified that act in the autonomous pathway, including the RNA-binding or RNA-processing proteins, FCA, FPA, FLK, and FY, and chromatin modifiers, FVE and FLD [13]. However, no studies have reported the involvement of membrane trafficking in the regulation of FLC expression. Our results highlighted an unexpected link between the vacuolar SNARE and autonomous regulation of flowering in A. thaliana.

Results

syp22-1 was Defective in Determination of Floral Meristem Identity

Previously, syp22-1 was reported to exhibit a broad range of phenotypes, including weak late flowering under continuous light (CL) (Fig. 1A) [9]. First, we investigated whether there was a functional link between SYP22 and floral meristem identity determination. Under normal growth conditions (CL, 23°C), determination of the floral meristem identity was not affected in syp22-1 (Fig. 1B). However, when grown under CL at 16°C, or under short-day conditions (SD: 8 h light and 16 h dark) at 23°C, the floral meristems of syp22-1 were frequently converted to inflorescence meristems (Fig. 1C, D), and aerial rosettes were occasionally produced (Fig. 1E). A similar phenotype has been reported for other late flowering mutants, including fld-2, a mutant defective in the autonomous pathway [17].

We then examined whether the abnormality in determination of floral meristem identity was related to the regulatory mechanism involving LEAFY (LFY), a key determinant of meristem identity. We generated double mutants between syp22-1 and either a weak (lfy-2) or null (lfy-1) allele. The syp22-1 mutation exaggerated the lfy-2 phenotype, which suggested that the two genes operated in a synergistic manner; the double mutant syp22-1 lfy-2 exhibited an indistinguishable phenotype from lfy-1, even when the double mutant plants were grown at 23°C in CL (Fig. 2A). On the other hand, the double mutant syp22-1 lfy-1 exhibited the same phenotype as lfy-1. Similar synergistic enhancement of weak alleles of lfy mutations was reported in double mutants of lfy combined with other late flowering mutations, fl, flr, and fld [17,18,19].

We then analyzed the morphology of 15 proximal axillary organs that formed along the stems of wild-type and mutant plants. Compared with wild type and syp22-1, the lfy-2 plants generated a larger number of lateral shoots, with or without a bract, instead of flowers. This was also synergistically enhanced in the double mutant, syp22-1 lfy-2 (Fig. 2B, p<0.01, Student’s t-test). These results indicated that impairment of SYP22 function caused a deleterious effect on the determination of meristem identity through a pathway that involved LFY.

syp22-1 Responded Normally to Gibberellin and Vernalization

The four genetically distinguishable pathways that regulate the flowering time of A. thaliana are integrated by the expression of flowering pathway integrators, including the LFY gene [20]. To examine whether the syp22-1 mutant was defective in these regulatory pathways, we first tested the syp22-1 response to gibberellic acid (GA3). We found that GA3 treatment significantly promoted the flowering of syp22-1 (Fig. 3A and Fig. S1A, p<0.01, Student’s t-test); this indicated that syp22-1 retained responsiveness to gibberellin. Thus, the late flowering phenotype of syp22-1 did not seem to be caused by impairment in the gibberellin-dependent pathway.

Next, we tested the syp22-1 response to the photoperiod. The wild-type plants (Columbia accession) used in this study flowered after generating 12.1±1.1 leaves under CL conditions and after generating 42.7±14.8 leaves under SD. On the other hand, the syp22-1 mutant generated a significantly larger number of leaves (79.8±23.4 leaves) when grown under SD than the wild type plants grown under SD (Fig. 3B and Fig. S1B, p<0.01, Student’s t-test). In contrast, the late flowering mutants defective in the photoperiod-dependent pathway are known to be insensitive to changes in photoperiod [21]; thus, the photoperiodic pathway did not seem to be markedly affected by the syp22-1 mutation.

We then demonstrated that syp22-1 responded normally to vernalization; vernalization treatment for 4 weeks at 4°C promoted flowering of syp22-1 under SD (Fig. 3B, p<0.01, Student’s t-test). These results indicated that SYP22 was involved in the regulation of flowering time through regulatory pathways other than the above three pathways. Furthermore, we confirmed that the late flowering phenotype could be rescued by introducing the GFP-SYP22 chimeric gene into the syp22-1 mutant, under the regulation of its own promoter (Fig. S2); this confirmed that the late flowering phenotype was due to a loss of function in SYP22.

