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RESEARCH PAPER

HDA6 is required for jasmonate response, senescence and flowering in *Arabidopsis*

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Abstract

Post-translational modifications of histones, including acetylation, play a key role in modulating dynamic changes in chromatin structure and gene activity. Histone acetylation is modulated through the action of histone acetyltransferases and deacetylases. HDA6 is a RPD3-type histone deacetylase in *Arabidopsis*. The *Arabidopsis HDA6* mutant, *axe1-5,* and *HDA6* RNA-interfering (*HDA6*-RNAi) plants displayed higher levels of acetylated H3 compared with wild-type, suggesting that HDA6 affects histone acetylation levels globally. The expression of the jasmonate responsive genes, *PDF1.2*, *VSP2*, *JIN1*, and *ERF1*, was down-regulated in *axe1-5* and *HDA6*-RNAi plants. Furthermore, *axe1-5* and *HDA6*-RNAi plants displayed increased leaf longevity compared with the wild type. The expression of the senescence-associated genes, *SAG12* and *SEN4*, was down-regulated in the *axe1-5* and *HDA6*-RNAi plants. In addition, *axe1-5* and *HDA6*-RNAi plants displayed late-flowering. The expression of *FLC* was up-regulated and hyperacetylated in *axe1-5* and *HDA6*-RNAi plants, suggesting that HDA6 is required to deacetylate *FLC* chromatin and thereby repress its expression. Our results suggest that HDA6 is involved in jasmonate response, senescence, and flowering in *Arabidopsis*.

Key words: HDA6, flowering, histone deacetylases, jasmonic acid, senescence.

Introduction

In higher organisms, many different patterns of gene expression are required for proper development to occur and to allow for specific responses to environmental cues. Transcriptional regulation of gene expression plays a fundamental role in plant response to environmental stimuli. A fundamental mechanism controlling gene expression is the ability of many transcription factors to access the genome of eukaryotes. Post-translational modifications of histones, including acetylation, methylation, phosphorylation, and ubiquitinylation play a key role in modulating dynamic changes in chromatin structure and gene activity (Berger, 2002; Reyes *et al*., 2002). Histone acetylation levels are determined by the action of histone acetyltransferases and histone deacetylases (HDACs). Plant HDACs can be grouped into four different classes, namely, RPD3-class, HDA1-class, SIR2-class, and HD2-class (Pandey *et al*., 2002). RPD3-class HDACs are the homologues of the yeast protein, RPD3 (Taunton *et al*., 1996). Four RPD3-class HDACs, HDA19 (also called AtRPD3A or AtHD1), HDA6 (AtRPD3B), HDA7, and HDA9 (Wu *et al*., 2000; Murfett *et al*., 2001; Tian and Chen, 2001; Pandey *et al*., 2002) were identified in *Arabidopsis*. Mutations in *HDA6* affected transgene expression, DNA methylation, and regulation of rRNA genes (Murfett *et al*., 2001; Aufsatz *et al*., 2002; Probst *et al*., 2004; Earley *et al*., 2006). Antisense suppression and T-DNA disruption of *HDA19* expression resulted in a range of developmental abnormalities including apical defect, reduced fertility, delayed flowering and altered light response (Wu *et al*., 2000; Tian and Chen, 2001; Tian *et al*., 2003; Benhamed *et al*., 2006; Long *et al*., 2006). More recently, it was found that HDA18, a HDA1-class HDAC, is required for normal cellular patterning of the *Arabidopsis* root epidermis (Xu *et al*., 2005).

There is increasing evidence indicating that histone acetylation is involved in the plant response to abiotic and biotic stresses (Stockinger *et al*., 2001; Devoto *et al*., 2002; Jang *et al*., 2003; Kim *et al*., 2004; Song *et al*.,

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2005; Zhou *et al*., 2005). It was suggested that histone acetyltransferases may be recruited through transcription factors, such as CBF1, to cold-induced genes, through multiprotein complexes similar to those found in other eukaryotes (Stockinger *et al*., 2001). Recently, it was demonstrated that HDA6 can interact with COI1, an F-box protein, that is involved in jasmonate (JA)-mediated plant defence responses, suggesting a possible role for *HDA6* in plant–pathogen interaction (Devoto *et al*., 2002). Our recent studies indicate that the expression of *Arabidopsis HDA19* and *HDA6* can be induced by ethylene and JA (Zhou *et al*., 2005). In addition, overexpression of *HDA19* in *Arabidopsis* induced ethylene- and JA-regulated *PR* gene expression and resulted in increased resistance to the pathogen *Alternaria brassicicola*. These studies provide evidence that RPD3-type HDACs, HDA19, and HDA6, may play an important role in ethylene- and JA-signalling and pathogen responses. A recent study by Song *et al*. (2005) provides direct evidences that HDACs are involved in ABA and abiotic stress responses. It was found that an AP2/EREBP transcription repressor, AtERF7, interacts with the *Arabidopsis thaliana* homologue of a human global corepressor of transcription, AtSin3, which, in turn, may interact with a HDAC.

