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RESEARCH PAPER

MGOUN3: evidence for chromatin-mediated regulation of *FLC* expression

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Abstract

The *MGOUN3*(*MGO3*)/*BRUSHY1*(*BRU1*)/*TONSOKU*(*TSK*) gene of *Arabidopsis thaliana* encodes a nuclear leucine–glycine–asparagine (LGN) domain protein that may be implicated in chromatin dynamics and genome maintenance. Mutants with defects in *MGO3* display a fasciated stem and disorganized meristem structures. The transition to flowering was examined in *mgo3* mutants and it was found that, under short days, the mutants flowered significantly earlier than the wild-type plants. Study of flowering-time associated gene expression showed that the floral transition inhibitor gene *FLC* was under-expressed in the mutant background. Ectopic expression of the flower-specific genes *AGAMOUS* (*AG*), *PISTILLATA* (*PI*), and *SEPALLATA3* (*SEP3*) in *mgo3* vegetative organs was also detected. Western blot and chromatin immunoprecipitation experiments suggested that histone H3 acetylation may be altered in the *mgo3* background. Together, these data suggest that MGO3 is required for the correct transition to flowering and that this may be mediated by histone acetylation and associated changes in *FLC* expression.

Key words: *Arabidopsis thaliana*, BRU1, chromatin dynamics, FLC, histone modifications, MGO3, TSK.

Introduction

The continuous development of plants relies on the activity of shoot and root apical meristems (SAM and RAM). These are organized pools of dividing and progressively differentiating cells located at the stem and at the root tips, generating shoot and root tissues continuously, while maintaining their integrity. A complex network of transcription factors and signal proteins ensures the co-ordination of cell division and cell differentiation in these structures (for a review see Veit, 2004). In addition, numerous genes involved in chromatin dynamics have been shown to participate in SAM and RAM maintenance and activity (for a review see Guyomarc'h *et al.*, 2005). Chromatin modifiers are required to generate the chromatin structure in which DNA is wrapped or to modulate its conformation during development, favouring or inhibiting the expression of specific loci in different developmental phases (for review see Hsieh and Fischer, 2005).

In *Arabidopsis thaliana*, the transition to flowering is controlled by four major pathways, namely the autonomous, vernalization, gibberellic-acid-dependent and long-day pathways (for a review see Boss *et al*., 2004). These pathways converge to induce the expression of two main targets: *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (for reviews see Bolt *et al*., 2004; Parcy, 2005). Among multiple regulators involved in this control, the MADS-box floral repressor gene *FLOWERING LOCUS C* (*FLC*) emerges as a convergent point for the control of flowering time by the vernalization and autonomous pathways. Regulation of *FLC* expression involves putative transcription factors, RNA-processing factors, and chromatin modifiers (He and Amasino, 2005). In the autonomous pathway, homologues of histone deacetylase complex subunits *FLOWERING LOCUS D* (*FLD*) and *FVE* have been shown to repress *FLC* expression by means of histone H3 and H4

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deacetylation around the translation start site and in the first intron of the gene (He *et al.*, 2003; Ausín *et al.*, 2004; Kim *et al.*, 2004). Conversely, histone H3 acetylation in *FLC* is associated with high expression levels of this gene (Bastow *et al.*, 2004; Sung and Amasino, 2004). In addition, *Arabidopsis* relatives of subunits of the yeast RNA polymerase II (Pol II) Associated Factor 1 (PAF1) complex were recently shown to participate in *FLC* activation via histone H3 Lysine 4 trimethylation at the translation start site of this locus (He *et al*., 2004; Oh *et al*., 2004).

A new gene, *MGOUN3* (*MGO3*), has previously been described, mutations of which affect both SAM and RAM organization and maintenance (Guyomarc'h *et al.*, 2004). This gene was also identified as *BRUSHY1* (*BRU1*) and *TONSOKU* (*TSK*) by other groups (Suzuki *et al.*, 2004; Takeda *et al.*, 2004). The *mgo3/bru1/tsk* mutants display stem fasciation, aberrant phyllotaxy, perturbation in leaf and flower morphogenesis, and shorter roots. These developmental defects can be linked to the loss of the cellular organization of both SAM and RAM and perturbations in the expression pattern of some key meristem regulators such as *WUSCHEL* (*WUS*) in the SAM, *SCARECROW* (*SCR*) in the RAM, and *AGAMOUS* (*AG*) in flower meristems (Guyomarc'h *et al.*, 2004; Suzuki *et al.*, 2004). Defects in cell cycle progression and increased levels of cyclin B1 have also been recently reported in *tsk* mutant seedlings (Suzuki *et al.*, 2005).

