The Flowering Repressor SVP Underlies a Novel Arabidopsis thaliana QTL Interacting with the Genetic Background

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Abstract

The timing of flowering initiation is a fundamental trait for the adaptation of annual plants to different environments. Large amounts of intraspecific quantitative variation have been described for it among natural accessions of many species, but the molecular and evolutionary mechanisms underlying this genetic variation are mainly being determined in the model plant Arabidopsis thaliana. To find novel A. thaliana flowering QTL, we developed introgression lines from the Japanese accession Fuk, which was selected based on the substantial transgression observed in an F2 population with the reference strain Ler. Analysis of an early flowering line carrying a single Fuk introgression identified Flowering Arabidopsis QTL1 (FAQ1). We fine-mapped FAQ1 in an 11 kb genomic region containing the MADS transcription factor gene SHORT VEGETATIVE PHASE (SVP). Complementation of the early flowering phenotype of FAQ1-Fuk with a SVP-Ler transgen demonstrated that FAQ1 is SVP. We further proved by directed mutagenesis and transgenesis that a single amino acid substitution in SVP causes the loss-of-function and early flowering of Fuk allele. Analysis of a worldwide collection of accessions detected FAQ1/SVP-Fuk allele only in Asia, with the highest frequency appearing in Japan, where we could also detect a potential ancestral genotype of FAQ1/SVP-Fuk. In addition, we evaluated allelic and epistatic interactions of SVP natural alleles by analysing more than one hundred transgenic lines carrying Ler or Fuk SVP alleles in five genetic backgrounds. Quantitative analyses of these lines showed that FAQ1/SVP effects vary from large to small depending on the genetic background. These results support that the flowering repressor SVP has been recently selected in A. thaliana as a target for early flowering, and evidence the relevance of genetic interactions for the intraspecific evolution of FAQ1/SVP and flowering time.

Introduction

Flowering initiation is an essential developmental transition in plant life because it determines the timing of sexual reproduction. This transition is regulated by different environmental signals that synchronize reproduction with the most favourable season for seed production. Hence, the timing of flowering is a crucial adaptive trait in annual plants, since it will affect their survival and reproductive yield [1]. Supporting this relevance, considerable intraspecific quantitative variation has been classically described for flowering time among natural accessions or crop varieties for many annuals, which is presumed to reflect adaptation to local environments [2,3]. In the past fifteen years there has been an unprecedented advance in our understanding of the molecular mechanisms of flowering regulation, mostly achieved by genetic studies of artificially induced mutants in the model plant Arabidopsis thaliana [4]. More than 100 flowering genes have been identified whose analyses are defining a complex regulatory network that involves several flowering pathways integrating different environmental signals. This network includes, among others, the photoperiod, the vernalization and the autonomous pathways, as well as various regulatory genes that play a role as pathway integrators, such as FT and SOC1 [5–7]. Presently, a major aim in plant biology is to decipher the molecular and evolutionary bases of the naturally-existing genetic variation, for which A. thaliana has also become a promising model species [1,8–10].

A. thaliana is broadly distributed as a native species in Eurasia, whereas it has been later introduced in North America and Japan, as well as in Australia and South America (reviewed in [11]). The large amount of natural genetic variation that has been described for flowering time is likely involved in adaptation to the contrasting climates that are covered by A. thaliana geographic distribution because this variation has been associated with latitude, altitude and climatic factors [12–16]. A. thaliana accessions have been qualitatively classified for long time as winter- or summer-annuals depending on their extreme late or early flowering behaviours and their high or low response to vernalization, respectively [17]. Mendelian genetic analyses identified two flowering repressors, FRI and FLC, as major determinants of such qualitative flowering differences [18,19]. In addition, numerous quantitative trait locus (QTL) analyses have been carried out with different sorts of experimental mapping populations including F2 families [20],...
**Author Summary**