Expression Level of FLC was Elevated in syp22-1

The results mentioned above strongly suggested that the late flowering of syp22-1 was caused by impairment in the last of a series of major regulatory pathways, the autonomous promotion pathway. FLC is a key regulator of the autonomous pathway. FLC encodes a MADS transcription factor that represses the expression of the floral pathway integrators, FT, SOC1, and LFY [13]. Thus, we reasoned that, if delayed flowering of syp22-1 was due to a defect in autonomous flowering promotion, then the mRNA level of FLC might be altered in this mutant. Accordingly, we examined the expression level of FLC and the floral pathway integrators in 14-day-old seedlings of the syp22-1 mutant by quantitative RT-PCR (qRTPCR). In syp22-1, FLC mRNA expression exceeded that of wild type by about three-fold (Fig. 4, p<0.01, Student’s t-test). Moreover, the mRNA expression of FT was significantly reduced compared to wild type (Fig. 4, p<0.01, Student’s t-test). In the syp22-1 mutant transformed with GFP-SYP22, accumulation of mRNA for FLC was comparable to that in wild type (Fig. S2C). These results strongly suggested that the late flowering phenotype of syp22-1 could be ascribed to elevated expression of FLC. We also confirmed this genetically; we reasoned that, if the elevated expression of FLC mRNA in syp22-1 had directly caused its late
flowering phenotype, then the elimination of FLC activity should suppress this phenotype. To test this, we generated a double mutant of syp22-1 and flc-3, a null allele of flc, in a Columbia background. The endogenous expression of FLC was low in the Columbia accession, and flc-3 exhibited a phenotype similar to wild type when grown in CL (Fig. 5) [22]. On the other hand, as expected, the flc-3 syp22-1 double mutant generated a significantly smaller number of leaves (14.2 ± 3.4 leaves) before flowering than syp22-1 (23.7 ± 3.9 leaves, p<0.01, Student’s t-test) under CL conditions; thus, the double mutant was comparable to the wild type (12.3 ± 1.1 leaves) and flc-3 (11.4 ± 1.7 leaves) plants (Fig. 5). These results clearly demonstrated that the elevated level of FLC mRNA expression conferred a late flowering phenotype on syp22-1. Among the pleiotropic phenotypes of syp22-1, the flc-3 mutation suppressed only the late flowering phenotype; in contrast, other phenotypes, like wavy leaves and semi-dwarfism, were not remedied in the flc-3 syp22-1 double mutant (Fig. 5A). This indicated that the late flowering phenotype of syp22-1 was conferred by a mechanism distinct from that responsible for other phenotypes, which are most likely due to impairments in polar auxin transport.

Mutations in BIG, a Putative Regulator of Membrane Trafficking, and ARA6 also Altered FLC Expression

The results described above strongly suggested that vacuolar and/or endocytic trafficking pathways were involved in autonomous regulation of flowering. We then examined whether other membrane trafficking mutants exhibited a similar phenotype. We tested whether a genetic mutant of BIG, which encodes a Calossin-like protein, might also exhibit elevated FLC expression. BIG was
originally identified as the gene responsible for the doc1 mutation [23]. BIG was also reported to be required for the auxin-mediated inhibition of endocytosis [24]. Furthermore, treatment with naphthalphthalamic acid (NPA), an inhibitor of polar auxin transport, altered subcellular localization of PIN1 in the doc1 mutant [23]. Based on these results, mutations in BIG were implicated in impairments of membrane trafficking pathways, including the endocytic and/or vacuolar transport pathways. Intriguingly, some mutant alleles of BIG have been also reported to exhibit the late flowering phenotype [25,26]. These results implied that BIG was also involved in regulation of FLC expression. Among the big mutations, the allele that caused the most severe late flowering phenotype was doc1-1 [26]. Under our CL conditions, doc1-1 exhibited a late flowering phenotype (13.9 ± 1.2 leaves, Fig. 6A, C) compared with wild type (12.0 ± 0.8 leaves); this phenotype was similar to that of syp22-1 (16.3 ± 1.7 leaves, Fig. 6A, C). We then generated a doc1-1 syp22-1 double mutant, which exhibited a more severe late flowering phenotype (21.0 ± 3.6 leaves, Fig. 6A–C, p < 0.01, Student’s t-test). A qRT-PCR analysis also indicated that the level of FLC mRNA expression was slightly increased in doc1-1, and this was enhanced in the double mutant, doc1-1 syp22-1 (Fig. 6C). Thus, BIG and SYP22 could be involved in the same regulatory pathway for FLC expression.

We then examined the effect of a mutation in the other membrane trafficking regulator, ARA6, which is a plant-unique Rab GTPase involved in trafficking between multivesicular endosomes and the plasma membrane [27]. We have already reported that the ara6-1 mutation suppressed the late flowering phenotype of syp22-1. We examined whether the elevated expression level of FLC in syp22-1 is also suppressed by ara6-1, and found that FLC expression was significantly reduced in ara6-1 syp22-1 (Fig. 6D, p < 0.05, Student’s t-test). This result also
supports a possible link between membrane trafficking and flowering regulation.