Although the MGO3/BRU1/TSK protein structure does not allow any speculation about its function, the increased intrachromosomal homologous recombination, the hypersensitivity to DNA-damaging agents, and the release of transcriptional gene silencing observed in *bru1* mutants, suggested that the MGO3/BRU1/TSK protein is involved in chromatin organization and dynamics and in genome maintenance (Takeda *et al.*, 2004). Moreover, the mutant phenotype is very close to those of *fasciata1* (*fas1*) and *fasciata2* (*fas2*) mutants that also show enlarged and disorganized SAM and RAM, resulting in stem fasciation, irregular phyllotaxy, and a shorter root (Kaya *et al.*, 2001; Leyser and Furner, 1992). The cellular arrangement and the expression pattern of cell-identity regulators such as *WUS* and *SCR* are misregulated in *fas1* and *fas2* apical meristems. The *FAS1* and *FAS2* genes encode subunits of the *Arabidopsis thaliana* counterpart of the Chromatin Assembly Factor-1 (CAF-1) complex (Kaya *et al.*, 2001). In yeast, CAF-1 acts as a histone-chaperone complex and participates in the reconstitution of chromatin after DNA replication and repair (Polo *et al.*, 2004).

In order to understand the function of MGO3 in *Arabidopsis* development better, analysis of the *mgo3* phenotype was continued by focusing on particular developmental pathways. In this work, it is reported that *mgo3* mutants showed an early-flowering phenotype under short days as well as under-expression of *FLC*, associated with histone H3 hypoacetylation at this locus. These data suggest that MGO3 is required for the chromatin modifications involved in the regulation of *FLC* expression.

Materials and methods

Plant material and growth conditions

All three *mgo3* lines used in this study were described in Guyomarc'h *et al*. (2004). The *mgo3-1* allele is in the Landsberg *erecta* (L*er*) background, *mgo3-2* is in the Wassilewskija (WS) background, and the *mgo3-4* allele is in the Columbia (Col-0) background. All *mgo3* lines were back-crossed at least twice with the corresponding wild-type parent.

Plants were grown in chambers at 20 °C on soil or on sterile half-strength MS medium supplemented with 10 g l^{-1} sucrose and 1.5% agar in short-days (8 h of light; SD) or long-days (16 h of light; LD) conditions. To assess the vernalization response, plants were first grown at 4 °C under SD for 6 weeks before being placed into SD at 20 °C. In all cases, bolting time are given as the number of days in normal growth (20 °C) SD or LD conditions.

For double mutant analysis, the *flc-3* mutant (Col-0 background) was kindly provided by Richard M Amasino (Michaels and Amasino, 1999).

Genetic characterization

Double mutants (*flc-3 mgo3-4*) were selected from an F_2 population using molecular markers on individual F_2 plants. The *MGO3-4* genotype was determined with primers 5′-GCTGACATTTGGAGCCACC-3′ and 5′-ACGGAATATGGATAGACACC-3′, which give a PCR product for the wild-type allele but not for the mutant allele. The *FLC-3* genotype was determined with primers 5′-TATCGCCGGAGGAGAAGC-3′ and 5′-TAGAAAGAAATAAAGCGAGAAAAGGA-3′, which amplify a 300 bp fragment for the wild-type allele and a 196 bp fragment for the *flc-3* allele because *flc-3* has a 104 bp deletion (Michaels and Amasino, 1999).

Expression analysis by RT-PCR and microarrays

Total RNA was extracted from rosette leaves, flower buds or whole seedlings following the TRIzol® protocol (Invitrogen/Life Technologies). After quantification by spectrophotometry, RNA integrity was checked by electrophoresis.

To study gene expression in leaves, 1 μg of total RNA was reverse transcribed using the RNase H- Superscript™ II DNA polymerase (Invitrogen/Life technologies). The cDNA solution was diluted four times and 1 μl was used to test candidate gene amplification by PCR. The primers used were *WUS*:5′-GTAGCCATGTCTATGGATCTATGG-3′ and 5′-ACCGGCGTAAGAGCTAGTTCAGAC-3′, *AG*: 5′-GTTGATTTGCATAACGATAACCAGA-3′ and 5′-TTCACTGATACAACATTCATGGGA-3′, *PI*: 5′-GAGGAGGAATGGATTGGTGA-3′ and 5′-CCGCCATCATCTTCTCATTT-3′. Amplification with the *ACTIN* primers (5′-GGTAACATTGTGCTCAGTGGTGG-3′ and 5′-AACGACCTTAATCTTCATGCTGC-3′, Charrier *et al.*, 2002) was used as a positive control.

