The B-Box Family Gene STO (BBX24) in Arabidopsis thaliana Regulates Flowering Time in Different Pathways

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Abstract

Flowering at the appropriate time is crucial for reproductive success and is strongly influenced by various pathways such as photoperiod, circadian clock, FRIGIDA and vernalization. Although each separate pathway has been extensively studied, much less is known about the interactions between them. In this study we have investigated the relationship between the photoperiod/circadian clock gene and FRIGIDA/FLC by characterizing the function of the B-box STO gene family. STO has two B-box Zn-finger domains but lacks the CCT domain. Its expression is controlled by circadian rhythm and is affected by environmental factors and phytohormones. Loss and gain of function mutants show diversiform phenotypes from seed germination to flowering. The sto-1 mutant flowers later than the wild type (WT) under short day growth conditions, while over-expression of STO causes early flowering both in long and short days. STO over-expression not only reduces FLC expression level but it also activates FT and SOC1 expression. It also does not rely on the other B-box gene CO or change the circadian clock system to activate FT and SOC1. Furthermore, the STO activation of FT and SOC1 expression is independent of the repression of FLC; rather STO and FLC compete with each other to regulate downstream genes. Our results indicate that photoperiod and the circadian clock pathway gene STO can affect the key flowering time genes FLC and FT/SOC1 separately, and reveals a novel perspective to the mechanism of flowering regulation.

Introduction

There are several key developmental changes during the plant lifecycle. One of these is flowering, the correct timing of which is critical for reproductive success [1]. Physiological and genetic studies have shown that multiple pathways can promote or repress flowering [1] [2] [3]. The floral pathway integrators FLOWERING LOCUS C (FLC), FLOWERING LOCUS T (FT), SUPPRESSOR OF CONSTANS (SOC1) and LEAFY (LFY) are all involved in this transition. The MADS box gene FLC is a central repressor of flowering in Arabidopsis [4] [5], and its expression is regulated by vernalization and an autonomous pathway involved in chromatin regulation, transcription level and co-transcriptional RNA metabolism [6]. Vernalization pathway genes (VRN1, VRN2, VRN3, VIL1/VRN5, atPRMT5) repress FLC expression by histone modification of FLC during and after the cold treatment [7] [8] [9] [10] [11] [12].

Arabidopsis has two antagonistic pathways that regulate FLC expression. The FRIGIDA (FRI) pathway is a positive regulator, while a group of genes that belong to the autonomous floral-promotion pathway are negative regulators (i.e., LD, FLD, FCA, FT, FVE). FRI is a unique plant gene that encodes a nuclear-localized protein with a coiled-coil domain [13]. The functional allele of FRI is only found in the winter-annual Arabidopsis, which requires vernalization to flower rapidly in the spring through repression of FLC. In rapid-cycling Arabidopsis there is no functional FRI; FLC expression is kept at low levels and the photoperiod pathway accelerates flowering. FLC directly binds and represses the two important flowering genes FT and SOC1 [14]. FT is the “florigen” acting as a long distance signal that is transported from leaves to the shoot meristem [15] [16] [17] [18]. The MADS-box gene SOC1 was initially cloned as a suppressor of the CONSTANS (CO) overexpressor [19] [20]. SOC1 regulation integrates inputs from multiple flowering pathways including photoperiod, vernalization, aging and GA [12] [21]. Besides GA, other phytohormones (BR, ethylene and ABA) also play important roles in flowering, but the underlying mechanisms are less well understood [22] [23] [24]. SOC1 can also be considered as a meristem-identity gene because it maintains the meristem in a floral state [25]. Both FT and SOC1 are regulated by different flowering pathways [20] [26]. The effect of day length within the photoperiod pathway has been extensively studied [21] [27].