In many plant species, the timing of flowering initiation shows abundant quantitative variation among natural varieties, which reflects the importance of this trait for adaptation to different environments. Currently, a major goal in plant biology is to determine the molecular and evolutionary bases of this natural genetic variation. In this study we demonstrate that the central flowering regulator SHORT VEGETATIVE PHASE (SVP), encoding a MADS transcription factor, is involved in the flowering natural variation of the model organism Arabidopsis thaliana. In particular, we prove that a structural change caused by a single amino acid substitution generates a SVP early flowering allele that is distributed only in Asia. Furthermore, genetic interactions have been shown to be a component of the natural variation for many important adaptive traits. However, very few studies, either in animals or plants, have systematically addressed the extent of genetic interactions among specific alleles responsible for the natural variation of complex traits. Our study shows that the flowering effects of SVP natural alleles depend significantly on the genetic background; and, subsequently, we demonstrate the relevance of epistasis for the evolution of this crucial transcription factor and flowering time.


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**Results**

**FAQ1** is a novel flowering QTL affecting the photoperiod response

In order to uncover natural genetic variation for flowering initiation that is not detected by direct phenotypic comparisons of wild accessions, we quantified transgressive segregation in F2 populations derived from crosses between several accessions and the reference strain Landsberg erecta (Ler). Using this approach we selected the genotype Fukuyama (Fuk) because 36% of the F2 individuals showed transgressive flowering times that duplicate the phenotypic variation observed between both parents (Figure 1A). To identify the loci responsible for this variation we developed introgression lines by phenotypic selection for flowering time during four backcross generations (see Materials and Methods). Two early flowering lines, II-2 and II-FAQ1, carrying single Fuk introgressions from chromosome 2 (of ~9 and ~2 Mb, respectively) in an otherwise Ler genetic background, were characterized for their flowering behaviour (Figure 1B). On average, the two lines flowered two days earlier and with two leaves fewer than Ler under long-day (LD) photoperiod. In contrast, under short-day (SD), both ILs flowered 21 days earlier and with 28 leaves less than the reference strain, which indicates that, similar to Fuk accession, these lines have a reduced response to photoperiod (Figure 1B). F1 hybrids derived from Ler and the ILs showed towards-early intermediate flowering phenotypes suggesting incomplete dominance (Table S1). Thus, we identified a new large effect locus contributing to the natural variation for flowering initiation and its photoperiodic response, which was named as **Flowering Arabidopsis QTL 1 (FAQ1)**.

**SVP** is the gene underlying **FAQ1**

Fine mapping using an F2 (Ler×II-2) population of 2988 individuals located FAQ1 within a genomic interval of 11 kb where...
Col reference genome sequence contains only two open reading frames (Figure 1C). One of them, At2g22540, corresponded to the previously known flowering gene SHORT VEGETATIVE PHASE (SVP), encoding a MADS-box transcription factor [50]. To test if SVP might be FAQ1, we generated two SVP genomic constructs corresponding to Ler and Fuk SVP alleles, and used them to transform plants of the early flowering line IL-Fuk (Figure 2A and 2B). Homozygous transgenic lines carrying SVP-Fuk transgene did not differ in their flowering behaviour from IL-Fuk, indicating that this allele, in this genetic background, has no effect on flowering initiation. By contrast, most transgenic lines for SVP-Ler flowered significantly later than control plants, under SD and/or LD photoperiods (Figure 2A and 2B). Since SVP-Ler, but not SVP-Fuk, transgenes largely complemented the early flowering and the reduced photoperiod response of IL-Fuk, it was concluded that SVP underlies FAQ1.

A single amino acid substitution is the SVP/FAQ1 causal polymorphism

Sequencing of SVP in the parental accessions identified 50 single nucleotide polymorphisms (SNPs) and small indel polymorphisms differing between Ler and Fuk (Figure 2C). Most polymorphisms were detected in non-coding genomic regions and only one non-synonymous SNP was found, which was located in the middle of the MADS domain. This mutation is predicted to change Ala32 to Val32, and appeared conserved in all SVP-like proteins (Figure S1). To evaluate the functional effect of this substitution we developed two additional chimerical SVP genomic constructs corresponding to Ler and Fuk alleles where we replaced by directed mutagenesis Ala32 with Val32, and vice versa. In IL-Fuk SVP genetic background, homozygous transgenic lines carrying SVP-Ler-Val32 transgene flowered similar to IL-Fuk and did not differ from transgenic lines for SVP-Fuk allele (P>0.05; Figure 2D and 2E). However, most transgenic lines bearing SVP-Fuk-Ala32 transgenes flowered significantly later than control plants, under LD and SD photoperiod conditions. These results demonstrated that this single amino acid substitution strongly alters SVP function, Val32 from Fuk generating a SVP loss-of-function allele that displays no effect on flowering initiation, while Ler Ala32 renders SVP functional and delays flowering initiation.