In the present study, it is reported that HDA6 is required for the JA response, senescence, and flowering in *Arabidopsis*. The expression of the JA-responsive genes, *PDF1.2*, *VSP2*, *JIN1*, and *ERF1*, was down-regulated in the *Arabidopsis HDA6* mutant, *axe1-5*, and *HDA6*-RNA interfering (*HDA6*-RNAi) plants. Furthermore, *axe1-5* and *HDA6*-RNAi plants displayed increased leaf longevity compared with the wild type. The expression of the senescence-associated genes, *SAG12* and *SEN4*, was also down-regulated in the *axe1-5* and *HDA6*-RNAi plants. In addition, *axe1-5* and *HDA6*-RNAi plants displayed late-flowering. The expression of *FLC* was up-regulated and hyperacetylated in *axe1-5* and *HDA6*-RNAi plants, suggesting that HDA6 is required to deacetylate *FLC* chromatin and thereby repress its expression.

Materials and methods

Plant materials

Arabidopsis thaliana was grown in a growth chamber under long-day (LD, 16 h light and 8 h dark) or short-day (SD, 8 h light and 16 h dark) conditions after a 2–4 d stratification period. For growth under sterile conditions, seeds were surface-sterilized [15 min incubation in 5% (v/v) sodium hypochlorite, and rinsed three times in sterile distilled water] and sown on half-strength Murashige and Skoog (MS) salts (Sigma, St Louis, MO) supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar in Petri dishes.

Plasmid construction

To generate the *HDA6:GUS* construct, a 1.3 kb promoter for *HDA6* was PCR amplified by using the primer pairs, 5′-TCCAGATCTG-CAGTTGTAGG-3′ and 5′-GCCTCCATCTCCGTCTCTCACT-CAGAATC-3′. The resulting PCR product was then digested by *Pst*I and *Nco*I and subcloned into the pCAMBIA1381 binary vector (Cambia, Canberra, Australia). To generate *35S:HDA6-GFP*, the *HDA6* coding region was PCR amplified using the primer pairs, 5′-AATTTCCCGGGCATGAGGCAGACGAAAGCGGCA-3′ and 5′-AATTGAGCTCTTAAGACGATGGAGGATTCACG-3′, to replace *AtHD2A* in the *AtHD2A-GFP* construct (Zhou *et al*., 2004). DNA and protein sequence analysis was carried out using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) and the Vector NTI Suite program (InforMax Inc., Bethesda, MD).

Plant transformation and selection

Plant transformation plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 as described by Shaw (1995). The *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* was performed as described by Clough and Bent (1998). T_1 seeds were harvested, dried at 25 °C, and germinated on sterile medium containing 40 μg ml^{-1} hygromycin to select the transformants. Surviving T_1 plantlets were transferred to soil to set seeds (T_2).

β-glucuronidase assays and GFP localization

For histochemical GUS assay, *Arabidopsis* tissues were incubated in a 0.5 mg ml^{-1} solution of 5-bromo-4-chloro-indolyl β-D-glucuronide (X-Glu) in 100 mM sodium phosphate buffer, pH 7.0, and incubated at 37 °C overnight, followed by washing with 70% ethanol to remove the chlorophyll (Jefferson, 1988).

For GFP localization, transgenic seeds were germinated on a MS medium. Protoplasts were isolated from transgenic *Arabidopsis* seedlings as described by Weigel and Glazebrook (2002). The fluorescence photographs of protoplasts were taken using an Olympus florescent microscope (Tokyo, Japan) fitted with fluorescein isothiocyanate filters (excitation filter, 450–490 nm; emission filter, 520 nm; and dichroic mirror, 510 nm).

Semi-quantitative RT-PCR analysis

One microgram of total RNA was used for the first-strand cDNA synthesis after incubation at 65 °C for 10 min. cDNA was synthesized in a volume of 20 μl that contained MoMLV reverse transcriptase buffer (Promega, Madison, Wisconsin, USA), 10 mM dithiothreitol, 1.5 μM poly(dT) primer, 0.5 mM dNTPs, 2 U of MoMLV reverse transcriptase at 37 °C for 1 h. All PCR reactions were performed with 0.5 U of *Taq* polymerase (PGC Scientific, Gaithersburg, Maryland, USA), the buffer provided by the supplier, 0.2 mM dNTPs, and a pair of primers (0.1 μM each) in a final volume of 20 μl. PCR parameters differed for each gene: thermocycling conditions were 94 °C for 2 min followed by 25–35 cycles of 94 °C for 1 min, 55–68 °C for 1 min, and 72 °C for 2 min, with a final polymerization step at 72 °C for 10 min. The gene-specific primer pairs used for the RT-PCR are listed in the Table 1.

