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Regulation of flowering time by *Arabidopsis MSI1*

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The transition to flowering is tightly controlled by endogenous programs and environmental signals. We found that *MSI1* is a novel flowering-time gene in *Arabidopsis*. Both partially complemented *msi1* mutants and *MSI1* antisense plants were late flowering, whereas ectopic expression of MSI1 accelerated flowering. Physiological experiments revealed that *MSI1* is similar to genes from the autonomous promotion of flowering pathway. Expression of most known flowering-time genes did not depend on MSI1, but the induction of *SOC1* was delayed in partially complemented *msi1* mutants. Delayed activation of *SOC1* is often caused by increased expression of the floral repressor *FLC*. However, MSI1 function is independent of *FLC*. MSI1 is needed to establish epigenetic H3K4 di-methylation and H3K9 acetylation marks in *SOC1* chromatin. The presence of these modifications correlates with the high levels of *SOC1* expression that induce flowering in *Arabidopsis*. Together, the control of flowering time depends on epigenetic mechanisms for the correct expression of not only the floral repressor *FLC*, but also the floral activator *SOC1*.

KEY WORDS: *Arabidopsis*, Flowering, Chromatin, *MSI1*, *SOC1*

INTRODUCTION

Deciding when to flower is a crucial choice for plants to ensure successful reproductive development. Therefore, the transition to flowering is tightly controlled by both endogenous programs and environmental signals (for reviews, see Komeda, 2004; Putterill et al., 2004). Genes involved in the control of flowering have been grouped into genetic pathways with assigned functions based on physiological experiments. The photoperiod, the vernalization and the autonomous pathways are now understood in detail (for reviews, see Boss et al., 2004; Searle and Coupland, 2004; Simpson, 2004). The photoperiod pathway responds to changing day lengths, and the vernalization pathway responds to long exposure of imbibed seeds or seedlings to low temperatures. These two pathways contribute to the initiation of flowering in winter-annual plants in the favourable conditions of spring and summer. By contrast, the promotion of flowering by the autonomous pathway is independent of environmental signals, ensuring that winter-annual plants flower even after a mild winter. Additional pathways, which are less well understood, promote flowering in response to other internal or external factors, such as gibberellins, light quality and ambient temperature.

In the model plant *Arabidopsis thaliana*, the major flowering-time pathways converge to regulate the expression of at least three genes that promote flowering: the pathway integrators *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*, or *AGL20*), *FLOWERING LOCUS T* (*FT*) and *LEAFY* (*LFY*) (Weigel et al., 1992; Kardailsky et al., 1999; Kobayashi et al., 1999; Lee et al., 2000; Onouchi et al., 2000). In the photoperiod pathway, for instance, the transcription factor CONSTANS (CO) activates the expression of *SOC1* through *FT* (Samach et al., 2000; Wigge et al., 2005; Yoo et al., 2005). The activation of *SOC1* expression has to overcome the repressive action of one of the most potent inhibitors of flowering in *Arabidopsis* – the MADS-domain protein encoded by *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999). Because the vernalization and autonomous pathways negatively regulate the expression of *FLC*, these two pathways promote flowering by releasing *SOC1* from the repression by FLC. Once expressed, SOC1 and the other pathway integrators activate downstream target genes, including the transcription factor *APETALA1* (*AP1*), which cause the transformation of the vegetative shoot apical meristem (SAM) into an inflorescence meristem that produces floral meristems (Krizek and Fletcher, 2005).

Mutants of genes from the autonomous pathway, such as *luminidependens* (*ld*) and *fve*, are late flowering because they fail to reduce the expression of *FLC*. Introducing an *flc*-null allele completely rescues the late-flowering phenotype of autonomous pathway mutants (Michaels and Amasino, 2001). Interestingly, although the autonomous pathway in *Arabidopsis* converges on FLC, this gene appears to be restricted to the Brassicaceae family, and seems to be absent from other dicotyledonous plants and from monocotyledonous plants (Searle and Coupland, 2004). Because monocotyledonous plants are of broad economic and agricultural importance, it is of great interest to better understand the mechanisms that promote autonomous flowering independently of *FLC*.

MSI1-like proteins are a family of WD40 proteins in eukaryotes that form subunits of several protein complexes acting on chromatin (for a review, see Hennig et al., 2005). *Arabidopsis* has five *MSI1*-like genes, *MSI1-MSI5* (Ach et al., 1997; Kenzior and Folk, 1998; Hennig et al., 2003). *Arabidopsis MSI1* is essential for gametophyte and seed development, and is a member of the Fertilisation independent seed (FIS) complex, which is similar to the *Drosophila* Polycomb repressive complex PRC2 (Köhler et al., 2003a; Guitton et al., 2004). In addition, *MSI1* has been suggested to be part of a second PRC2-like complex, the CURLY-LEAF (CLF) complex (Chanvivattana et al., 2004; Hennig et al., 2005). In addition to MSI1, the function of its homolog MSI4 was discovered when the autonomous pathway mutant *fve* was mapped to the *MSI4* locus (Koornneef et al., 1991; Ausin et al., 2004; Kim et al., 2004). It was suggested that MSI4/FVE acts together with a histone deacetylase to repress transcription of the floral repressor FLC (Ausin et al., 2004).

Here, we show that *Arabidopsis* MSI1 is an *FLC*-independent activator of the floral transition. MSI1 acts genetically upstream of the floral activator *SOC1* in a pathway parallel to its homolog MSI4/FVE. These results suggest that MSI1 participates in a novel mechanism to promote flowering, which is similar to the autonomous pathway but independent of *FLC*.

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MATERIALS AND METHODS

Plant material and growth conditions

In this study, we used the *msi1-1* mutant, the line *msi1OEc2*, which ectopically expresses MSI1, and the line *msi1Asb7* (*msi1-as*), which carries a *MSI1* antisense construct (Hennig et al., 2003; Köhler et al., 2003a) (V. Exner, W.G., L.H. and P. Taranto, unpublished). To complement the *msi1-1* mutant with wild-type MSI1, a 2000 bp fragment of the *MSI1* promoter fused to the *MSI1* cDNA was inserted into vector pCAMBIA1380. To complement the *msi1* mutant with tagged MSI1, the 2000 bp fragment of the *MSI1* promoter fused to the *MSI1* cDNA was inserted into a pCAMBIA1380 vector that was modified for carboxyterminal tagging of proteins by insertion of oligonucleotides coding for a tandem affinity purification (TAP) tag between *Bgl*II and *Nco*I (Rigaut et al., 1999) (see Table S2 in the supplementary material). Heterozygous *msi1-1* plants were transformed by floral dip and transgenic plants were selected on hygromycin. T1 plants were assayed for complementation of seed abortion and several complementing lines were obtained. To ectopically express *SOC1*, the *SOC1* cDNA was amplified by PCR and inserted into the pK7WG2 binary destination vector downstream of the cauliflower mosaic virus (CaMV) 35S promoter using Gateway technology (Invitrogen) (Karimi et al., 2002). Wild-type plants were transformed by floral dip and selected on kanamycin. T1 plants were assayed for early-flowering time and the *35S::SOC1* transgene was introduced by crossing into *msi1-tap1* plants. The *flm-3*, *fve-5*, *flc-6* and *clf-29* mutants are null alleles (see Fig. S2 in the supplementary material), which were obtained from the SAIL and SALK collections of T-DNA insertion lines (SALK_141971, SAIL_1167E5, SALK_41126 and SALK_N521003) (Sessions et al., 2002; Alonso et al., 2003). Seeds of the *soc1-2* mutant were kindly provided by Professor Ihla Lee (Lee et al., 2000). All plants used in this study are in the Columbia background.