SVP allelic interaction explains FAQ1 incomplete complementation

Even though most IL-Fuk transgenic lines carrying Ler Ala32 in SVP transgene flowered later than IL-Fuk, quantitative analysis of these lines showed that on average they flowered earlier than Ler (Figure 2A and 2B). Therefore, FAQ1 complementation with SVP transgenes was incomplete. To test if this was due to the existence of an additional gene linked to SVP that might contribute to FAQ1, or to an interaction between the transgenic and the endogenous copies of SVP, we used the four SVP genomic constructs to transform also Ler plants (Figure 2F–2I). The four classes of Ler transgenic lines showed the same overall flowering patterns observed in IL-Fuk background. However, most transgenic lines carrying Fuk Val32 flowered earlier than Ler, while most lines carrying Ler Ala32 flowered significantly later than Ler under SD and/or LD photoperiods (Figure 2F and 2G). The effect of SVP alleles was estimated in each background by comparing the transgenic lines carrying Ler and Fuk transgenes (Table 1). Thus, SVP effect in Ler background was significantly larger than in IL-Fuk (P<0.05) and similar to FAQ1 effect estimated by comparing Ler and IL-Fuk control lines. These results indicated that SVP accounts for most FAQ1 effect but SVP transgenes interact with the genetic background. Since both backgrounds, Ler and IL-Fuk, differed only in the small introgression containing SVP gene, the SVP transgene most likely interact with the endogenous allele of SVP.
**SVP/FAQ1 flowering effects involve epistatic interactions**

To further evaluate the genetic-background-dependency of FAQ1/SVP effect, we used the two SVP genomic constructs corresponding to Ler and Fuk alleles to transform three additional accessions (Fuk, Pak-1 and Pak-3) carrying similar loss-of-function FAQ1/SVP-Fuk allele (see later). A total of 108 homozygous transgenic lines were selected in all five backgrounds and grown together under LD and SD photoperiods (Figure 3). The joint analysis of these lines showed strong additive effects of SVP transgenes and genetic backgrounds (P<0.001; Table S2). However, this quantitative analysis also detected significant SVP transgene by background interaction (P<0.01; Table S2) indicating that the allelic effect of SVP depends on the genetic background. This interaction was mainly determined by the small effect of SVP transgenes in Pak-1, since significant interactions were detected (P<0.05) in all pair comparisons of Pak-1 transgenic lines with the rest of backgrounds. As shown in Figure 3, in Pak-1, the two allelic classes of SVP transgenic lines differed weakly under both photoperiods (Table 1). In contrast, both classes of transgenic lines showed larger differences in the other backgrounds, the largest SVP allelic effect appearing in Ler (Figure 3). Furthermore, the three-way interaction among SVP transgene, genetic background and photoperiod was significant (P<0.01; Table S2) evidencing that the effect of SVP on the flowering photoperiod response also depends on the genetic background. This is illustrated with the comparable SVP effect observed in Fuk, Pak-3 and IL-FAQ1 lines when grown under SD, but not under LD photoperiod where Fuk lines displayed larger SVP allelic effect (Figure 3 and Table 1). Therefore, the differential behaviour of transgenic lines in backgrounds bearing the same endogenous FAQ1/SVP allele indicates that SVP transgenes interact with one or several genomic regions other than SVP locus, as well as with the photoperiodic environment.

**SVP/FAQ1 loss-of-function allele shows a regional distribution in Asia**

Genotyping of a world-wide collection of 289 *A. thaliana* accessions with a CAPS marker specific for SVP causal polymorphism detected six additional accessions carrying Fuk Val32, two from Pakistan and four from Japan (Figure 4A). This showed that SVP causal polymorphism is geographically structured, Fuk loss-of-function allele appearing as rare at a global scale (<2.5% frequency) but common at a regional scale in Japan, where it displayed a frequency of ~15%.