**Discussion**

The *syp22* mutants have exhibited pleiotropic phenotypes, including late flowering, semi-dwarfism, waved leaves, immature leaf vascular tissues, and excessive idioblast differentiation. All these phenotypes, except late flowering, have been thought to result from a misdistribution of the phytohormone, auxin [9,10,11]. In contrast, the reason that mutations in *SYP22* engendered late flowering remained unclear. In the present study, we demonstrated that elevated expression of *FLC* in the *syp22-1* mutant was a direct cause of the late flowering phenotype (Figs. 4 and 5). The *syp22-1* mutant was capable of responding to vernalization treatment (Fig. 3D), which suggested that the *syp22-1* mutation resulted in impairment in the autonomous promotion pathway. We further demonstrated that the expression level of *FLC* was elevated in another membrane trafficking mutant, *doc1-1* (Fig. 6A–C), and that *ara6-1*, a mutation in the gene encoding a plant-unique Rab GTPase, suppressed elevated expression of *FLC* in *syp22-1* (Fig. 6D). These results indicated that endocytic and/or vacuolar trafficking pathways were involved in the autonomous promotion of flowering in the Columbia accession of *A. thaliana*.

Our findings clearly indicated that the vacuolar SNARE, SYP22, was required for proper regulation of *FLC*, a key regulator of flowering time. This result may elucidate the recent finding that *TERMINAL FLOWER 1* (*TFL1*), a homolog of *FT*, which is required for repression of the transition from the inflorescent meristem to the floral meristem, played a critical role in membrane trafficking to protein storage vacuoles (PSVs) [28]. Based on that finding, the authors proposed a novel function of the

![Figure 3. The *syp22-1* mutant responded normally to gibberellic acid, photoperiodic flowering induction, and vernalization treatment. (A) The number of rosette leaves in wild type (WT) and *syp22-1* mutants treated with (red) or without (blue) GA$_3$. Results are presented as the means ± S.D. (n = 6 plants). (B) The total leaf numbers in wild type and *syp22-1* under SD with (red) or without (blue) vernalization. Results are presented as the means ± S.D. (n = 17 plants for SD and n = 10 plants for SD+ vernalization). Flowering of *syp22-1* was delayed in SD, which was suppressed by vernalization treatment (8 weeks at 4 °C). doi:10.1371/journal.pone.0042239.g003](image1)

![Figure 4. Expression level of *FLC* was elevated in *syp22-1* mutants. The expression levels of *FLC*, *FT*, *LFY*, and *SOC1* in 14-day-old wild type (WT), *syp22-1*, and *fve-4* seedlings were examined by qRT-PCR. In *syp22-1* plants, expression levels of *FLC* were elevated, which resulted in downregulation of downstream flowering pathway integrators. *fve-4*, an autonomous pathway mutant, was used as a control. Results are presented as means ± S.D. (n = 3–12). doi:10.1371/journal.pone.0042239.g004](image2)
PSV: the storage of factors necessary for flowering and meristem maintenance. Other genetic studies indicated that TFL1 acted downstream of FVE and FCA, which are upstream regulators of FLC in the autonomous pathway [19,29]. In addition, tfl1-1, a loss-of-function mutant of TFL1, exhibited an early flowering phenotype and reduced expression of FLC [30]. Those results suggest that TFL1 may also be involved in autonomous promotion of flowering. In our previous study, we found that the syp22 mutant also exhibited defects in the biogenesis of PSVs in A. thaliana [6]. Taken together, these results suggest the hypothesis that syp22-1 is defective in transport or storage of the flowering factors harbored in PSVs that affect autonomous promotion of flowering via modulation of FLC expression. Our present results clearly indicated that the flowering phenotype of syp22-1 was caused by a mechanism genetically separable from that responsible for other phenotypes, like semi-dwarfism and wavy leaves, which are most likely due to abnormal polar auxin transport. However, the predicted flowering regulators could be transported via the same trafficking pathway as the efflux carriers of auxin, because mutants of both SYP22 and BIG/DOC1 also exhibited abnormalities in polarized localization of the PIN1 protein [10,23].