For measuring flowering time, seeds were plated on Murashige and Skoog (MS) medium (Duchefa, Haarlem, The Netherlands), stratified for 2 days at 4°C, and grown on plates for 10 days before transfer onto soil. Plants were kept in Conviron growth chambers with mixed cold fluorescent and incandescent light (110 to 140 μmol/m^2s, 21±2°C) under long day (16 hour) photoperiods, unless indicated otherwise. The flowering time was measured as the number of total rosette leaves longer than 0.5 cm at bolting for at least 14 plants, except for four plants for the *msi1-tap1 fve* double mutant. Graphs show means±s.e.m. For the GA treatment, plantlets were grown for 10 days on MS medium containing 100 μM GA_3. After transfer onto soil, plants were sprayed weekly with 100 μM GA_3. For the vernalization treatment, seeds were plated on MS medium and kept in continuous light for one day before being exposed to 4°C for 6 weeks. After the vernalization treatment, plants were transferred into growth chambers under long day (16 hour) or short day (8 hour) photoperiods, as indicated.

RNA isolation and RT-PCR

RNA was extracted using TRIzol, as previously described (Hennig et al., 2003). For RT-PCR analysis, 2 μg total RNA were treated with DNaseI. The DNA-free RNA was reverse-transcribed using an oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen). Aliquots of the generated cDNA, which equalled 100 ng total RNA, were used as a template for PCR with gene-specific primers (see Table S3 in the supplementary material).

Array hybridization and evaluation

Experimental design

Seedlings of *Arabidopsis thaliana* (L.) Heynh. (Accession Columbia) were grown on MS medium for 8 days in growth chambers at 21°C under long day photoperiods (16 hours light, 8 hours darkness). Seedlings were pooled from three individual plates for each replicate. The entire experiment was performed twice, providing independent biological replicates.

Array design, samples, hybridizations and measurements

Affymetrix Arabidopsis ATH1 GeneChips were used in the experiment (Affymetrix, Santa Clara, CA). The exact list of probes present on the arrays can be obtained from the manufacturer's website (http://www.affymetrix.com). Analysis was based upon annotations compiled by TAIR (http://www.arabidopsis.org). Labelling of samples, hybridizations and measurements were performed as described (Hennig et al., 2004). Data were deposited into the ArrayExpress database (Accession number E-MEXP-513).

Evaluation, normalization and data analysis

Signal values were derived from the Affymetrix *.cel files using the GCRMA algorithm (Wu et al., 2003). Data were processed with the statistical package R (version 1.9.1) that is freely available at http://www.r-project.org/. Significantly different gene expression was detected based on the rank-product algorithm implemented in R (Breitling et al., 2004). This algorithm inherently corrects for multiple testing. Genes were considered as being differentially expressed if $P<0.05$. To enrich for biologically relevant changes, only genes with a minimal fold change of 1.5 in all experiments were selected.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed as previously described (Köhler et al., 2003b; Ausin et al., 2004). Chromatin was isolated from fifteen day-old seedlings grown on MS in short day photoperiods. After cross-linking with 1% formaldehyde, the chromatin was sonicated to obtain DNA fragments of 200 to 1000 bp. The chromatin was then immunoprecipitated using anti-dimethyl-histone H3 Lys4 antiserum (Upstate, Charlottesville, VA) and anti-acetyl-histone H3 Lys9 polyclonal IgG (Upstate). PCR was performed to amplify fragments presented in Fig. 6 (for primers used, see Table S4 in the supplementary material). For each fragment of the *SOC1* locus, signals were normalized to that of the phosphofructokinase gene *At4g04040*.

RESULTS

MSI1 is needed for the transition to flowering

In previous work, we and others have found that the *Arabidopsis* protein *MSI1* is essential for gametophyte and seed development (Köhler et al., 2003a; Guitton et al., 2004; Guitton and Berger, 2005). In a project addressing the function of MSI1 in seed development, we complemented the embryo-lethal *msi1-1* allele with constructs containing 2 kb of *MSI1* promoter sequences driving the *MSI1* cDNA either alone or fused to a carboxyterminal TAP (tandem affinity purification) tag. Several independent transgenic *MSI1/msi1-1* lines showed the expected complementation of seed lethality and of fertilization-independent seed development (Table 1; data not shown). Among the progeny of the transformed *MSI1/msi1-1* plants, we identified homozygous *msi1-1/msi1-1* individuals that were entirely reliant on the complementing *MSI1* or *MSI1-TAP* transgenes. These complemented *msi1-1/msi1-1* plants could successfully complete their sporophytic development to produce normal amounts of viable seeds, which was in striking contrast to *msi1* mutants and to plants with strongly reduced MSI1 levels (Hennig et al., 2003). Although the seed phenotype of the *msi1* mutant was complemented by expression of the *MSI1* transgenes, all complemented *msi1-1/msi1-1* lines flowered later than wild type, suggesting that the transgenes could not fully complement the *msi1* mutant (Fig. 1A-C). All lines expressing either tagged or untagged MSI1 were late flowering, suggesting that the *MSI1* promoter fragment-cDNA fusion used was not sufficient for normal *MSI1* expression. Because protein levels appeared unchanged in extracts from whole *msi1-tap1* (*msi1/msi1* P_{MSI1}*::MSI1-TAP* line 1) leaves and flowers, or from entire *msi1-notap3* (*msi1/msi1* P_{MSI1}*::MSI1* line 3) seedlings (data not shown), the promoter fragment-cDNA fusion that was used probably failed to sustain normal amounts of MSI1 protein only at specific developmental stages, or only in specific tissues. Subsequently, we focused our experiments on the *msi1-tap1* transgenic line, which had only a single inserted transgene and was thus well suited for genetic analysis. In *msi1-tap1* plants, the transition to flowering was delayed both in the number of rosette leaves and in the number of days at

Table 1. Seed abortion in *msi1* mutants and complemented lines

Name	Genotype	Normal (%)	Aborted (%)
Wild type	*MSI1/MSI1*	397 (98.0)	8 (2.0)
msi1-1	*MSI1/msi1-1*	204 (50.0)	204 (50.0)
msi1-tap1	*msi1-1/msi1-1* P_{MSI1}*::MSI1-TAP/*P_{MSI1}*::MSI1-TAP**	325 (96.2)	13 (3.8)
msi1-notap1	*msi1-1/msi1-1* P_{MSI1}*::MSI1/*P_{MSI1}*::MSI1**	169 (100.0)	0 (0.0)
msi1-notap2	*msi1-1/msi1-1* P_{MSI1}*::MSI/*P_{MSI1}*::MSI1**	126 (98.4)	2 (1.6)

*P_{MSI1} refers to a 2 kb fragment of regulatory sequences from the *MSI1* promoter.

bolting (Fig. 1B,D). However, *msi1-tap1* plants were more affected in leaf number than in days. Such a slightly faster rate of leaf initiation was also observed for mutants of the autonomous pathway (Koornneef et al., 1998).