To monitor the expression of flowering-time genes, total RNA was extracted from aerial parts of seedlings and reverse transcription was performed as described above. Semi-quantitative RT-PCR was then carried out; in each case the semi-quantitative property of the amplification was checked by comparing the results obtained with one volume and twice this volume of template. The primers used were *FLC*: 5′-ATGTGAGTATCGATGCTCTTG-3′ and 5′-AGATATACAAACGCTCGCCC-3′, *CO*: 5′-CTCCTCGGCTTCGATTTCTC-3′ and 5′-CATTAACCATAACGCATACATTTC-3′, *FT*: 5′-TACGAAAATCCAAGTCCCA-3′ and 5′-AAACTCGCGAGTGTTGAAGTTC-3′, *SOC1*: 5′-AATATGCAAGATACCATAGATCG-3′

and 5′-TCTTGAAGAACAAGGTAACCCAAT-3′. Amplification of the *ACTIN2* cDNA (5′-CTAAGCTCTCAAGATCAAAGGCTTA-3′ and 5′-TTAACATTGCAAAGAGTTTCAAGGT-3′, Bertrand *et al.*, 2003) was used as a semi-quantitative control. The bands were scanned with Molecular Image FX Pro (Bio-Rad), and normalized to actin mRNA signals by using Bio-Rad Quantity One 1-D Analysis software.

To compare precisely gene expression between *mgo3* and wild-type leaves, a transcriptome analysis was performed using CATMA arrays containing 24 576 gene-specific tags from *Arabidopsis thaliana* (http://www.catma.org). Total RNA was extracted from the three first rosette leaves of at least 25 14-d-old seedlings using the TRIzol® extraction kit (Invitrogen/Life technologies) followed by two ethanol precipitations, and then checked for integrity with the Bioanalyser from Agilent (Waldbroon, Germany). cRNA were produced from 2 μg of total RNA from each sample using the Message Amp aRNA® kit (Ambion). Then 5 μg of cRNA were reverse transcribed in the presence of 300 units of Superscript™ II DNA polymerase (Invitrogen/Life technologies), cy3-dUTP and cy5-dUTP (PerkinElmer Life Sciences) for each slide. Three plant replicates were analysed, allowing the statistical analysis of hybridization results with respect to experimental and biological variabilities as described by Lurin *et al.* (2004). Functional data or predictions given by the Gene Ontology database (http://www.geneontology.org) were used to classify in a functional way genes expressed differentially between *mgo3* and wild-type leaves.

Histone acetylation analysis by western blot and chromatin immunoprecipitation

Nuclear proteins were extracted from aerial parts of 12-d-old seedlings grown under SD as described by Gendrel *et al.* (2002). After quantification with the Bradford method, the same amounts of protein were resolved by SDS-PAGE, and then transferred to a PVDF membrane (Bio-Rad) using a Mini-Protean® 3 Cell (Bio-Rad). Western analysis was performed using primary polyclonal antibodies raised against histones H3 acetylated on Lysine 18 (H3acK18; Cell Signaling) or on any residue between 4 and 18 (H3acK4-18; Upstate), or raised against all forms of histones H3 (Upstate), and then secondary antibodies conjugated to alkaline phosphatase. Antibody complexes were detected by chemiluminescence using the Immun-Star™ AP Substrate kit (Bio-Rad).

Chromatin immunoprecipitation experiments were performed as described by Gendrel *et al.* (2002), starting from 2 g of entire 12-d-old seedlings grown *in vitro* in SD. Antibodies raised against acetylated histone H3 (Upstate) were used for immunoprecipitation. DNA fragments associated with the immune complexes were dissolved in water and detection of target sequences was carried out by PCR using primers kindly provided by Ausín *et al.* (2004). Three independent experiments were performed, and PCR tests were repeated at least twice. The bands were scanned with Molecular Image FX Pro (Bio-Rad), and normalized to actin signals using Bio-Rad Quantity One 1-D Analysis software.

Results

Mutations in MGO3 *result in early flowering in short days*

In studies on *Arabidopsis*, the transition to flowering is usually dated at bolting or measured with the number of rosette and cauline leaves produced by the SAM before flowering (Pouteau *et al.*, 2004). The strong developmental defects of the *mgo3* mutants prevented us from using leaf numbers as a criterion for floral transition, and in most experiments, only the bolting day was used. In addition, these *mgo3* developmental defects sometimes led to heavily fasciated plants which did not bolt or flower. This feature led us to state previously that the transition to flowering was often delayed in the *mgo3* mutant (Guyomarc'h *et al.*, 2004). However, a precise study of the *mgo3* plants revealed that, in the absence of very strong developmental defects in the vegetative phase (heavy fasciation, possible meristem abortion), the *mgo3* mutants flowered significantly earlier than the wild-type plants. Thus, a minority of *mgo3* individuals showing heavy fasciation in the vegetative phase were excluded in the studies presented here.

When grown on soil in SD, plants with the *mgo3-1*, *mgo3-2*, and *mgo3-4* alleles bolted significantly earlier than the wild-type (respectively 3.5, 19.7, and 6.0 d earlier than the corresponding wild-type ecotype; Table 1; Fig. 1). Vernalization still accelerated the transition to flowering in the *mgo3-2* background (Table 1).