Arabidopsis senses changes in day length by the circadian clock, which in turn regulates the transcription factor CO. CO belongs to a subfamily of the zinc finger protein family that is now known as the B-Box Zinc Finger Family (BBX) [28]. This family consists of 32 genes divided into five structural groups from I to V. Proteins in group I (including CO) all contain a B-box B1, a B-box B2, and a C-terminal CCT (CO/COL/TOC1) domain. Group II members are similar to group I, and contain both B1, B2 and CCT domains, but have minor differences in their B2 domains. Proteins from group III only contain a B1 and CCT domain, while group
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Materials and Methods

Growth conditions and plant material

Plants were grown in soil under controlled conditions of LD (16 h light/8 h dark) or SD (8 h light/16 h dark) at 22°C. The level of photosynthetic active radiation was 60 μmol photons m⁻²s⁻¹ under both LD and SD conditions. Plants were grown on MS plates, 4 d in dark at 4°C before moved to LD or SD at 22°C. The Columbia (Col-0) ecotype was used. In the STO over-expression studies, phenotypic analysis of all transgenic lines and expression studies, phenotypic analysis of all transgenic lines and expression analysis by real-time PCR, all the samples were performed with at least three independent RNA samples. For cDNA synthesis, the poly-dT primer was used. The quantitative real-time PCRs were used using the primers described in Table S2. For cDNA synthesis, the poly-dT primer was used. The quantitative real-time PCRs were performed with at least three independent RNA samples. For STO expression analysis by real-time PCR, all the samples were harvested 6 h into the photoperiod when the peak level of STO expression is reached.

DNA extraction

Plant tissue (200 mg) was ground to a fine paste in 500 μl of CTAB buffer. The CTAB/plant extract mixture was transferred to a microfuge tube and incubated for 15 min at 55°C in a recirculating water bath. After incubation, the CTAB/plant extract mixture was centrifuged at 12000 g for 5 min to pellet cell debris. The supernatant was transferred to clean microfuge tubes and 250 μl of chloroform/isoamylalcohol (24:1) was added to each, and then mixed by inversion. After mixing, tubes were centrifuged at 13000 g for 10 min. The upper aqueous phase containing the DNA was transferred to a clean microfuge tube and placed at −20°C for 1 h after the addition of ethanol to precipitate DNA. The precipitated DNA was then pelleted, washed twice in 70% ethanol and then resuspended in sterile DNase-free water.

Results

Developmental phenotypes of the STO loss- and gain-of-function mutants

STO was originally found to increase the tolerance of yeast to both Li and Na ions [33]. STO, along with STH (BBX25) interacts with the WD40 domain of COP1 [34], with COP1 repressing the transcription of STO and contributing to STO protein destabilization in etiolated seedlings [35]. Overall, STO acts as a negative regulator in the early photomorphogenesis response to red, far-red, blue and UV-B light signaling [32] [35] [36]. To further uncover the biological function of STO, we examined the phenotypes of the sto-1 T-DNA insertion knockout mutant (SALK_067475) and STO over-expression line STO-OE (33::STO) at different developmental stages. Early in development, STO appears to repress the rate of seed germination (24 h after transfer to light), with the sto-1 mutant exhibiting faster and the STO-OE line slower germination rates than the WT (Figure 1A). Despite this variation, 90–100% of seeds for all lines studied germinated after 48 hours. At the seedling stage, sto-1 and STO-OE hypocotyls were shorter and longer than WT, respectively (Figure 1B). The difference in hypocotyl length between sto-1 and STO-OE appears due to elongation post germination rather than to the rate of
germination itself, and STO plays an important role in this process. Moreover, both the sto-1 and STO-OE lines lost sensitivity to low and moderate concentrations of BL (0–100 nM), but all responded to a higher concentration (1000 nM) (Figure 1C and E). Under dark conditions, sto-1 had slightly longer hypocotyls than the WT and was less sensitive to low concentrations of ACC (0–0.1 μM) (the precursor of ethylene). However, sto-1 still showed a triple-response phenotype at higher concentrations (Figure 1D and F); it should be noted that the STO-OE germination rate was very poor in darkness and ACC treatments. In the adult rosette leaves, sto-1 accumulated much more anthocyanin on the abaxial surface than WT, while STO-OE had much less anthocyanin than WT. In STO-OE, the purple color concentrated in the main vascular tissue, while the remaining leaf blade was much greener than WT. STO affects not only mature rosette leaf pigmentation but also leaf morphology. In sto-1, adult leaves were narrower and more curled than WT, but less serrated, whereas the STO-OE leaves had much deeper serrations than WT (Figure 1G).