To test whether late flowering of *msi1-tap1* plants was indeed caused by incomplete complementation of the *msi1* mutant, we crossed late-flowering *msi1-tap1* plants with plants that ectopically express MSI1 from a 35S promoter-*MSI1* cDNA construct (line *msi1OEc2*) (Hennig et al., 2003). Plants that are hemizygous for the *35S::MSI1* transgene (*msi1-oe*) strongly overexpress *MSI1*, whereas plants that are homozygous for this transgene (*msi1-cs*) suffer from strongly aberrant development caused by the co-suppression of endogenous and transgenic *MSI1* genes (Hennig et al., 2003). Here, we analyzed only plants that ectopically express *MSI1* and did not exhibit the *msi1-cs* phenotype (see Fig. S1E in the supplementary material). Ectopic expression of the *MSI1* cDNA construct did not only completely rescue the late-flowering phenotype of *msi1-tap1* plants, but even caused these plants to flower slightly but significantly earlier than wild-type plants (Fig. 1E). Similarly, ectopic expression of *MSI1* accelerated flowering in a wild-type background under continuous light and in long days (Fig. 1F, Fig. 3B). Because the delay of flowering observed in the partially complemented *msi1-tap1* plants could be fully complemented by increasing the MSI1 protein dosage, it was most likely to be caused by insufficient MSI1 protein amounts at specific developmental stages or in specific tissues. In addition, plants in which general MSI1 levels were reduced to 50% by expression of a *MSI1* antisense RNA construct (see Fig. S1A in the supplementary material) flowered later than wild-type plants (Fig. 1E), demonstrating that normal MSI1 levels are required to attain the correct flowering time. Plants heterozygous for the *msi1* mutation were never late flowering in our conditions (see Fig. S1B in the supplementary material), probably because they contain similar levels of MSI1 protein to wild type (see Fig. S1D in the supplementary material). Together, our results show that reduced levels of MSI1 delay flowering, and that increased levels of MSI1 accelerate flowering.

Epigenetic defects in flowering time can arise in mutants with defective chromatin dynamics like *ddm1* and *met1*, and such defects can subsequently be stably transmitted (Kakutani, 1997; Kankel et al., 2003). Therefore, we tested whether the late-flowering trait of *msi1-tap1* plants could be transmitted independently of the mutated *msi1* allele. However, after crossing *msi1-tap1* with wild-type plants, late flowering was never observed in the progeny that carried a wild-type *MSI1* allele (see Fig. S1C in the supplementary material). This was similar to plants heterozygous for the *msi1* mutation (see Fig. S1B in the supplementary material). In addition, we tested whether *msi1-tap1* plants could accumulate epi-mutations similar to *ddm1* or *met1* mutants, but in six subsequent generations no alterations of the phenotype were observed. In late-flowering *ddm1* and *met1* plants, late flowering is usually attributed to increased *FWA* expression. In

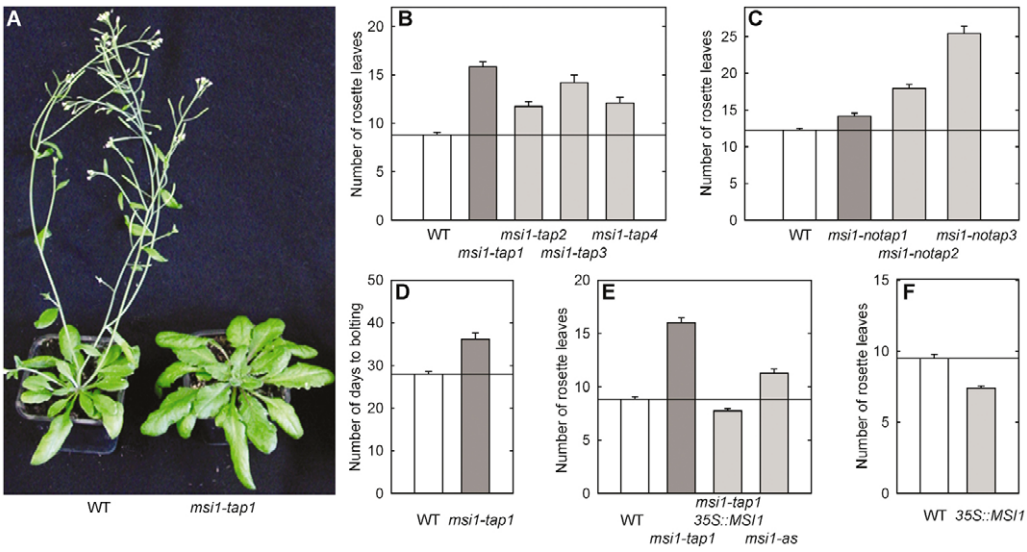

Fig. 1. MSI1 is an activator of floral transition. (**A**) 35-day-old wild-type (WT) and *msi1-tap1* plants grown in long days (LD). Note, *msi1-tap* represents *msi1-1/msi1-1* P_{MSI1}*::MSI1-TAP*. (**B**) Analysis of the flowering time of four independent *msi1* lines complemented with a TAP-tagged MSI1 protein grown in LD. (**C**) Analysis of the flowering time of three independent *msi1* lines complemented with untagged MSI1 protein grown in LD. Note, *msi1-notap* represents *msi1-1/msi1-1* P_{MSI1}*::MSI1*. (**D**) Flowering time of *msi1-tap1* grown in LD presented in number of days to bolting. (**E**) Analysis of the flowering time of the *msi1-tap1* line complemented with a *35S::MSI1* construct, and of *MSI1* antisense (*msi1-as*) plants grown in LD. (**F**) Analysis of the flowering time of wild-type plants carrying the *35S::MSI1* construct grown in continuous light. Graphs show means±s.e.m. of total rosette leaves at bolting (B,C,E,F) or days to bolting (D).

contrast to *ddm1* and *met1*, *FWA* expression was not increased in *msi1-tap1* plants (data not shown). Together, these results established that the late flowering of *msi1-tap1* plants was not caused by heritable, epigenetic defects at other loci, but that MSI1 is directly involved in the transition to flowering.