Sequencing analysis revealed that all seven accessions with Fuk Val32 carried the same SVP loss-of-function allele because they only differed in the length of a short AT-microsatellite located in the first intron. Further SVP sequencing in 18 accessions covering the world distribution (Figure 4B and 4C) showed an overall low nucleotide diversity in SVP coding region (π-silent = 0.0038), which increased up to average genome levels [51] only in the 5’ and 3’ flanking regions. Non-synonymous diversity was especially low because only the Ala32 to Val32 substitution was found, and no other polymorphism with obvious potential effect on SVP function was detected (Table S3). To determine the genetic relationships among accessions carrying SVP loss-of-function alleles we genotyped a sample of 54 Asian accessions for a set of 237 genome-wide SNPs (Figure 4D). The five Japanese accessions carrying Fuk Val32 were nearly identical with an average proportion of allelic differences (genetic distance) of 1.6%. However the two Pakistan

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**Figure 2. Flowering phenotypes of transgenic lines for parental and chimerical SVP alleles.** Leaf number of independent homozygous *T₂* transgenic lines carrying parental (A, B, D and G) or chimerical (D, E, H and I) SVP genomic constructs in IL-FAQ1 (A, B, D and E) or Ler (F–I) genetic backgrounds. Lines were grown under long-day (LD) (A, D, F and H) or short-day (SD) (B, E, G, and I) photoperiods. C) Nucleotide polymorphisms found between SVP genomic sequences of Ler and Fuk. Parental and chimerical SVP transgenes derived from Fuk (red colour) and Ler (green colour) are depicted in the upper part of each panel. Bars are means ± SE of 10–15 plants per line. Mean ± SE of all lines carrying the same transgene are shown above the bars. Dashed lines delimit the 95% confidence intervals of the leaf number observed in untransformed IL-FAQ1 (red colour) and Ler (green colour) control lines, as established by Bonferroni tests. doi:10.1371/journal.pgen.1003289.g002
Table 1. FAQ1/SVP allelic effects on flowering initiation in different genetic backgrounds.

| Genetic background | Endogenous SVP allele | Transgenes | # of Fuk/Ler transgenic lines | Experiment | LD FAQ1 effect | SD FAQ1 effect |
|--------------------|-----------------------|------------|------------------------------|------------|----------------|----------------|
| Ler<sup>1</sup>    | Ler, Fuk              | no transgene | -                            | 1          | 3.3            | 23.2           |
| IL-FAQ1            | Fuk                   | SVP-Ler, SVP-Fuk | 13/10                        | 1          | 3.4            | 14.3           |
| Ler                 | Ler                   | SVP-Ler, SVP-Fuk | 14/10                        | 1          | 5.6            | 22.5           |
| Ler<sup>1</sup>    | Ler, Fuk              | no transgene | -                            | 2          | 3.4            | 21             |
| Pak-1              | Fuk                   | SVP-Ler, SVP-Fuk | 15/10                        | 2          | 0.9            | 1.8            |
| Pak-3              | Fuk                   | SVP-Ler, SVP-Fuk | 10/6                         | 2          | 6.8            | 13.6           |
| Fuk                 | SVP-Ler, SVP-Fuk      | 14/13       | 2                            | 11.5       | 9.5            |                |
| IL-FAQ1            | Fuk                   | SVP-Ler, SVP-Fuk | 10/10                        | 2          | 3.5            | 10.3           |
| Ler                 | SVP-Ler, SVP-Fuk      | 10/10       | 2                            | 5.9        | 18             |                |

For each background is shown: the endogenous and transgenic SVP alleles analysed, the number of independent homozygous transgenic lines evaluated, and the average FAQ1/SVP allelic effects in long-day (LD) and short-day (SD) photoperiod. Allelic effects were estimated in two experiments as the mean difference between the leaf number of transgenic lines carrying SVP transgenes from Ler and Fuk. Only transgenic lines differing significantly from the corresponding untransformed control were used for allelic effect estimates.

1: The allelic effect of the original FAQ1 locus (detected in Ler and IL-FAQ1 lines) was estimated as the leaf number difference between Ler and IL-FAQ1 untransformed plants.