In addition to changes in leaf characteristics, flowering time was also affected by STO in both LD and SD conditions (Figure 1H–K). Over-expression of STO promoted flowering in both LD (Figure 1H and J) and SD (Figure 1I and K) while loss of STO delayed flowering relative to the WT but only in SD; both sto-1 and WT were almost bolting at the same time in LD. These results confirm that STO is an important gene affecting different developmental stages throughout the plant lifecycle.

**STO expression has diurnal transcript characteristics and is affected by environmental factors and phytohormones**

To gain insights into the biological function of STO, its spatial and temporal expression characteristics were analyzed by qPCR. STO is expressed in major tissues of Arabidopsis and the highest expression level was found in the shoot apex (Figure S4). Previous studies have shown that expression of STO is under circadian clock control [36]. The STO transcript has diurnal characteristics in both LD and SD conditions (Figure 2A). The highest STO expression level was at 6 h into the photoperiod under both LD and SD conditions, after which a rapid decline in LD and SD was observed. During the dark period, however, STO expression increased earlier in SD than LD. In the STO-OE line, STO expression was maintained at high levels in both light and dark conditions (Figure 2B). STO expression in WT was also up-regulated by exposure to low temperature (Figure 2C), similar to that previously shown for UV-B treatment [32]. This cold-induced increase in STO transcripts, however, was reversed upon returning the plants to the standard growth temperature of 22°C, dropping almost to the levels observed in the control plants (Figure 2C). In contrast, STO expression was repressed by treatment with the phytohormone BR (Figure 2D). BR is an essential hormone that regulates a wide range of developmental and physiological processes including cell expansion, vascular differentiation, etiolation, flowering and male fertility [37]. Light and BR antagonistically regulate the developmental process in de-etiolation of plants [38]. When seedlings were grown in 1/2 MS medium and treated with BL (100 nM) for 3 h and 4 d, respectively, STO expression was repressed during both exposure times (Figure 2D). Taken together, these phenotypes and expression characteristics indicate that STO is an important gene during the development of the plant and its response to certain environmental cues.

**STO represses FLC expression**

Although the role of STO in early photomorphogenesis has been well studied [32] [35] [36], little is known about its function during the late developmental stages. Earlier microarray analysis revealed that the level of FLC expression increases in sto-1 etiolated seedlings (unpublished data), results which were confirmed in this study by qPCR at different time points in etiolated seedlings (Figure S1). Both sto-1 and STO-OE have flowering phenotypes; FLC was selected for further investigation to examine how this Box family gene affects Arabidopsis flowering time.

We investigated FLC expression in different lines and light conditions (i.e., LD, SD, darkness) (Figure 3A). We found that over-expression of STO strongly repressed FLC expression in all three light conditions, whereas FLC expression in sto-1 seedlings was higher than in WT Col-0 and STO-OE in SD and darkness. In the LD condition, however, the level of FLC expression in sto-1 was similar to that in WT seedlings. Moreover, over-expression of STO in FRIGIDA and fbl-3, which have high levels of FLC transcripts, did not further repress FLC expression (Figure 3B). At same time, in FRI and fbl-3/FRI background, STO expression level did not show any statistically significant difference compared with WT (Figure S2). These results suggest STO is an upstream regulator that represses FLC expression.

**STO promotes FT and SOC1 expression**

In addition to FRI and FLC, the circadian clock system, photoperiod pathway (CO), and flowering integrators (FT, SOC1) also play important roles in the transition from vegetative to reproductive phases. The circadian clock consists of at least three interlocked transcriptional feedback loops. The LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) proteins are important components of the circadian clock system and have partially redundant functions [39] [40]. LHY and CCA1 not only repress the floral transition under LD and SD conditions, they also accelerate flowering in continuous light by promoting FT expression [41]. FT and SOC1 are both CO target genes. CO, FT and SOC1 are deeply affected by the circadian clock system at different levels [40].