MSI1 is similar to genes from the pathway for the autonomous promotion of flowering

Several pathways suppress or promote flowering in response to environmental conditions. Therefore, we analyzed flowering time of *msi1-tap1* plants under different growth conditions (Fig. 2). First, we tested the effect of the exogenous application of gibberellic acid (GA), which can strongly reduce flowering time in wild-type plants (Chandler et al., 1996). We found that flowering time was strongly reduced by GA in *msi1-tap1* plants as well (Fig. 2A). Wild-type and *msi1-tap1* plants responded similarly to low concentrations of GA, but *msi1-tap1* responded slightly more to high concentrations of GA than did wild type (Fig. 2A, see Fig. S1F in the supplementary material). This is similar to late-flowering mutants, such as *fca* and *fld*, that are not in the GA pathway (Chandler et al., 1996; Chou and Yang, 1998). Second, we compared flowering time in long-day and short-day photoperiods. Mutants defective in the photoperiod pathway flower at similar times in short or long days (Koornneef et al., 1991). By contrast, *msi1-tap1* plants flowered much later in short days than in long days (Fig. 2C). Third, we tested a potential role of MSI1 in the thermo-sensory pathway of flowering control. This pathway requires the *MSI1*-homolog *MSI4*/*FVE* (Blazquez et al., 2003). We compared the flowering time of *msi1-tap1* plants at 16°C and at 23°C, and found that, unlike a *fve* mutant, *msi1-tap1* plants flowered significantly later at 16°C than at 23°C (Fig. 2B). Finally, we tested whether *msi1-tap1* plants can respond to vernalization. Vernalization of *msi1-tap1* plants at 4°C for 6 weeks accelerated flowering but failed to fully suppress the late-flowering phenotype (Fig. 2D). This is similar to late-flowering mutants such as *gi*, *ft* or *co* that are not in the vernalization pathway (Moon et al., 2005). Together, we conclude that *msi1-tap1* plants have no major defects in the promotion of flowering by gibberellic acid, photoperiod, ambient temperature or vernalization. Instead, the physiology of *msi1-tap1* plants was most similar to that of mutants from the autonomous pathway.

MSI1 and MSI4 are not redundant

Both *fve* mutants (Ausin et al., 2004; Kim et al., 2004) and *msi1-tap1* plants (this study) are late flowering, suggesting that the two homologous proteins MSI1 and MSI4/FVE are not completely redundant. Alternatively, *MSI1* and *MSI4/FVE* could have distinct, specific expression patterns and fulfil similar functions in different tissues. However, neither *MSI1* nor *MSI4*/*FVE* has a highly specific expression pattern (Hennig et al., 2003; Ausin et al., 2004). Moreover, the expression of these two genes was highly correlated in a large collection of microarray experiments profiling *Arabidopsis* development (Schmid et al., 2005) (Fig. 3A). The observed correlation (Pearson correlation coefficient=0.92) was among the top 0.028% of the highest values found in this data set (see Fig. S3 in the supplementary material). Despite generally very similar expression patterns, it is possible that MSI4/FVE is essential in a restricted domain where *MSI1* is not expressed. To test this hypothesis, we crossed a *fve* mutant with the line *msi1OEc2*, in which MSI1 is ectopically expressed and which can fully complement the *msi1-tap1* plants (Fig. 1E). Although ectopic expression of *MSI4*/*FVE* under the control of the 35S promoter can complement the *fve* mutant (Ausin et al., 2004), we found that ectopic expression of *MSI1* under the control of the 35S promoter could not complement the *fve* mutant (Fig. 3B). Although ectopic *35S::MSI1 fve* plants flowered slightly later than *fve* alone in the experiment shown in Fig. 3B, this effect was not observed in independent experiments. These results demonstrate that MSI1 cannot replace MSI4/FVE, i.e. that MSI1 and MSI4/FVE are not redundant.

The promotion of flowering by MSI1 is independent of CURLY LEAF

Arabidopsis MSI1 could potentially interact with many partners (for a review, see Hennig et al., 2005), but only its function in the PRC2-like FIS-complex has been confirmed in plants (Köhler et al.,

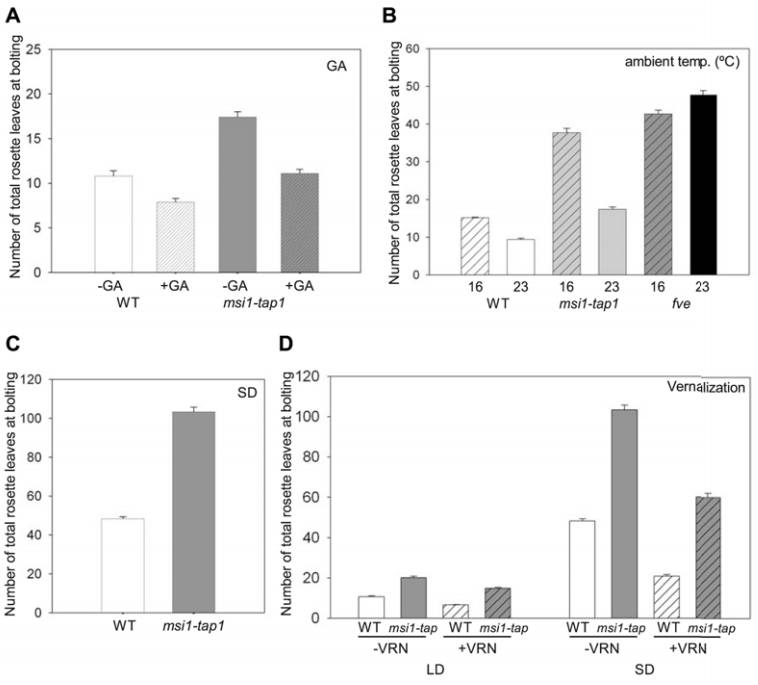

Fig. 2. Physiology of *msi1-tap1* plants is similar to that of autonomous pathway mutants. (**A**) Analysis of the flowering time of *msi1-tap1* without (–GA) or with (+GA) gibberellic acid treatment in LD. (**B**) Analysis of the flowering time of *msi1-tap1* and *fve* at 16°C and 23°C in LD. (**C**) Analysis of the flowering time of *msi1-tap1* grown in short days (SD). (**D**) Analysis of the flowering time of *msi1-tap1* without (–VRN) or with (+VRN) a vernalization treatment in LD (left) and SD (right).

2003a). The FIS-complex functions specifically during gametophyte and seed development. Other homologs of PRC2 subunits in *Arabidopsis*, such as *CLF*, are expressed in sporophytic tissues during later stages of development, suggesting the existence of additional PRC2-like complexes (Chanvivattana et al., 2004; Hennig et al., 2005). Therefore, we measured flowering time in *clf*, *msi1-tap1* and *clf msi1-tap1* double mutants. In agreement with observations by others (Goodrich et al., 1997; Chanvivattana et al., 2004), *clf* flowered earlier than wild type (Fig. 3C). By contrast, the *clf msi1-tap1* double mutant flowered considerably later than *clf* alone, although not as late as *msi1-tap1*. Thus, MSI1 function to promote flowering does not involve CLF.

