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. Thus, 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 (API), 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 lumindependens (ld) and fve, are late flowering because they fail to reduce the expression of FLC. Introducing an fve-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; Guitteny 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 (Aisin 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.
MATERIALS AND METHODS

Plant material and growth conditions

In this study, we used the msil-1 mutant, the line msilOec2, which ectopically expresses MSII, and the line msilAsb7 (msil-as), which carries a MSII antisense construct (Hennig et al., 2003; Köhler et al., 2003a) (V. Exner, W.G., L.H. and P. Taranto, unpublished). To complement the msil-1 mutant with wild-type MSII, a 2000 bp fragment of the MSII promoter fused to the MSII cDNA was inserted into vector pCAMBIA1380. To complement the msil mutant with tagged MSII, the 2000 bp fragment of the MSII promoter fused to the MSII 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 BglII and NcoI (Rigaut et al., 1999) (see Table S2 in the supplementary material). Heterozygous msil-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 pKW2 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 msil-tap1 plants. The fms-3, fve-5, fcl-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_116765, SALK_41126 and SALK_N521003) (Sessions et al., 2002; Alonso et al., 2003). Seeds of the socl-2 mutant were kindly provided by Professor Inbala 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/m²s, 21±2°C) under long day (16 hour) photoperiods, unless indicated otherwise. The flowering time was measured as the number of total rosette leaves remaining than 0.5 cm at bolting for at least 14 plants, except for four plants for the msil-tap1 fve double mutant. Graphs show means±s.e.m. For the GA treatment, plantslet were grown on MS medium containing 100 μM GA3. After transfer onto soil, plants were sprayed weekly with 100 μM GA3. 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 experiments. 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 (Bretliing 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 Act4g04040.

RESULTS

MSII is needed for the transition to flowering

In previous work, we and others have found that the Arabidopsis protein MSII 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 MSII in seed development, we complemented the embryo-lethal msil-1 allele with constructs containing 2 kb of MSII promoter sequences driving the MSII cDNA either alone or fused to a carboxyterminal TAP (tandem affinity purification) tag. Several independent transgenic MSII/msil-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 MSII/msil-1 plants, we identified homozygous msil-1/msil-1 individuals that were entirely reliant on the complementing MSII or MSII-TAP transgenes. These complemented msil-1/msil-1 plants could successfully complete their sporophytic development to produce normal amounts of viable seeds, which was in striking contrast to msil mutants and to plants with strongly reduced MSII levels (Hennig et al., 2003). Although the seed phenotype of the msil mutant was complemented by expression of the MSII transgenes, all complemented msil-1/msil-1 lines flowered later than wild type, suggesting that the transgenes could not fully complement the msil mutant (Fig. 1A-C). All lines expressing either tagged or untagged MSII were late flowering, suggesting that the MSII promoter fragment-cDNA fusion was used not sufficient for normal MSII expression. Because protein levels appeared unchanged in extracts from whole msil-tap1 (msil/msil PMSII::MSII-TAP line 1) leaves and flowers, or from entire msilnotap3 (msil/msil PMSII::MSII line 3) seedlings (data not shown), the promoter fragment-cDNA fusion that was used probably failed to sustain normal amounts of MSII protein only at specific developmental stages, or only in specific tissues. Subsequently, we focused our experiments on the msil-tap1 transgenic line, which had only a single inserted transgene and was thus well suited for genetic analysis. In msil-tap1 plants, the transition to flowering was delayed both in the number of rosette leaves and in the number of days at
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Table 1. Seed abortion in msi1 mutants and complemented lines