To further investigate how STO regulates flowering time, we next examined how it affects expression of the aforementioned genes. Several different time points under the SD condition, where the sto-1 knock-out mutant and STO over-expressor showed significant differences in flowering time were investigated (Figures 1 H–K). CCA1 expression level did not change either in amount or rhythm in Col, sto-1 and STO-OE (Figure 4A). In sto-1, CO expression level was equal to that in Col during most time points and slightly lower in the dark (15–18 h). In the STO-OE line, CO expression level was higher compared to that in Col and sto-1 at the end of the day period (after 6–8 h) but lower during the dark (Figure 4B). The expression level of FT and SOC1 first peaked in the middle of the photoperiod (6 h) and then again in the dark (15 h) (Figures 4C and D). Since both CO and STO proteins are degraded by COP1 in the dark [32] [34] [42], the second peak of FT and SOC1 expression in STO-OE might be a result of the extra STO protein overwhelming the ability of COP1 to degrade it, thereby activating FT and SOC1 expression. Also in the STO-OE line, FT and SOC1 expression increased earlier than CO and started to decrease at the time when CO had only just begun to increase (i.e., after 6 h; Figures 4C and D). These results suggest that STO activates FT and SOC1 expression in a CO and circadian rhythm-independent manner, although this does not exclude the possibility that CO affects STO at the protein level. In the STO-OE line, moreover, the promotion of FT expression mainly occurred in cotyledons, whereas the expression of FT in sto-1 cotyledons was lower than in Col. Furthermore, the level of FT expression did not differ in the shoot apex, hypocotyl and root (Figure S3).
STO competes with FLC to promote FT and SOC1 expression

Since FLC is known to block FT by directly binding to its chromatin [14], there are two possible explanations why STO can promote flowering: that either STO directly represses FLC and activates FT and SOC1 expression simultaneously, or that STO only represses FLC that then leads to the activation of both FT and SOC1. To investigate these two scenarios, we overexpressed STO in different genetic backgrounds (Figure S3) to hopefully reveal the regulatory relationship between STO and FLC (Figure 5A). In the flc-3/FRI lines, which lack full length FLC but have functional FRI, FT and SOC1 expression was slightly higher than in FLC/FRI under SD conditions. In the STO-OE/flc-3/FRI lines (individual lines 4 and 15), FT and SOC1 expression significantly increased (Figures 5B and C), showing that STO activates FT and SOC1 expression independently of the repression of FLC. If STO activates FT and SOC1 expression by only reducing FLC expression, there should be similar amounts of FT and SOC1 transcripts in both the STO-OE/flc-3/FRI and flc-3/FRI lines. Moreover, these two lines (STO-OE/flc-3/FRI 4 and 15) also flowered earlier under SD conditions (Figure 5D and Table S3). In contrast, in the STO-OE/FLC/FRI line (line11) that has high levels of FLC and over-
expresses STO, the level of FT expression was only slightly higher than in the FRI line, and SOC1 transcripts were no longer upregulated (Figures 5B and C).

Discussion

In this work, we have examined in detail the biological function of STO from seed germination to flowering by the use of loss- and gain-of-function mutants. STO loss- and gain-of-function mutants both lost sensitivity to low concentrations of phytohormones such as BR and ethylene. Both BR and ethylene affect flowering time. We showed that STO was involved in those signaling pathways at least in the seedling stage (Figure 1C–F). In Arabidopsis, BR biosynthetic and signaling pathway mutants exhibit delayed flowering phenotypes [43] [44]. BR can promote flowering time by affecting circadian clock and FLC at both the transcription and chromatin modification levels [44] [45]. BR and STO have similar functions in the repression of photomorphogenesis, increased hypocotyl length and promotion of flowering (Figure 1 and [24]). However, the relationship between STO and BR signaling is rather complicated, as STO expression was repressed by BL treatment (Figure 2D). The reason for this conflicting observation could be that BL treatment results in accumulation of STO protein and feedback inhibition of STO transcription. Another explanation is that they may have common downstream genes, such as GATA2 and GATA4, which are positive regulators of photomorphogenesis and are repressed by BR signaling [38]. GATA2 and GATA4 expression levels in sto-1 and STO-OE were higher and lower than WT, respectively (unpublished data). BR signaling could through repressing STO achieve the appropriate expression level of...
downstream genes. In addition, STO expression was also regulated by environmental factors, such as the photoperiod and cold temperature (Figure 2B and C). All of those environmental and endogenous factors substantially affect plant flowering time. Important was the observation that over-expression of STO produced an early flowering phenotype under both LD and SD conditions, whereas loss of STO caused late flowering under SD. Altogether, STO appears to function more than just a negative regulator of flowering time.