MSI1 is needed for controlled expression of the floral activator *SOC1*

Many flowering genes are known and can serve as molecular markers to identify affected pathways in flowering time mutants. We used Affymetrix ATH1 microarrays, which simultaneously probe the expression of 71 known flowering genes, to test whether gene expression patterns support the conclusions from the physiological experiments. RNA was extracted from 8-day-old wild-type and *msi1-tap1* seedlings that were grown in long days and harvested at the beginning of the light period and one hour after end of the light period. We chose 8-day old seedlings because the pathway integrators SOC1 and FT accumulate at this stage of development (Kardailsky et al., 1999; Samach et al., 2000). The two sampling times were used because important flowering genes such as *CO* and *FT* have their circadian expression peaks at these times (Suarez-Lopez et al., 2001; Yanovsky and Kay, 2002). The expression data reflected the reported circadian expression pattern of clock-associated genes (for a review, see Eriksson and Millar, 2003), but there was no significant change in the expression of known clock-associated genes between *msi1-tap1* and wild-type plants, suggesting that the circadian clock functions normally in *msi1-tap1* plants. This is consistent with the observation that the photoperiod pathway of flowering control, which depends on the circadian clock, is not affected in *msi1-tap1* plants. Statistical analysis of the four independent microarray data sets (see Materials and methods for details) identified 106 genes that were upregulated and 18 genes that were downregulated in *msi1-tap1* plants (see Table S1 in the supplementary material). These genes fall into diverse functional categories, including several genes related to stress responses. Notably, some late-flowering *Arabidopsis* mutants have increased tolerance to drought stress, and it was found that both *FRI* and *FLC* pleiotropically affect flowering time and water use efficiency (McKay et al., 2003). It remains to be tested whether late-flowering mutants also affect tolerance to other types of stress and what role the genes play that have changed expression in *msi1-tap1* plants.

Most of the 71 flowering genes probed by the ATH1 array were similarly expressed in wild type and *msi1-tap1*, except for *SOC1*, which had significantly reduced expression in both the evening and the morning (4.3-fold and 2.7-fold, respectively; see Table S1 in the supplementary material). Interestingly, neither *FT* nor *LFY*, which are two other pathway integrators, showed significant expression differences in *msi1-tap1* plants. Most importantly, there were no changes in the expression of *FLC*, the major repressor of *SOC1* transcription. In order to confirm and extend the microarray results on *SOC1*, we performed a time-course experiment using independently prepared RNA. In wild-type seedlings grown in long days, *SOC1* transcripts accumulate between five and eleven days after germination (Kardailsky et al., 1999). When comparing the dynamics of *SOC1* transcription between wild type and *msi1-tap1*, we found that *SOC1* transcripts accumulated about four days later in *msi1-tap1* than in wild-type plants (Fig. 4A,B). Because flowering was much more delayed in short days than in long days (Fig. 2), we hypothesized that differences in *SOC1* expression should also be more robust in short day conditions. Indeed, we found strong differences in *SOC1* expression between wild type and *msi1-tap1* at 15 and 18 days after germination in short days (Fig. 4C). This delay in *SOC1* accumulation correlates with the delay in flowering. Similarly, we found a strong reduction of *SOC1* expression 14 days after germination in *msi1-as* and *msi1-notap3* (Fig. 4D). In contrast to *SOC1*, transcript levels of *FT* and *FLC* were altered only in *fve* mutants and not in *msi1-tap1* plants (Fig. 5A,B; see Fig. S2E in the supplementary material). The model that MSI1 is needed for the activation of *SOC1* assumes expression of *MSI1* in the shoot apex, where *SOC1* is believed to act. Indeed, *MSI1* is widely expressed throughout the plant (Hennig et al., 2003), including the shoot apex (Fig. 4E; see Fig. S4 in the supplementary material). Together, these results demonstrate that MSI1 is required for the control of *SOC1* expression during the induction of flowering.

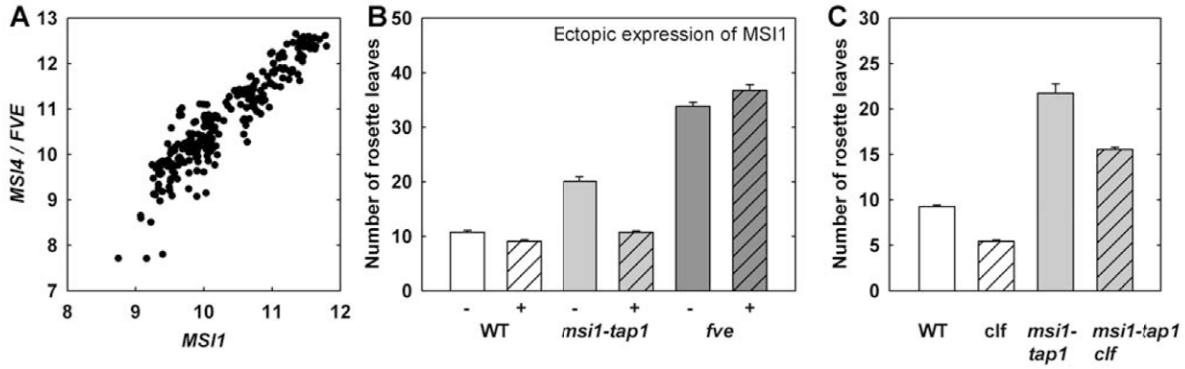

Fig. 3. MSI1 and MSI4/FVE are non-redundant. (**A**) *MSI1* and *MSI4/FVE* expression profiles are strongly correlated. Data are based on measurements from 238 microarrays profiling *Arabidopsis* development (Schmid et al., 2005), which were processed using the GCRMA algorithm. The observed correlation between *MSI1* and *MSI4/FVE* (Pearson correlation coefficient=0.92) was among the top 0.028 % of the highest values found in this data set. (**B**) Analysis of the flowering time of *msi1-tap1* and *fve* plants in LD either without (–) or with (+) introduction of the construct *35S::MSI1*. (**C**) Analysis of the flowering time of *msi1-tap1*, *clf* and *clf msi1-tap1* double mutants in LD.