The early-flowering phenotype was also observed for these three alleles of *mgo3* mutants grown under short days *in vitro* (Table 2). It was also apparent in LD, on soil, for the *mgo3-2* allele (*mgo3-2* plants bolted after 18.21±0.54 d, compared with 22.25±0.69 d for WS; n=28).

mgo3 *mutations cause under-expression of* FLC *and early induction of* FT *expression*

The early-flowering phenotype of *mgo3* in SD was reminiscent of plants under-expressing *FLC*, a repressor of *SOC1* and *FT* expression, or over-expressing *CONSTANS* (*CO*), a transcription factor responsible for *FT* and *SOC1* induction in LD (Michaels and Amasino, 1999; Sheldon *et al.*, 1999; Samach *et al.*, 2000). RT-PCR was used to compare the expression of *FLC*, *CO*, *FT*, and *SOC1* in *mgo3-2* and wild-type seedlings grown *in vitro* in LD for 6, 9, or 12 d or grown in SD for 12 d or 19 d, which, in each case, roughly corresponds to the flowering inductive period for the wild-type in this study's conditions. In both conditions, at all stages tested, levels of *FLC* mRNA were

Table 1. *Bolting time of wild-type and mutant plants grown under short days on soil*

Genotype	Without vernalization		With vernalization	
	n[a]	Days before bolting	n	Days before bolting
WS	23	61.87±1.33	21	34.52±1.12
mgo3-2	25	42.08±1.42	21	26.81±1.23
L*er*	21	65.67±0.89		ND[b]
mgo3-1	24	62.13±1.05		ND
Col-0	24	60.33±1.25		ND
mgo3-4	22	54.32±0.97		ND
flc-3	24	52.42±1.02		ND
flc-3 mgo3-4	6	55.86±1.36		ND

[a] n, Number of plants analysed.
[b] ND, not determined. Bolting times are shown as mean of days before bolting ±standard deviation of the mean.

Fig. 1. Early-flowering phenotype of *mgo3* mutant. Comparison between wild type (A) and a homozygous *mgo3-2* plant (B) grown under SD on soil for 6 weeks.

Table 2. *Bolting time of wild-type and* mgo3 *mutant plants grown under short days* in vitro

Genotype	n^a	Days before bolting
WS	45	28.33±1.03
mgo3-2	61	19.69±0.24
L*er*	47	35.11±0.90
mgo3-1	47	33.98±0.60
Col	37	32.57±1.23
mgo3-4	40	26.03±0.88

[a] *n*, Number of plants analysed. Bolting times are shown as mean of days before bolting ±standard deviation of the mean.

dramatically reduced in *mgo3-2* compared with the wild type (Fig. 2A, B). A slight up-regulation of *FT* expression was also detected in the mutant background both under LD (Fig. 2A) and SD (Fig. 2B). By contrast, no significant difference in *CO* (Fig. 2A) or *SOC1* (Fig. 2A, B) expression levels was detected between the two genotypes.

A strong and slight decrease of *FLC* mRNA level could also be detected respectively in the *mgo3-2* and in the *mgo3-4* alleles compared with the wild type, when grown for 12 SD, on soil *(*Fig. 2C). In the L*er* wild-type ecotype, the *FLC* gene is naturally expressed at very low levels that prevented meaningful comparison with the *mgo3-1* mutant allele (Fig. 2C; see also Gazzani *et al.*, 2003; Michaels *et al.*, 2003).

mgo3 *mutants show ectopic expression of floral meristematic genes in leaves*

Defects in leaf morphogenesis in the *mgo3* mutants were reminiscent of those observed when meristem-specific genes or flower-specific genes are ectopically expressed in vegetative organs (Goodrich *et al.*, 1997; Ori *et al.*, 2000). In addition, ectopic expression of the floral-organ identity gene *AG* was previously shown to cause early flowering (Mizukami and Ma, 1992; Goodrich *et al.*, 1997). To test whether the early-flowering phenotype of *mgo3* was correlated with ectopic expression of MADS-box genes in leaves, RT-PCR analysis of wild-type and *mgo3* rosette