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Functional allelic variation at SVP/FAQ1 most likely originated in Japan

Sequence and genotypic analyses identified YGU as a Japanese genotype that is very close to the five Japanese accessions bearing Fuk Val<sup>32</sup>, for the overall genetic background (genetic distance of 5.6%) and for SVP haplotypes (Figure 4C and 4D). However, YGU carried the active Ala<sup>32</sup> SVP allele, the only other SVP nucleotide difference corresponding to the length of the first intron microsatellite. Furthermore, YGU flowered significantly later than Fuk and the remaining Val<sup>32</sup> accessions (Table S1), suggesting that SVP accounts for these flowering differences. This was strongly supported by cosegregation analysis in an F<sub>2</sub> (Fuk x YGU) population grown under LD photoperiod, where SVP causal polymorphism explained 43% of the flowering phenotypic variance (Figure 4E). Thus, in this Fuk/YGU homogeneous genetic background, SVP/FAQ1 displayed a large LD effect, in agreement with the behaviour of Fuk transgenic lines. Therefore, SVP loss-of-function allele was probably generated recently in Japan, and after outcrossing and recombination it expanded to Middle Asia.

Discussion

FAQ1/SVP sets MADS transcription factors as the main gene family accounting for natural flowering variation in A. thaliana

Despite the large number of flowering time QTL identified in A. thaliana, the molecular bases of only a dozen of them have been determined until now (see Introduction). In this work, we have isolated FAQ1, a new QTL identified as a large effect locus in a population highly trangressive for flowering initiation. Most previous studies have used permanent RIL populations or F<sub>2</sub> families to detect and map QTL [9,10,20]. However, we identified this locus in a population of introgression lines developed by phenotypic selection in a homogeneous reference genetic background. Although the construction of such biological materials requires considerable time, they facilitated the later characterization, the fine mapping and the molecular isolation of FAQ1, showing the power of phenotype-based ILs as an alternative mapping resource to standard experimental populations.
We have demonstrated that the well-known regulator SVP encoding a MIKC-type MADS transcription factor [50,52] contributes to the natural variation for flowering initiation in A. thaliana. It has been previously shown that SVP is a flowering repressor that affects the photoperiod response by negatively regulating several integrator genes such as FT and SOC1 [53,54]. SVP appears regulated by the circadian clock and by the autonomous, the thermosensory and the gibberellin pathways [53,55,56], which suggests that SVP is also a flowering pathway integrator. Network and protein interaction studies have further revealed that SVP is down-regulated by AP1 and interacts with AP1 and other floral MADS transcription factors like CAL and SEP3 [57–59] thereupon showing the close regulation between SVP and the flower identity genes. In addition, SVP binds to the promoters and regulates the expression of other transcriptional regulators including miR172 and several floral repressors of the AP2 family [60]. In this study we have proven that the natural amino acid substitution Ala32 to Val32, in the MADS domain, generates a SVP loss-of-function allele that cause early flowering, in agreement with the phenotypes described for artificial svp mutants [50,53]. MADS domains are required for DNA binding but the Ala32 , highly conserved among species, has been shown to participate also in MADS protein dimerization [61]. These functions suggest that SVP-Fuk-Val32 is likely unable to properly bind and repress SOC1 and/or FT promoters, leading to the early flowering and reduced photoperiod sensitivity observed in Fuk accession. In addition, the specificity and uniqueness of this natural structural mutation suggest that most SVP structural modifications are likely deleterious and that SVP protein is essential for A. thaliana survival in nature.

Natural regulatory and structural polymorphisms in three additional MADS-box genes, FLC, FLM and MAF2, have been shown to affect flowering in A. thaliana [41–43,62,63]. In addition, a natural amino acid substitution in the MADS-box gene AGL6 has been recently demonstrated to alter shoot branching in a flowering time dependent manner [64]. Moreover, an extensive A. thaliana genome-wide association study [32] has found SVP as associated with several flowering related traits, which suggests that additional SVP polymorphisms might affect flowering initiation. Hence, MIKC-type MADS transcription factors appear as the main class of genes accounting for the flowering natural variation in this species. Interestingly, another MADS-box gene homologous to AP1 was found to contribute to the natural variation for vernalization flowering response in cereals [65]. Several studies have shown that SVP-like genes in different families of mono- and dicotyledonous plants display partially conserved functions in the photoperiod and vernalization flowering pathways [66–71] despite substantial copy number variation for SVP-like genes among species. Therefore, MADS transcription factors in general, and SVP in particular, appear as important candidate genes to explain the natural variation for flowering time or related traits also in plant families that are phylogenetically distant from A. thaliana [72].