Another potential hypothesis is that SYP22 could be involved in transcriptional regulation of FLC via chromatin remodeling. In mammalian cells, an adaptor protein containing PH domain, PTB

Figure 5. The late flowering phenotype of syp22-1 was suppressed by flc-3. (A) Wild type (WT), syp22-1, flc-3, and flc-3 syp22-1 plants were grown in CL for 30 days at 23°C. (B) Numbers of rosette leaves are shown for wild type (WT) and mutant plants grown under the same conditions as those in (A). Results are presented as means ± S.D. (n = 6 or 7 plants). The flc-3 mutation suppressed only the late flowering phenotype of the syp22-1 phenotypes.
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the nucleus [31]. APPL1 was also shown to interact with the RAB5 effector, which transmits a signal from the endosomes to the cell membrane. APPL1 domain, and leucine zipper motif 1 (APPL1) has been identified as a RAB5 effector, which transmits a signal from the endosomes to the nucleus [31]. APPL1 was also shown to interact with the effector, which transmits a signal from the endosomes to the cell membrane. APPL1 domain, and leucine zipper motif 1 (APPL1) has been identified as a RAB5 effector, which transmits a signal from the endosomes to the nucleus [31]. APPL1 was also shown to interact with the

The elevated expression level of FLC was suppressed by the ara6-1 mutation. Expression levels of FLC were normalized by the expression of TUA3. Results are presented as means ±S.D. (n = 5 experiments).

Materials and Methods

Plant Materials and Growth Conditions

A. thaliana (Columbia accession) seeds were sown on MS plates after sterilization. After a 2-day incubation at 4°C, plants were grown at 23°C under CL or SD (8 h light, 16 h dark). After 14 days (CL) or 21 days (SD) of incubation on plates, plants were transferred to the soil. For the vernalization treatment, plants were grown for 3 weeks at 4°C under CL, then incubated at 4°C for 3 weeks in SD.

The lfy-1 and lfy-2 mutants were obtained from ABRC; the fve-4 [33] and fve-3 mutants [22] were kindly provided by T. Araki (Kyoto Univ.). The doc1-1 mutant [35] was a kind gift from S. Sawa (Univ. of Tokyo). The ara6-1 mutant [33] was a kind gift from S. Sawa (Univ. of Tokyo). syp22-1, doc1-1 and ara6-1 syp22-1 8-week-old seedlings were examined by qRT-PCR.

GA3 Treatment

Seeds were sown on MS plates and cultured for 14 days under CL conditions. Plants were transferred to the soil, and then sprayed with 20 μM GA3 (Wako) or water twice per week.

qRT-PCR

RNA was extracted from the aerial parts of 14-day-old plants with the RNAqueous-4 PCR kit (Ambion). Superscript III (Invitrogen) was used for reverse transcription. qRT-PCR was performed on a Light cycler 480 system (Roche) with specific sets of primers, including FLC: FLCf. 5’-ttctcaacaagcttcaacatgag-3’ and FLCr. 5’-cttcctaaagttctctcgacta-3’ and FLCr. 5’-cttcctaaagttctctcgacta-3’ and FLCr. 5’-cttcctaaagttctctcgacta-3’. Primers and probes were selected according to the Universal Probe Library (Roche). Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are: At5g14680 (STP22/VAM3/ SGR3), At5g61850 (FT), At1g54080 (BIF), At2g45660 (SOC1), At5g10140 (FLC), At2g19520 (FVE), At3g34840 (ARA6), and At3g02260 (BIG/DOC1).

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Figure 6. Effect of doc1-1 and ara6-1 mutations on expression of FLC in syp22-1. (A) Wild type (WT), syp22-1, doc1-1, and doc1-1 syp22-1 plants were grown in CL for 41 days at 23°C. (B) doc1-1 syp22-1 plants were grown in CL for 41 days at 23°C. (C) Numbers of rosette leaves (blue) and expression levels of FLC (red) are shown for wild type (WT), syp22-1, doc1-1, and doc1-1 syp22-1 plants grown in CL at 23°C. Results are presented as means ±S.D. (n = 5 experiments). (D) Numbers of rosette leaves (blue) and expression levels of FLC (red) are shown for wild type (WT), syp22-1, doc1-1, and ara6-1 syp22-1 plants grown in CL at 23°C. Results are presented as means ±S.D. (n = 5 experiments).
Supporting Information

Figure S1 The syp22-1 mutant responded normally to gibberellic acid and photoperiodic flowering induction. (A) The syp22-1 mutant was grown in CL for 30 days at 23°C, with (right) or without (left) GA3 treatment. (B) Wild-type (left) and syp22-1 mutant (right) grown under short-day conditions (SD, 8 h light/16 h dark) for 65 days at 23°C.

Figure S2 Phenotypes of syp22-1 were rescued by expressing GFP-SYP22. (A) Wild type (WT), syp22-1 (c), and two independent transgenic syp22-1 lines (#3-1 and #6-2) rescued with GFP-SYP22 expression under the regulation of the authentic promoter (proSYP22::GFP-SYP22) were grown under SD for 60 days at 23°C. (B) Numbers of rosette leaves are shown for wild type (WT), syp22-1, and syp22-1 rescued with GFP-SYP22 expression. Results are presented as means ± S.D. (n = 6 plants).

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