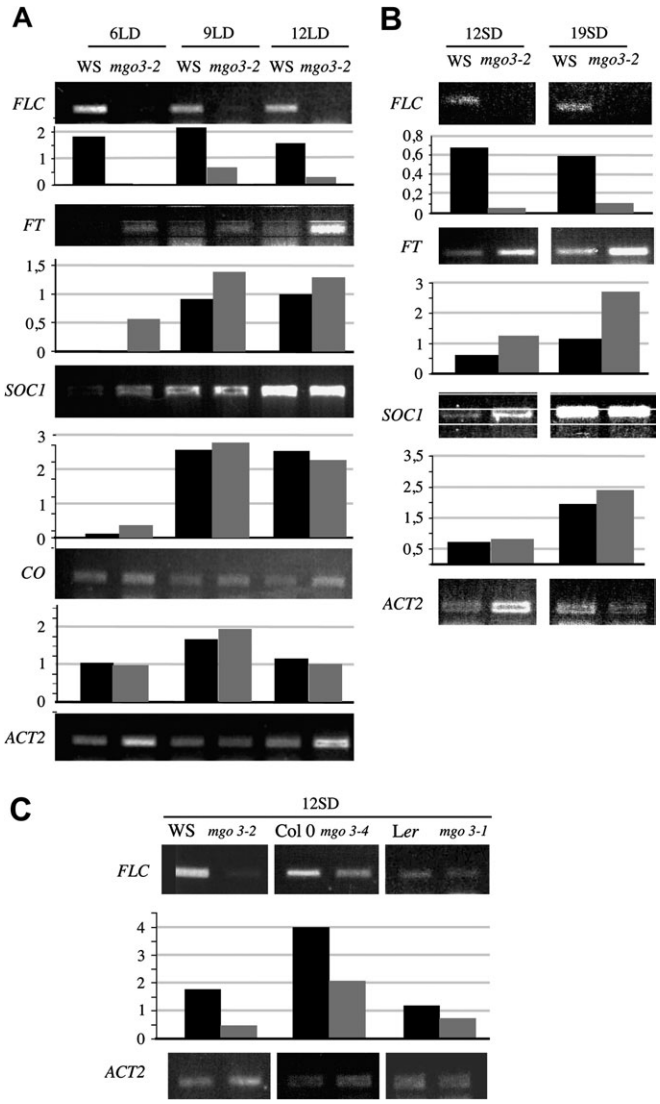

Fig. 2. Expression of flowering-time regulatory genes in *mgo3* and wild-type plants. RT-PCR analysis of *FLC*, *FT*, *SOC1*, *CO*, and *ACTIN2* mRNA levels in 6-, 9-, 12-, and 19-d-old wild-type (WS or Col-0), *mgo3-1*, *mgo3-2*, and *mgo3-4* plants grown under LD or SD, *in vitro* (A, B) or in soil (C). Quantification data after normalization with the actin signals are shown under each band.

leaves were performed. Transcripts of two flower-specific MADS-box genes *AG* and *PISTILLATA* (*PI*) were detectable in *mgo3* but not in wild-type leaves (Fig. 3). No change was detected in the steady-state transcript level of a known *AG* regulator, *WUS* (Fig. 3; Lenhard *et al.*, 2001; Lohmann *et al.*, 2001).

The transcriptomes of *mgo3* and wild-type leaves were compared using the Complete *Arabidopsis* Transcriptome MicroArray (CATMA) technology (http://www.catma.org). CATMA chips containing 24 576 genes of the *Arabidopsis* genome were hybridized with cDNA probes synthesized from total RNA harvested from 14-d-old rosette leaves of wild-type and *mgo3-2* plants in three separate experiments. Based on the statistical test (see Materials and

methods), 1.46% of the 24 576 genes were revealed as differentially expressed between the mutant and the control. The affected genes are listed under Supplementary Materials. Complete data files were deposited to ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) under experiment accession number E-MEXP-196. Of the affected genes, 34% were repressed and 66% were induced. In these experiments over-expression of *AG* was not detected but transcript levels of two other flower-specific MADS-box genes, namely *PI* and *SEPALLATA3* (*SEP3*), were significantly higher in *mgo3-2* leaves compared with those of wild-type plants.

Genetic interaction between MGO3 *and* FLC *genes*

In order to analyse genetic interactions between *MGO3* and *FLC* genes, a cross between *mgo3-4* and null *flc-3* mutants was performed. The F_2 progeny of the cross, as well as the parents, were grown under short-days on soil and the flowering time was measured. Both *flc-3* and *mgo3-4* single mutants flowered earlier than the wild-type Col-0 plants, and both flowered approximately at the same time compared with each other (Table 1). In the F_2 progeny, *flc-3 mgo3-4* double mutants were identified using molecular markers. All *flc-3 mgo3-4* plants flowered earlier than the wild-type, at the same time as the *mgo3-4* and *flc-3* single mutants (Table 1). No new flowering phenotype was detected among the F_2 progeny. Moreover, all the double mutants displayed a *mgo3* phenotype with perturbations of shoot morphogenesis and organogenesis. These results are consistent with an epistasy of *MGO3* over *FLC* with regard to the early-flowering phenotype, which suggests that FLC is one of the targets of MGO3.