Genetic interactions determine the effects of natural SVP variation

Although FAQ1/SVP was detected as a large effect flowering QTL, quantitative analysis of transgenic lines shows that FAQ1/SVP effects vary from large to rather small as consequence of its genetic interactions. On the one hand, transgenic lines differing only in a small introgression indicate that SVP effect depends on the natural alleles in a genomic region located around SVP, which strongly suggests allelic interactions. This is best illustrated with the lack of flowering effects observed for SVP-Fuk-Val32 transgenes in
the SVP loss-of-function background of IL-FAQI, whereas these transgenes accelerated flowering in the near isogenic background of Lr. Thus, the flowering repression of active SVP-Lr alleles seems to be reduced by the presence of SVP-Fuk loss-of-function alleles. This result is in agreement with the incomplete dominance observed in hybrid plants derived from IL-FAQI and Lr, which cannot be explained simply by a SVP dosage effect [50]. Since the function of MADS transcription factors involves homo- and hetero-dimers [57,58] it can be speculated that in plants bearing both natural SVP alleles, protein complexes containing SVP-Val129 directly or indirectly, reduce the overall SVP transcriptional repressing capacity. On the other hand, transgenic lines in different genetic backgrounds carrying the same endogenous loss-of-function SVP allele show that SVP effects depend on the natural alleles in other genomic region(s), which implies significant SVP epistatic interactions. Interestingly, SVP interacts physically with several MADS transcription factors like FLC, AP1, SOC1 and AGL6 [55,56,57]. This suggests that the functional basis of the observed SVP genetic interaction is the physical interaction between SVP protein and other MADS transcription factors involved in multiple complexes. Such interactions could also account for the genetic-background-dependency observed for the incomplete dominance of SVP alleles because, in contrast to the behavior in F1(Lr×IL-FAQI) plants, SVP-Fuk allele behaved nearly as recessive in the F2(Fuk×YGU) population (Figure 4E).

All flowering QTL isolated so far correspond to large effect alleles [9,10], which has hampered our understanding of the molecular mechanisms involved in the natural variation for flowering initiation mediated by small effect QTL [73]. The genetic-background-dependency of FAQI/SVP shows that QTL that are primarily detected as large effect loci may have varying effects owing to genetic interactions. Thus, epistasis appears as an important component of QTL effect estimation, which is often neglected in Fisher’s views of natural quantitative variation that assume the existence of series of alleles with different additive effects [39,74]. This result brings the possibility that some of the natural flowering alleles previously isolated might also underlie flowering QTL detected with small effect, a hypothesis whose testing requires the analysis of genetic interactions in multiple backgrounds, as shown here for FAQI/SVP. In particular, natural variants of gene families that participate in multimer protein complexes, such as the MADS genes [57], are expected to show significant genetic interactions [39], as described for numerous artificial mutant alleles of these genes including SVP, FLM and FLC [55,56,73–78]. This view is also supported by the recent identification of a natural allele of AGL6 that affects axillary bud formation in an epistatic manner [64]. It can then be speculated that the natural SVP interacting partners are any of the MADS genes FLM, FLC, MAF2 or AGL6, as supported by their segregation in nature and their participation in SVP genetic and physical interactions, although we cannot discard other genes. Thus, our study shows the usefulness of quantitative analyses of transgenic lines in multiple genetic backgrounds as a general approach to uncover any order (di- and higher-order) genetic interactions with specific natural alleles. Nevertheless, given the significant variation found among transformants, this method demands the generation of large numbers of independent transgenic lines.