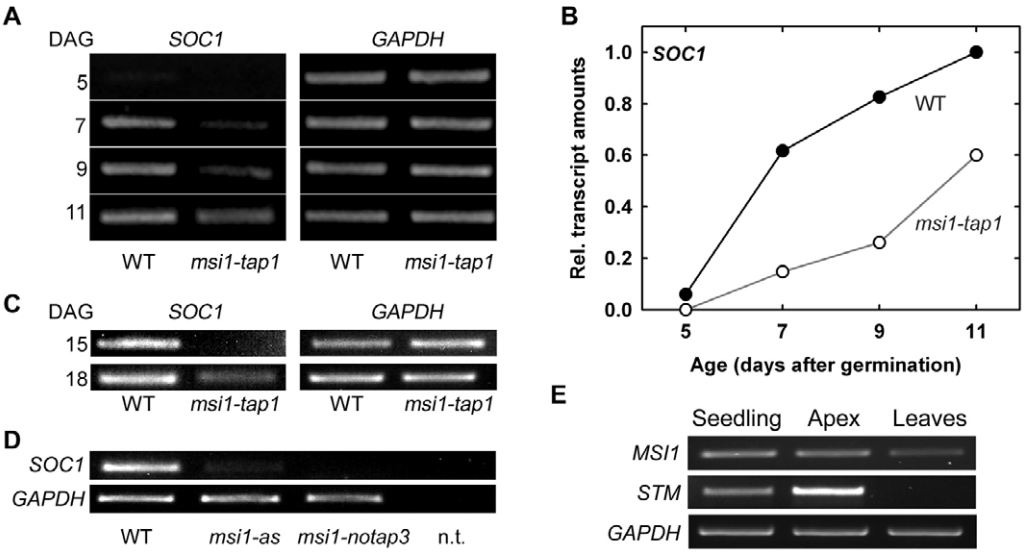

Fig. 4. Expression of the floral activator *SOC1* is delayed in *msi1-tap1*, *msi1-as* and *msi1-notap3*. (**A**) Expression analysis of *SOC1* in wild-type and *msi1-tap1* during seedling development in LD. Plants were grown for 5 to 11 days after germination (DAG) on plates containing MS medium in LD. RNA was extracted from total seedlings grown on plates containing MS medium in LD and harvested every second day at 4 hours after start of the light period. (**B**) Quantification of the results in A. Values were normalized to the corresponding GAPDH control. The maximal value in wild-type was set to 1.0. Black symbols, wild type; white symbols, *msi1-tap1*. (**C**) Expression analysis of *SOC1* in *msi1-tap1* in SD. RNA was extracted from total 15- and 18-day-old seedlings grown on plates containing MS medium in SD harvested at 1 hour before the end of the light period. (**D**) Expression analysis of *SOC1* in *msi1-as* and *msi1-notap3* in SD. RNA was extracted from total 14-day-old seedlings grown on plates containing MS medium in SD harvested at 1 hour before the end of the light period. (**E**) *MSI1* expression in the shoot apex. RNA was isolated from dissected 18-day-old seedlings grown in SD. *STM* was used an apex-specific control. *GAPDH* was used as control in A,C-E. n.t., no template control.

MSI1 promotes flowering independently of *FLC* and *FLM*

Our results show that MSI1 functions to promote flowering in an autonomous-like pathway. In contrast to other known members of the autonomous pathway, which all promote flowering by reducing *FLC* transcript levels (Simpson, 2004), *FLC* transcript levels were not changed in *msi1-tap1* plants, suggesting that *MSI1* acts independently of *FLC*. In order to test this hypothesis, we crossed *msi1-tap1* plants with an *flc* null mutant (Fig. 5C; see Fig. S2C in the supplementary material). If *MSI1*, like the known autonomous pathway genes, acted by controlling *FLC* activity, complete loss of *FLC* in the *msi1-tap1* background should suppress the late-flowering phenotype. However, the flowering time in *msi1-tap1* and in *msi1-tap1 flc* double mutant plants was similar. This demonstrates that *MSI1* acts downstream of *FLC* or in a parallel pathway. *FLM* is a homolog of *FLC* that acts as a repressor of flowering in the photoperiod pathway (Scortecci et al., 2001; Scortecci et al., 2003). To test whether *MSI1* acts upstream of *FLM* and to confirm that *MSI1* is not involved in the photoperiod pathway, we crossed *msi1-tap1* plants with an *flm* mutant, which lacks all four major alternatively spliced *FLM* transcripts (Fig. 5C, see Fig. S2D in the supplementary material). Similar to *flc*, the *flm* mutation failed to fully rescue the late-flowering phenotype of *msi1-tap1* plants, thus demonstrating that *MSI1* and *FLM* act in parallel pathways.

If *MSI1* acts in a parallel pathway to *FLC*, then increased expression of *FLC*, as observed in *fve* plants, should have an additive effect on the flowering time of *msi1-tap1* plants. To test this hypothesis, we constructed *msi1-tap1 fve* double mutants. The double mutants flowered extremely late, and much later than either *msi1-tap1* plants or *fve* mutants alone (Fig. 5D). In order to test whether the synergistic effect of *msi1-tap1* and *fve* on flowering time was caused by a synergistic effect on *FLC* expression, we measured *FLC* transcript levels in the wild-type, *msi1-tap1*, *fve* and *msi1-tap1 fve* plants. As described above, *FLC* expression was similar in wild-type and *msi1-tap* plants, but was much higher in *fve*. Importantly, expression of *FLC* was not higher in *msi1-tap1 fve* double mutant plants than in the *fve* single mutant (Fig. 5B). This observation strongly supports our conclusion that MSI1 and MSI4/FVE have non-redundant functions and act in separate genetic pathways to control flowering. Together, we conclude that MSI1 functions to promote flowering independently of *FLC*.

MSI1 acts upstream of *SOC1*

The expression data demonstrate that MSI1 is required for the activation of *SOC1* at the floral transition (Fig. 4). Therefore, we genetically tested whether *SOC1* is the major target of MSI1 for flowering time control, i.e. whether delayed expression of *SOC1* causes the delayed flowering of *msi1-tap1* plants. If MSI1 indeed acted genetically upstream of *SOC1*, loss of *SOC1* should not, or should only weakly, enhance the late-flowering phenotype of *msi1-tap1* plants. Alternatively, MSI1 could act in a parallel pathway to *SOC1* or by controlling targets other than *SOC1* (like FLC that represses *SOC1* and *FT*). In this case, complete loss of *SOC1* in the *msi1-tap1* background should strongly enhance the late-flowering phenotype, similar to the loss of *SOC1* in *fve* or *ft* (Moon et al., 2005). Using the *SOC1* loss-of function allele *soc1-2* (Lee et al., 2000), we constructed *msi1-tap1 soc1* double mutants and measured their flowering time. Both, *msi1-tap1* and *soc1* single mutants had a similar delay in flowering, and the *msi1-tap1 soc1* double mutant flowered only slightly later than the single mutants (Fig. 5D). This increase was much smaller than the increase observed in *msi1-tap1 fve* double mutants. Because we observed before that *MSI1* is required for correct expression of *SOC1* (Fig. 4), we conclude that *MSI1* acts genetically upstream of *SOC1*, and possibly of at least one unidentified additional flowering-time gene as well.