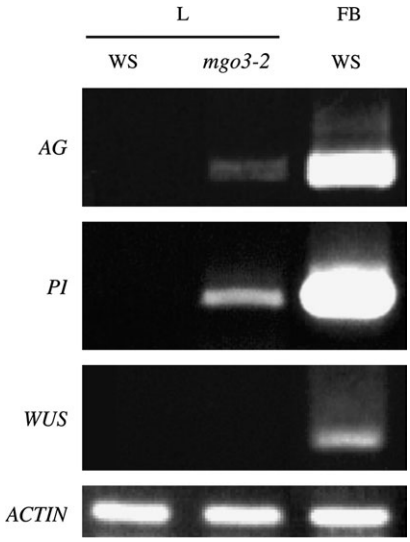

Fig. 3. Expression of meristematic regulatory genes in *mgo3-2* and wild-type (WS) plants. RT-PCR analysis of rosette leaves (L) or flower buds (FB) RNA with primer sets specific to *AG*, *PI*, *WUS*, and *ACTIN* genes.

mgo3 *mutations affect global histone acetylation, especially in* FLC *chromatin*

The recent characterization of *FLC* repressors and activators has shown that some of these regulatory proteins are involved in covalent modifications of *FLC* chromatin such as acetylation or methylation of specific histones (reviewed by He and Amasino, 2005). Previous results suggest that the MGO3 protein may be required for the stability of heterochromatin (Takeda *et al.*, 2004). Therefore, it was asked whether the deregulation of *FLC* expression observed in *mgo3* plants could be due to defects in epigenetic modifications of histones at the *FLC* locus and, particularly, loss in histone H3 acetylation.

To address this, global histone H3 acetylation levels were first compared between wild-type and mutant plants after 12 SD. Nuclear proteins were extracted and analysed by immunoblotting using antisera specific for histone H3 acetylated on lysines 4, 9, 14, or 18. For the same amount of nuclear proteins loaded, a significantly weaker signal was obtained for *mgo3* compared with wild type (Fig. 4). Similar results were obtained using antibodies raised specifically against histone H3 acetylated on lysine 18 (Fig. 4). No difference in the level of histone H3 was observed between both genotypes using the antibody specific to total histone H3 (Fig. 4) suggesting that acetylation of histones H3 is reduced in the *mgo3-2* plants compared with the wild type.

Next, chromatin immunoprecipitation assays were performed to detect histone H3 acetylation at the *FLC* locus in WS and *mgo3-2* plants after 12 SD. DNA released from chromatin fragments immunoprecipitated with antibodies raised against acetylated histone H3 was analysed by PCR with primers specific to four regions of the *FLC* locus (regions I–IV, Fig. 5A; see also Ausín *et al.*, 2004) covering the *FLC* promoter, the first exon and the first intron of the gene. No difference in immunorecovery, as judged by PCR, was detected for region IV. However, recovery and PCR using primers to the region around the *FLC* start codon (regions I–III) was somewhat lower in *mgo3-2* than in WS. The levels of precipitation and amplification of the *ACTIN2*

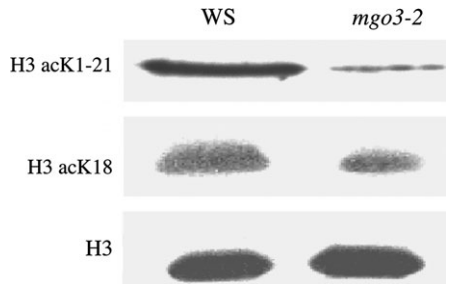

Fig. 4. Comparison of histone H3 acetylation level in *mgo3-2* and wild-type (WS) plants. Western blot analysis of nuclear proteins extracted from aerial parts of *mgo3-2* or WS seedlings after 12 SD. Analysis were performed using primary polyclonal antibodies raised against histone H3 acetylated on any residue between 4 and 18 (H3 acK4-18) or on lysine 18 (H3 acK18) or against all forms of histone H3 (H3).