SVp natural allelic variation is probably involved in A. thaliana adaptation

Most A. thaliana alleles that have been functionally demonstrated as contributing to the natural variation for flowering initiation are alleles found in a unique accession, which hampers inferences about their role in plant adaptation [9]. By contrast, the early flowering SVP-Fuk allele appears as a recent allele likely originated in Japan and distributed in Asia. Several arguments support that this genetic variant is involved in adaptation. First, its moderate frequency in Asia, in accessions that belong to genetically differentiated clades, indicates that this is not a deleterious allele to be purged from a unique local population. Phenotypic analysis of FAQI ILs did not detect any other obvious developmental alteration, further supporting flowering specificity and absence of pleiotropic effects of SVP-Fuk allele. Second, SVP-Val129 is the only detected amino acid substitution that has been maintained in nature at high regional frequency, whereas low silent and non-synonymous nucleotide diversities suggest that SVP is under purifying selection. Third, its early flowering phenotype is in agreement with the strong recent directional selection favouring earliness that has been described at the species level [2,49]. The significant SVP flowering effect in Fuk/YGU genetic background, in which most likely SVP-Fuk allele was originated, supports that natural selection could act through the SVP-Fuk earliness. Thus, in addition to FRI, FLC and MAF2 genes harbouring several frequent loss-of-function mutations [13,15,19,46–49] SVP represents another flowering repressor (or vegetative growth promoter) that might be under natural selection for early flowering, in agreement with previous predictions [2]. The limited regional distribution of SVP-Fuk is probably determined by its short demographical history in a non-native region that has been recently colonized [11]. However, SVP might be involved in adaptation to particular Asian local environments. The presence of this allele in a set of genetically related accessions suggests that such potential adaptive effect of SVP-Fuk depends on the genetic background, as supported by the genetic interactions described for SVP flowering effect. Conclusive demonstration of SVP contribution to adaptation awaits the analysis of the currently unknown environmental conditions where natural SVP alleles have evolved, as recently reported for other flowering genes in more extensively sampled and documented geographic regions [15,27].

Materials and Methods

Plant materials

The laboratory strain Landsberg erecta (Lr) and the wild accession Fuk, obtained from Sendai Stock Centre [JW116; http://www.brc.riken.jp/lab/epd/Eng/catalog/seedc.shtml] and originally collected around Fukuyama (Japan), were used as parental lines to develop a population of 31 introgression lines carrying Fuk genomic segments in Lr background. ILs were developed by phenotypic selection for early flowering time during four backcross generations, each backcross being followed by a selfing generation. Briefly, the four earliest plants of an F2 (Lr×Fuk) population of 120 plants were backcrossed to Lr to obtain four independent families. A single early plant was selected per family in each of the following selfing and backcross generations. After four backcrosses, 7–8 individual sister plants per family (a total of 31 ILs) were thoroughly genotyped with 100 AFLP, microsatellite and indel polymorphic markers previously described [26,79,80].

IL-2 carrying a single introgression fragment of ∼9 Mb in chromosome 2 was crossed to Lr to obtain a FAQI F2 mapping population. FAQI was fine mapped by genotyping 2986 F2 plants with 24 CAPS and indel markers developed from different sources. IL-FAQI, carrying an introgression of ∼2 Mb between physical positions 7.6 and 9.6, was derived from the mapping population. A world-wide collection of 189 accessions (Table S4) and a collection of 100 Iberian wild genotypes [81] were analysed for
flowering behaviour, for SVP sequence, and/or for SVP causal polymorphism.

**Growth conditions and measurements of flowering initiation**

Plants were grown in pots with soil and vermiculite at 3:1 proportion in an air-conditioned greenhouse at 21°C, supplemented with additional light to provide long-day photoperiod (16 h light:8 h darkness). For short-day photoperiods (8 h light:16 h darkness) plants were grown in a growth chamber illuminated with cool-white fluorescent lamps.

Flowering initiation was measured as leaf number and flowering time. Leaf number was calculated as the total number of rosette and cauline leaves in the main inflorescence. Flowering time was estimated as the number of days from the planting date until the opening of the first flower.