Figure 4. Over-expression of STO promotes FT and SOC1 expression independent of CO or CCA1. The level of CCA1 (A), CO (B), FT (C) and SOC1 (D) expression in eight-day-old seedlings under SD conditions. Data from two independent replicates are shown, with UBQ10 used as a control. White and black bars represent the light and dark periods, respectively.
doi:10.1371/journal.pone.0087544.g004

Figure 5. Over-expression of STO promotes FT and SOC1 expression independent of FLC repression. The level of STO (A), FT (B) and SOC1 (C) expression in eight-day-old seedlings of the indicated genotypes. In the analysis, the data from STO-OE/fc/FRI-4 and -15 were compared to that of fc/FRI, while the data for STO-OE/FLC/FRI-11 was compared to that of FRI. Data from three independent replicates are shown, with UBQ10 used as a control. (D) The number of rosette leaves in each indicated genotype at the time of flowering. All seedlings and mature plants were grown in SD. ** means p<0.01 in TTEST.
doi:10.1371/journal.pone.0087544.g005
regulator during early photomorphogenesis, and instead to play a crucial role connecting different signaling pathways throughout the plant lifecycle.

The observations from this study support the following conclusions: (i) STO represses the flowering repressor FLC; (ii) STO can up-regulate FT and SOC1 expression, and; (iii) STO competes with FLC in their regulation of FT and SOC1. STO can repress FLC in LD, SD and darkness. However, in the FRI and fld-3 genetic background, over-expressed STO can no longer repress FLC. Since STO lacks the transcriptional repression/activation domain, it could be that it interacts with another complex to regulate FLC expression. FLC is not only a repressor of flowering time, but it is also functional throughout the lifecycle of the plant [14], such as promoting seed germination [46], lengthening the circadian period, and vegetative development [47]. Interestingly, the Lo background line that has a low-expressing FLC allele exhibited high dormancy. A high-expressing FLC allele produced a significantly higher germination rate at cool temperatures (10°C), but only slightly higher at warmer temperature (22°C) [46]. We found that the STO-0E line which has low FLC expression level also displayed slower germination rates, whereas the sto-1 germination rate was slightly higher than the WT (Figure 1 A, B). As a consequence, we propose that STO as a photoperiod/circadian clock controlled gene is involved in the regulation of FLC, although the molecular mechanism by which this occurs remains unclear.

We have demonstrated that STO activates FT and SOC1 expression, thereby promoting flowering. Given that STO expression is controlled by photoperiod/circadian rhythm, it would appear that it is a new component within this regulatory pathway. Moreover, STO stimulation of FT and SOC1 expression does not rely on repression of FLC but rather STO competes with FLC to regulate FT and SOC1 expression and thereby promotes flowering. Overall, these characteristics of STO reveal new relationships between FRI/FLC and the photoperiod/circadian clock pathway.

The combination of phenotype, expression characteristics and genetic results extends our knowledge on the biological function of the B-box zinc finger family in plant development, especially in the transition from vegetative to reproductive phase. Given the central roles of FLC, FT and SOC1 in flowering-time regulation in Arabidopsis, these findings suggest that the B-box family gene STO, which is without the CCT domain, plays an important role in the control of flowering in Arabidopsis. The behavior of STO in promoting flowering gives us new clues in understanding of how plants integrate environmental and developmental signals. We can therefore expect that the relationship between the different pathways to be more complex than first suspected.