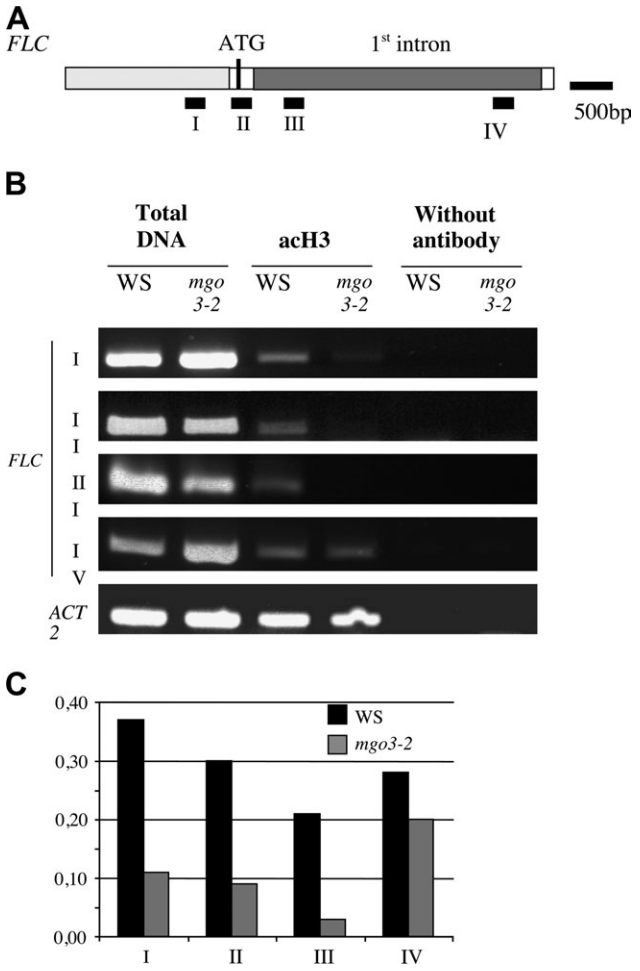

Fig. 5. Acetylation state of histones H3 at the promoter, first exon, and intron of *FLC* gene in *mgo3-2* and wild-type (WS) plants. (A) Regions of the *FLC* locus analysed. (B) Chromatin immunoprecipitation results. Nuclei were extracted after cross-linking from 12-d-old seedlings grown under SD, sonicated and immunoprecipitated with antibodies specific to acetylated histones (AcH3). The immunoprecipitates were analysed for the presence of DNA by PCR using primer sets specific for the four regions indicated in (A) or for the *ACTIN2* gene. The lane labelled 'total DNA' contains the product of PCR performed with the chromatin solution before immunoprecipitation. The lane labelled 'without antibody' corresponds to PCR performed with the result of an immunoprecipitation with no antibody. Similar results were obtained from three independents experiments. (C) Quantification data of the bands obtained in (B) with antibodies specific to acetylated histones after normalization with the actin signals.

promoter (used as an internal control) were comparable between both genotypes (Fig. 5B, C). These results suggest that the *mgo3* mutation may affect the accessibility of epitopes that reflect H3 acetylation in the proximal promoter region and in the beginning of the ORF of the *FLC* gene.

Discussion

MGO3 *is involved in the regulation of flowering time*

It has been shown that the *mgo3* mutants display an early-flowering phenotype and are still responsive to day length (they flower later under SD than under LD) and to vernalization treatment. Therefore, *mgo3* mutants seem to be mainly affected in the autonomous pathway of flowering-time regulation. A correlation between *FLC* transcript levels and the time to flowering has been established from the study of wild-type ecotypes and flowering mutants: most often the higher *FLC* is expressed, the later plants flower (Sheldon *et al.*, 2000; Rouse *et al.*, 2002; Gazzani *et al.*, 2003; Michaels *et al.*, 2003). Thus, the under-expression of *FLC* observed in the *mgo3* background is likely to contribute to the early flowering of the mutant. As this is the case with the null allele *flc-3*, this low expression of *FLC* in the *mgo3* background does not abrogate the vernalization response of the plants (Michaels and Amasino, 2001). The severity of the precocious-flowering phenotype is correlated with the differential of *FLC* expression between the wild-type and *mgo3* plants: strong in *mgo3-2*, weak in *mgo3-1*, and intermediate in *mgo3-4*. These variations in acceleration of flowering time might be due to differences in *mgo3* alleles, but also to differences in *FLC* alleles between the corresponding wild-type ecotypes. For instance, the relatively slight effect of *mgo3* mutation on flowering time in L*er* could be explained by the natural weak expression of *FLC* in this background, due to a transposon insertion in the first intron of the gene (Gazzani *et al.*, 2003; Michaels *et al.*, 2003). The fact that the *mgo3* mutation accelerates the flowering transition to almost the same extent in both L*er* and Col-0 backgrounds, despite the dramatic difference in strength of their respective *FLC* alleles, and that, conversely, the early-flowering phenotype of *mgo3* mutants is much more pronounced in the WS background than in the Col-0 background, strongly suggests that *MGO3* affects the activity of other flowering-time genes as well as *FLC*. Other members of the *FLC* family, such as *MADS AFFECTING FLOWERING 2* (*MAF2*), or *FLOWERING LOCUS M* (*FLM*), or other repressors of the transition to flowering, such as *SHORT VEGETATIVE PHASE* (*SVP*) could be candidate target genes (for a review see Boss *et al.*, 2004). In addition, the expression of downstream flower-specific genes, such as *AG*, *PI*, or *SEP3*, could also be up-regulated independently of FLC. However, the fact that *flc mgo3-4* double mutants flower as early as *flc* and *mgo3* single mutants does not support this hypothesis (see below).