**SVP sequences, constructs, and transgenic lines**

A SVP genomic fragment of 6.5 kb, including 3.2, 2.4 and 0.9 kb of the coding, the 5’ and the 3’ regions, respectively, were sequenced in Ler and Fuk. A 5.6 kb SVP segment was sequenced in 15 accessions (Table S4). Nine to 12 overlapping fragments of 0.8–1.3 kb were PCR amplified (Table S5) and products were sequenced using an ABI PRISM 3700 DNA analyzer. DNA sequences were aligned using DNASTAR v.8.0 (LaserGene) and alignments were inspected and edited by hand with GENEDOC [82]. Nucleotide diversity, recombination and linkage disequilibrium were estimated with DnaSP v.5 [83]. GenBank accession numbers of DNA sequences generated in this work are JX863084–JX863100.

The two 6.5 kb SVP genomic fragments from Ler and Fuk were cloned in pCAMBIA2300 binary vector (Cambia, Canberra, Australia) by standard molecular biology techniques. Briefly, three successive SVP segments were PCR amplified and cloned in appropriate cloning sites, and subsequently fused in the right orientation (Table S5). Two additional SVP chimerical constructs were derived by reciprocally replacing the SNP causing Ala32 to Val32 substitution. For that, site-directed mutagenesis of this SNP was performed by PCR using the spliced overlap extension method as described by Hepworth et al. [84]. Primers containing the nucleotide to be replaced are shown in Table S5. The two PCR products of each accession were purified, mixed, and subjected to 12 PCR cycles to be amplified with DnaSP v.5 [83]. GenBank accession numbers of the protein sequences included are as follow: SVP from Arabidopsis thaliana (ABU95408.1); SVP from A. lyrata (EFH54881); SVP from Brassica rapa (ABG24233.1); SVP from B. napus (AFG73587.1); SVP from B. juncea (AFG73588.1); SVP from Medicago truncatula (XP_003613054.1); SVP from Pseudosatsuma (AAX47170.1); SVP-like from Glycine max (ABY78023.1); JOINTLESS from Solanum lycopersicum (AAG09811.1); SVP-like 1 from S. tuberosum (AAB94006.1); SVP-like 2 from S. tuberosum (AAV65507.1); JOINTLESS from Malus domestica (ABD66219.2); SVP1 from Actinidia chinensis (AFA37967.1); SVP2 from A. chinensis (AFA37968.1); SVP3 A. chinensis (AFA37969.1); SVP4 from A. chinensis (AFA37970.1); SVP-like from Citrus trifoliate (ACJ09170.1); SVP-like 1 from Ficus microcarpa (XP_002269295.1); SVP-like 2 from V. vinifera (AFS96914.1); SVP-like 3 from V. vinifera (XP_002269295.1); SVP-like from Eucalyptus occidentalis (AAP40361.1); SVP-like from Coffea arabica (ADU56833.1); SVP-like from Marchantia polymorpha (ADB81895.1); SVP-like 1 from Ipomoea batatas (BAC155621.1); SVP-like 2 from I. batatas (BAC15561.1); SVP-like from Oryza sativa (Q9X66.1); SVP-like 1 from Hordeum vulgare (CAB97349.1); SVP-like 2 from H. vulgare (Q0201168.1); SVP-like from Zea mays (NP_001105481.1); SVP-like from Brassicathamnus distachyon (XP_003581663.1); SVP-like from Phyllosticta patens (XP_001779871.1); AGAMOUS from A. thaliana (AEE04111.1); APETALA 3 from A. thaliana (P56322.1); SRF from Homo sapiens (NP_001321.2); MEF2 from H. sapiens (NP_002388.2); MEF2 from Xenopus laevis (NP_001089962.1); SRF from Drosophila melanogaster (NP_726438.1); MEF2 from D. melanogaster (NP_95789.1); Mcm1p from Saccharomyces cerevisiae (CAA88409.1)

(TIF)

**Table S1** Flowering behaviour of genotypes with different natural SVP alleles.

(XLS)

**Table S2** General linear model testing the effects of SVP transgenes, the genetic background and the photoperiod in transgenic lines.

(XLS)

**Table S3** SVP nucleotide diversity.

(XLS)

**Table S4** A. thaliana natural accessions analyzed for SVP sequence and causal polymorphism.

(XLS)

**Table S5** Oligonucleotides used for SVP sequencing, accession genotyping, cloning and verification of transgenic lines.

(XLS)
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