MSI1 is needed to establish activating chromatin marks at the *SOC1* locus

Some flowering-time genes act by controlling the chromatin status of downstream genes (Gendall et al., 2001; Ye et al., 2003; Ausin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung and Amasino, 2004). Because MSI1-like proteins participate in various chromatin-

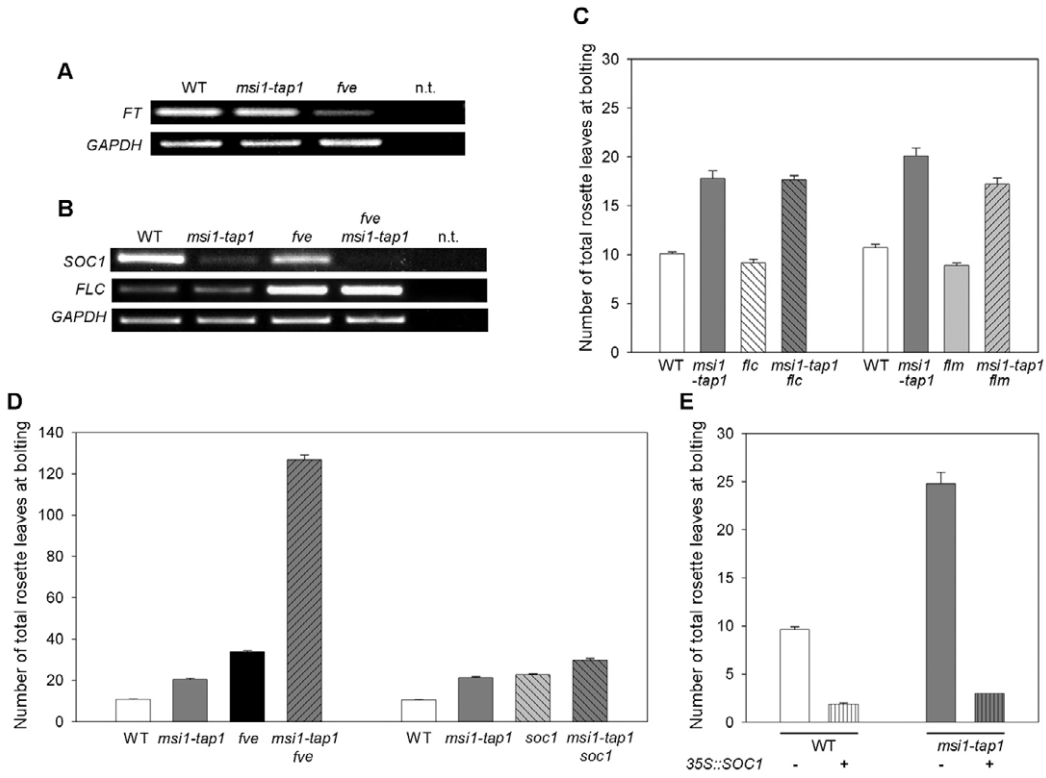

Fig. 5. Genetic interactions of *MSI1*. (**A**) Expression analysis of *FT* in *msi1-tap1* and *fve*. RNA was extracted from total 8-day-old seedlings grown on plates containing MS medium in LD. (**B**) Expression analysis of *SOC1* and *FLC* in *msi1-tap1 fve* double mutants. RNA was extracted from 8-day-old seedlings grown on plates containing MS medium in LD. *GAPDH* was used as a control in A and B. n.t., no template control. (**C**) Analysis of the flowering time of *msi1-tap1 flc* and *msi1-tap1 flm* double mutants in LD. (**D**) Analysis of the flowering time of *msi1-tap1 fve* and *msi1-tap1 soc1* double mutants in LD. (**E**) Analysis of the flowering time of *35S::SOC1* and *msi1-tap1 35S::SOC1* in LD.

modifying complexes, we tested whether *Arabidopsis* MSI1 is required to establish correct chromatin marks on the *SOC1* locus. We used whole seedlings at 15 days after germination in short days for Chromatin Immunoprecipitation (ChIP) experiments. At this stage, *SOC1* transcripts were clearly detectable in wild type but not in *msi1-tap1* plants. Because the methylation of lysine 4 at histone H3 (H3K4) is a major posttranslational modification known to facilitate transcription (for a review, see Peterson and Laniel, 2004), we tested the presence of H3K4 di-methylation on various regions of the *SOC1* locus by ChIP (Fig. 6). The ChIP results were normalized to a phosphofructokinase gene, which did not change expression in *msi1-tap1* plants (Fig. 6A). H3K4 di-methylation was similar between wild-type and *msi1-tap1* plants within the 5′ UTR and in the 3′ region, but it was significantly less abundant in the large second intron of *SOC1* (Fig. 6C), and the same result was found when ChIP data were normalized to a silenced Cinful-like retrotransposon gene instead of the phosphofructokinase gene (data not shown). Methylation of H3K4 was found to interfere with deacetylation at H3K9 (Nishioka et al., 2002). Therefore, we tested whether the acetylation of H3K9 was affected in *msi1-tap1* plants as well. Similar to H3K4 methylation, H3K9 acetylation was less abundant on *SOC1* chromatin in *msi1-tap1* than in wild-type plants (Fig. 6C). Interestingly, H3K9 acetylation was changed about 2-fold at *SOC1* in *msi1-tap1* plants, and this value is similar to the reported difference of H3K9 acetylation at *FLC* in *fve* [1.9-fold (Ausin et al., 2004)]. These results show that *MSI1* is required to establish chromatin marks that facilitate transcription at the *SOC1* locus.

DISCUSSION

We found that *Arabidopsis MSI1* is an activator of the floral transition. This novel function of *MSI1* became apparent because partially complemented *msi1* null mutants and plants in which MSI1 levels were reduced by the expression of a *MSI1* antisense RNA

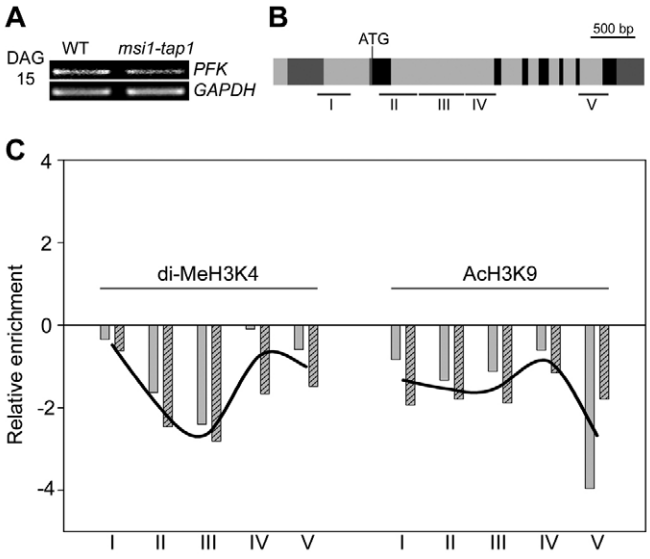

Fig. 6. Altered histone methylation and acetylation at the *SOC1* locus in *msi1-tap1* seedlings. (**A**) Scheme of the transcribed region of the *SOC1* locus and the fragments used for chromatin immunoprecipitation (ChIP). The gray and black boxes symbolize exons and introns, respectively, and the dark gray boxes, the untranslated exons. (**B**) Expression of the phosphofructokinase (*PFK*) gene *At4g04040* is not changed in *msi1-tap1* plants. RNA was extracted from total 15-day-old seedlings grown on plates containing MS medium in SD. (**C**) Quantification of PCR products after ChIP with anti-dimethyl-histone H3K4 and anti-acetyl-histone H3K9 antiserum. Values were normalized to *PFK* and are shown as relative enrichments in samples from *msi1-tap1* versus wild-type seedlings. The gray and hatched bars represent the results of two independent ChIP experiments. Chromatin was extracted from 15-day-old seedlings grown in SD harvested at 1 hour before the end of the light period.