FLC action on the transition to flowering is mainly mediated by repression of *FT* and *SOC1* (Lee *et al.*, 2000; Michaels and Amasino, 2001; Hepworth *et al.*, 2002), which are positive regulators of the expression of flower-specific genes like *AG* and *PI* (for a review see Parcy, 2005). Consistent with this model, an increase in *FT* expression was detected in the *mgo3* background, suggesting that FT may contribute to the early flowering of *mgo3* mutants. However, FT expression oscillates with a circadian rhythm in wild-type plants, so time-course experiments would be necessary to gauge precisely the

effect of *mgo3* mutations on *FT* expression. No changes were observed in *SOC1* expression in the *mgo3* mutants. It has been reported previously that, in addition to the down-regulation of *FLC*, a positive signal from the long-day pathway or from the gibberellic-acid pathway was required to stimulate *SOC1* expression (Moon *et al.*, 2003).

Ectopic expression of PI*,* SEP3*, and* AG *could be due to premature* FT *induction but also could be linked to chromatin modifications*

Over-expression of flower-specific genes during the vegetative phase is known to be associated with an early-flowering phenotype in several mutants (Goodrich *et al.*, 1997; Kotake *et al.*, 2003; Moon *et al.*, 2003, see below). Therefore mRNA accumulation of *AG*, *PI*, and *SEP3* genes in *mgo3* leaves could be also related to the early-flowering phenotype of the mutants. Their ectopic expression in vegetative organs of the *mgo3-2* mutant might be due to the premature repression of *FLC* and/or induction of *FT* expression in this mutant background. It is at present not clear why ectopic expression of *AG* could be detected by RT-PCR on leaves, but not by the microarray experiments on young seedlings. A variation in the severity of the phenotype between different populations or at different ages could explain this discrepancy.

Ectopic expression of *AG* has also been reported in leaves of *curly leaf* (*clf*), *incurvata2* (*icu2*), *embryonic flower1* and *2* (e*mf1* and *emf2*), *early bolting in short days* (*ebs*), *like heterochromatin protein1/terminal flower2* (*lhp1/tfl2*), *fertilization-independent embryo* (*fie*), and *vernalization independence4* (*vip4*) mutants (Chen *et al.*, 1997; Goodrich *et al.*, 1997; Serrano-Cartagena *et al.*, 2000; Gomez-Mena *et al.*, 2001; Kinoshita *et al.*, 2001; Zhang and Van Nocker, 2002; Moon *et al.*, 2003; Katz *et al.*, 2004; Kotake *et al.*, 2003). All these genes are involved in epigenetic processes regulating plant development. Most of them have been shown to influence chromatin conformation so as to repress the expression of target genes until the onset of the corresponding developmental programme, for example, flower-specific genes before the transition to flowering. Among them, *emf1*, *emf2*, *lhp1/tfl2*, and *fie* mutants also display ectopic expression of *PI* and *emf1*, *emf2*, *lhp1*/*tfl2* mutants show ectopic expression of *SEP3* (Kinoshita *et al.*, 2001; Kotake *et al.*, 2003; Moon *et al.*, 2003). However, no repression of *FLC* has been reported in these mutants. These observations suggest that chromatin-mediated repression of flower-specific genes contributes to the maintenance of the vegetative phase (Reyes and Grossniklaus, 2003; Wagner, 2003). Thus, the ectopic expression of *SEP3*, *AG*, and *PI* in *mgo3* leaves could result from *FLC* under-expression and/or from specific alterations of their chromatin-mediated repression, independently of FLC.

The mgo3 *mutation may be responsible for the loss of targeted histone H3 acetylation at the* FLC *locus*

The *mgo3* mutation has been shown here to be associated with a reduced recovery of acetylated histone H3. In addition, preliminary evidence has been presented that loss of histone H3 acetylation may occur around the translational start of the *FLC* gene. This region, corresponding to the 5′-untranslated region, the first exon, and the beginning of the first intron of the gene, was previously shown to be crucial for *FLC* expression and regulation (Sheldon *et al.*, 2002), and it is now known that this is mediated by histone modifications. Histone H3 lysine 4 trimethylation, histone H3 lysine 36 dimethylation, and histone H3 and H4 acetylation are associated with high *FLC* expression, whereas histone H3 and H4 deacetylation and H3 lysine 9 and 27 dimethylation correlate with low *FLC* expression (reviewed in He and Amasino, 2005; Zhao *et al.*, 2005). These results suggest that MGO3 is required for normal histone acetylation at the *FLC* locus, and that MGO3 might be a chromatin-associated protein required for the regulation of *FLC* expression during development. The fact that the *mgo3* mutation is epistatic to the *flc* mutation suggests that MGO3 function is necessary for correct functioning of the *FLC* gene. This is consistent with a role in *FLC* regulation during development via chromatin modifications. It is likely, however, that other loci might be subjected to histone H3 hypoacetylation in the *mgo3* background, which could lead to the pleiotropic phenotype of the mutant. This is consistent with the transcriptome analysis showing that a broad range of genes is affected in *mgo3-2*. All the developmental alterations, including the early-flowering phenotype, could be side-effects of major perturbations in chromatin dynamics, as suggested by Komeda (2004).

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