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Normal (%)</th>
<th>Aborted (%)</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>msi1/MSI1</td>
<td>397 (98.0)</td>
<td>8 (2.0)</td>
</tr>
<tr>
<td>msi1-1</td>
<td>msi1/msi1-1</td>
<td>204 (50.0)</td>
<td>204 (50.0)</td>
</tr>
<tr>
<td>msi1-tap1</td>
<td>msi1-1/msi1-1 P&lt;sup&gt;MSI1&lt;/sup&gt;-&gt;TAP P&lt;sup&gt;MSI1&lt;/sup&gt;-&gt;MSI1-TAP*</td>
<td>325 (96.2)</td>
<td>13 (3.8)</td>
</tr>
<tr>
<td>msi1-notap1</td>
<td>msi1-1/msi1-1 P&lt;sup&gt;MSI1&lt;/sup&gt;P&lt;sup&gt;MSI1&lt;/sup&gt;-&gt;MSI1*</td>
<td>169 (100.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>msi1-notap2</td>
<td>msi1-1/msi1-1 P&lt;sup&gt;MSI1&lt;/sup&gt;-&gt;MSI1 P&lt;sup&gt;MSI1&lt;/sup&gt;-&gt;MSI1*</td>
<td>126 (98.4)</td>
<td>2 (1.6)</td>
</tr>
</tbody>
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*P<sub>MSI1</sub> 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 msi1OE<sub>c2</sub>) (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 genetic 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<sup>MSI1</sup>->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<sup>MSI1</sup>->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 msil-tap1 plants (data not shown). Together, these results established that the late flowering of msil-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 msil-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 msil-tap1 plants as well (Fig. 2A). Wild-type and msil-tap1 plants responded similarly to low concentrations of GA, but msil-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 fdl, 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, msil-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 MS14/FVE (Blazquez et al., 2003). We compared the flowering time of msil-tap1 plants at 16°C and at 23°C, and found that, unlike a fve mutant, msil-tap1 plants flowered significantly later at 16°C than at 23°C (Fig. 2B). Finally, we tested whether msil-tap1 plants can respond to vernalization. Vernalization of msil-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 msil-tap1 plants have no major defects in the promotion of flowering by gibberellic acid, photoperiod, ambient temperature or vernalization. Instead, the physiology of msil-tap1 plants was most similar to that of mutants from the autonomous pathway.

**MSI1 and MS14 are not redundant**

Both fve mutants (Ausin et al., 2004; Kim et al., 2004) and msil-tap1 plants (this study) are late flowering, suggesting that the two homologous proteins MSI1 and MS14/FVE are not completely redundant. Alternatively, MSI1 and MS14/FVE could have distinct, specific expression patterns and fulfill similar functions in different tissues. However, neither MSI1 nor MS14/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 MS14/FVE is essential in a restricted domain where MSI1 is not expressed. To test this hypothesis, we crossed a fve mutant with the line ms1OEc2, in which MSI1 is ectopically expressed and which can fully complement the msil-tap1 plants (Fig. 1E). Although ectopic expression of MS14/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::MS14 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 MS14/FVE, i.e. that MSI1 and MS14/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.,

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**Fig. 2. Physiology of msil-tap1 plants is similar to that of autonomous pathway mutants.**

(A) Analysis of the flowering time of msil-tap1 without (+GA) or with (+GA) gibberellic acid treatment in LD. (B) Analysis of the flowering time of msil-tap1 and fve at 16°C and 23°C in LD. (C) Analysis of the flowering time of msil-tap1 grown in short days (SD). (D) Analysis of the flowering time of msil-tap1 without (−VRN) or with (+VRN) a vernalization treatment in LD (left) and SD (right).
Regulation of flowering time by MSI1

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.
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 a flc null mutant (Fig. 5C; see Fig. S2C in the supplementary material). If MSI1, like the known autonomous pathway genes, acts 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 fkm mutant, which lacks all four major alternatively spliced FLM transcripts (Fig. 5C; see Fig. S2D in the supplementary material). Similar to flc, the fkm 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-tap1 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 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; Auzin et al., 2004; Bastow et al., 2004; He et al., 2004; Sung and Amasino, 2004). Because MSI1-like proteins participate in various chromatin-
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

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**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 fve and msi1-tap1 soc1 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.

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**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 antisera. 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 MS14/FVE to promote flowering.

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; Chanivivattana 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 (Chanivivattana et al., 2004; Hennig et al., 2005). In contrast to ms1-tap1 plants, clf mutants are early flowering. In addition, only clf but not ms1-tap1 plants ectopically express MEA (see Fig. S2 in the supplementary material). Because clf ms1-tap1 double mutants have an intermediate phenotype between clf and ms1-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 ms1-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
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