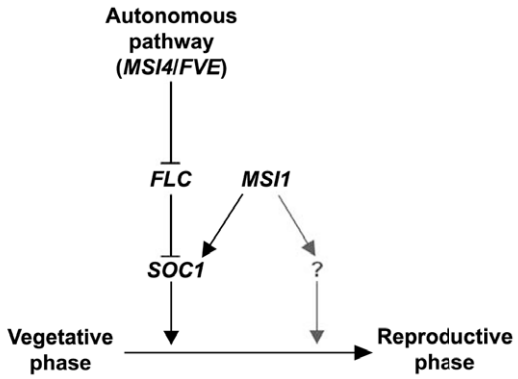

Fig. 7. Model of non-redundant functions of *MSI1* and *MSI4/FVE* to promote flowering.

construct were both late flowering. By contrast, transgenic plants with increased MSI1 levels were early flowering. Physiological experiments suggested that the function of *MSI1* in the control of flowering was most similar to that of the known genes from the autonomous pathway, such as *FVE*, *FCA*, *FPA*, *FY* and *LD*.

The genes of the autonomous pathway function to reduce expression of the flowering repressor *FLC*. In loss-of-function mutants of any of these genes, transcript levels of *FLC* are increased while transcript levels of the floral activators *SOC1* and *FT* are reduced (for a review, see Henderson and Dean, 2004). In contrast to the known autonomous flowering activators, MSI1 was required for the normal expression of *SOC1* only, and not for that of *FT* or *FLC*. The observations that the *soc1* mutant has a similar late-flowering phenotype to the *msi1-tap1* plants, and that the double mutant *msi1-tap1 soc1* flowers only slightly later than the single *msi1-tap1* or *soc1* mutants, is consistent with a model in which *MSI1* functions upstream of the floral integrator *SOC1*. The model that delayed flowering in *msi1-tap1* plants was caused by reduced *SOC1* expression was further confirmed by the observation that constitutive expression of *SOC1* completely rescues the late-flowering phenotype of *msi1-tap1* plants. *FT* and *SOC1* expression increase after vernalization even in an *flc* mutant background (Moon et al., 2003). As only *SOC1* expression was affected in *msi1-tap1* plants, the mechanisms for *FLC*-independent transcriptional activation might be different for *SOC1* and *FT*. The hypothesis that MSI1 promotes flowering independently of *FLC* was genetically tested. The fact that *msi1-tap1 flc* double mutants flowered as late as the *msi1-tap1* plants suggests that MSI1 functions completely independently of *FLC* in flowering control. Expression of *MSI1* is not strongly regulated during development. Similarly, the MSI1 protein could be detected in all tissues tested (Hennig et al., 2003). Therefore, the developmental activation of *SOC1* expression in wild-type plants is probably not caused by the transcriptional regulation of *MSI1*. Instead, MSI1 might be required to efficiently transmit a *SOC1*-activating signal. *SOC1* is a floral integrator and is activated by several pathways, including the GA pathway. Because *msi1-tap1* and wild-type plants responded similarly to non-saturating concentrations of GA, it is likely that MSI1 is not involved in the activation of *SOC1* by the GA pathway.

MSI1 is a homolog of MSI4/FVE, which functions in the autonomous flowering pathway that acts through *FLC*, but ectopically expressed MSI1 cannot replace MSI4/FVE. Alignment of their amino acid sequences (see Fig. S5 in the supplementary material) shows considerable sequence divergence between the two proteins (e.g. the long amino-terminal extension of MSI4/FVE), which could explain possible biochemical and functional differences. We suggest that MSI1 and MSI4/FVE act in two parallel pathways: MSI1 functions independently of *FLC* to activate *SOC1* and possibly at least one additional, unidentified gene, and MSI4/FVE functions through *FLC* to activate *SOC1* and *FT* (Fig. 7). The strong synergistic effect in *msi1-tap1 fve* double mutants supports this hypothesis. Currently, we do not know whether *SOC1* is a direct target gene of MSI1 or whether MSI1 indirectly stimulates *SOC1* expression. However, it will be important in future studies to clarify how and together with which other proteins MSI1 regulates *SOC1*.

MSI1-like proteins can be a part of many protein complexes (for a review, see Hennig et al., 2005). For *Arabidopsis* MSI1, however, participation only in the PRC2-like MEDEA-complex has been confirmed in plants (Köhler et al., 2003a). MEDEA has two homologs in *Arabidopsis*, *CLF* and *SWINGER* (*SWN*), of which *CLF* is developmentally more important because loss of SWN affects development only in a *clf* background and not in wild-type plants (Goodrich et al., 1997; Chanvivattana et al., 2004). Therefore, it has been proposed that in addition to the MEDEA-complex, which is involved in seed and embryo development (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999; Köhler et al., 2003a), a related PRC2-like CLF-complex functions in later stages of plant development (Chanvivattana et al., 2004; Hennig et al., 2005). In contrast to *msi1-tap1* plants, *clf* mutants are early flowering. In addition, only *clf* but not *msi1-tap1* plants ectopically express *MEA* (see Fig. S2 in the supplementary material). Because *clf msi1-tap1* double mutants have an intermediate phenotype between *clf* and *msi1-tap1*, it is likely that the promotion of flowering by MSI1 does not involve the CLF-containing sporophytic PRC2 complex. MSI1-like proteins are best characterized as subunits of transcriptional repressor complexes (for a review, see Hennig et al., 2005), but they can function in transcriptional activator complexes such as the *Drosophila* Nucleosome Remodelling Factor NURF as well (Mizuguchi et al., 1997; Martinez-Balbas et al., 1998). The fact that H3K4 di-methylation and H3K9 acetylation are reduced at the *SOC1* locus in *msi1-tap1* plants suggests that MSI1 is needed to establish a chromatin environment that correlates with the transcription of *SOC1*. Previous work has shown that H3K4, H3K9 and H3K27 methylation and H3K9 acetylation are involved in the regulation of *FLC* (for a review, see He and Amasino, 2005), and this work provides evidence that similar chromatin-modifications are involved in the regulation of the pathway integrator *SOC1*.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/9/1693/DC1

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