The homologous B-box zinc-finger family genes are widely conserved in higher plants [36] [48] [49]. Despite this, the study of flowering time only focuses on the first group (CO and C-like COLs [48]) because previous research has indicated that both B-box motifs and CCT domains are important for promoting flowering under LD conditions [50] [51]. CO and COL genes have been well studied. While resembling STO and STH1-3, they also show the opposite function. Flowering in the CO mutant is delayed in LD while overexpression of CO results in the acceleration of flowering in both LD and SD. Over-expression of COL5 can induce flowering in SD but col5 mutants do not show altered flowering [52]. Over-expression of COL9 resulted in delayed flowering, whereas the col9 mutant flowered earlier under LD conditions. COL9 negatively regulates CO expression, but it does not appear to directly affect flowering time [53]. Moreover, CO, COL3 and COL9 influence flowering time mainly through FT and SOC1 and no reports to date have shown that they can affect FLC expression. Interestingly, a recent study has shown that COL1 and -2, which are the most closely-related genes to CO, do not affect flowering time due to the amino acid differences coded for within the first exon [54]. The first B-box domain may have a more important role than others in affecting flowering time. COL genes (including STO) have evolved rapidly in the Brassicaceae family [48] [55]. It is possible that the effect of STO on flowering time independent of the CCT domain is not a unique case. It will be interesting to investigate if other members that lack the CCT domain within the B-box family are also involved in regulating flowering.

Supporting Information

Figure S1 Confirmation of microarray data. Col, sto-1 and STO-0E were grown in darkness, with FLC expression level checked at 2, 4 and 6 d. FLC expression levels of the indicated genotypes were checked, and all lines were grown under SD. (TIF)

Figure S2 STO expression levels in different lines. The level of STO expression in the indicated genotypes was checked in four-day-old seedlings grown under SD. Data from three independent replicates are shown, with UBQ10 used as a control. (TIF)

Figure S3 Double mutant genomic PCR and RT-PCR confirmation. (A) Genomic PCR test was performed in F2 FRI/ftp-3 x STO-0E, with the lines 4, 7, 10 and 15 being fte homozygous. (B) Functional FRI allele’s have a BsmFI restriction site. Restriction endonuclease BsmFI was used to test FRI homozygous in F2 FRI/ftp-3 x STO-0E, with the lines 4,7,10 and 15 having a functional FRI. (C) RT-PCR test of full-length FLC expression, with the lines 4, 10 and 15 having no FLC mRNA. (D) RT-PCR test of FLD expression level in F2 ftp-3 x STO-0E, with UBQ10 as the control. The lines 11, 13, 15, 19 and 21 were ftp homozygous. (E) Restriction endonuclease BsmFI was used to test FRI homozygous in F2 FRI x STO-0E. The lines 9, 11, 12, 15 and 17 were FRI homozygous. All lines were grown for two weeks under SD prior to genomic PCR or RT-PCR. (TIF)

Figure S4 Determination of STO expression levels in different plant tissues. The level of STO expression in different tissues of Arabidopsis was analyzed in ten-day-old seedlings (cotyledon, shoot apex, hypocotyl and root) and adult plants (rosetta leaf and flower meristem). Data from three or four independent replicates are shown, with UBQ10 used as a control. * means p<0.05 and ** means p<0.01 in TTEST. Plants were grown under LD. (TIF)

Figure S5 Increased expression of FT in cotyledons and hypocotyls. The level of FT expression in different tissues (cotyledon, shoot apex [including young leaf primordial], hypocotyl and root) of ten-day-old seedlings of Col, sto-1 and STO-0E. Data from three independent replicates are shown, with UBQ10 used as a control. * means p<0.05 and ** means p<0.01 in TTEST. Plants were grown under LD. (TIF)

Table S1 Seed list. (PDF)

Table S2 Primer list. (PDF)

Table S3 Rosette leaf number at flowering time for the indicated genotypes. (PDF)

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Acknowledgments

We thank Cornelia Spetka Wïkünd, Anders Nilsson, Lisa Adolfszon and Jenny Carlsson for critical reading and comments on the manuscript. We also thank Caroline Dean for kindly providing the flr-3 and FRI seeds. This article is dedicated to the memory of our colleague, fellow author and dear friend Magnus Holm, who sadly passed away in September 2012.

Author Contributions

Conceived and designed the experiments: FL MH. Performed the experiments: FL JS DW SB. Analyzed the data: FL JS DW SB AKC MH. Contributed reagents/materials/analysis tools: MH. Wrote the paper: FL AKC.

References