Protein gel blot analysis

Nuclear proteins were isolated as described by Weigel and Glazebrook (2002). 500 mg of *Arabidopsis* seedling tissues were homogenized in 1 ml of Honda buffer (2.5% Ficoll 400, 5% dextran T40, 0.4 M sucrose, 25 mM TRIS–HCl [pH 7.4], 10 mM $MgCl_2$, 10 mM β-mercaptoethenol, 100 μg ml^{-1} phenylmethylsulphonyl fluoride, 0.5 μg ml^{-1} antipain, 0.5 μg ml^{-1} leupeptin) and filtered through a 62 μm nylon mesh. 0.5% Triton X-100 was then added to the extract, which was incubated for 15 min on ice and centrifuged at 1500 *g* for 5 min. The pellet was washed with Honda buffer containing 0.1% Triton X-100, gently resuspended in 1 ml of Honda buffer, and centrifuged at 100 *g* for 5 min to pellet starch and cell debris. The supernatant was transferred to a microcentrifuge tube and centrifuged at 1800 *g* for 5 min to pellet the nuclear.

The nuclear extract was suspended in 200 μl of 5× SDS-PAGE loading buffer [0.2 M TRIS–HCl (pH 6.8), 25% SDS, 25% glycerol

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Table 1. *Primers used for RT-PCR*

Genes	Primers
HDA19	5′-ACAAGATGCCGGAGCATGAA-3′ and 5′-TTTAGGAGGAAACGCCTGCT-3′
HDA6	5′-TAGAGCCGGACAACAAACTC-3′ and 5′- TTCACGTCTGGCTCTGGGTT-3′
HDA7	5′-GGTGATCCGTTTGGTACATT-3′ and 5′-TCTTCTCCATGTCCACTTCC-3′;
HDA9	5′-TTACAGGAGGTGGAGGATAC-3′ and 5′-CGTTATCGTTGTCTCCATCG-3′
VSP2	5′-TTCTATGCCAAAGGACTTGC-3′ and 5′-GAGTGGATTTGGGAGCTTAA-3′
ERF1	5′-GTTCTCTTTGCTGCTTTCGAC-3′ and 5′-TTCTCCGTCTCATCGAGTGT-3′
PDF1.2	5′-ATGCTGGAACTACCACTACC-3′ and 5′-CCATGTTTGGCTCCTTCAAG-3′
JIN1	5′-TCGGTGACGCAATCGCTTAC-3′ and 5′-CTTGCTCTGAGCTGTTCTTG-3′
SAG12	5′-CAGCTGCGGATGTTGTTG-3′ and 5′-CCACTTTCTCCCCATTTTG-3′
SEN4	5′-TCTTCTTCACGACTCTTCTC-3 and 5′-TTGCCCAATCGTCTGCGTTC-3
RPS17	5′-ATGATAACGTCGTCCCTAAC-3′ and 5′-GCTGAGACTCCAAGGGAAGG-3′
FLC	5′-TTAGTATCTCCGGCGACTTGAACCCA-3′ and 5′-AGATTCTCAACAAGCTTCAACATGAG-3′
MAF1	5′- GCTAGGAAGGCAGAACTGAT –3′ and 5′-CCAATCCGTACATTCAGACAC-3′
MAF2	5′-TGTCCGTAACTAGAGCTAGG –3′and 5′-CCTTAATTTCCACATTGGCGC-3′
MAF4	5′-ATTAGGTCAGAAGAATTAGTCGGAGAAAAC-3′ and 5′-CTTGGATGACTTTTCCGTAGCAGGGGGAAG-3′
MAF5	5′-GGGGATTAGATGTGTCGGAAGAGTGAAG-3′ and 5′-GATCCTGTCTTCCAAGGTAACACAAAGG-3′
UBIQUITIN	5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′

and 12.5% 2-mercaptoethanol]. The protein samples were loaded on 15% polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was blocked in PBS containing 3% dry milk for 60 min and then incubated with 0.01–0.05 μg ml^{-1} of anti-acetyl-histone H3 K9 and K14 antibody (Catalogue no. 06–599, Upstate, Charlottesville, VA) for 2 h at room temperature. After washing, the primary antibody was detected with secondary anti-rabbit horseradish peroxidase coupled antibody (Amersham, Buckinghamshire, England) at room temperature for 45 min. Visualization was achieved using the ECL system (Amersham).

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was carried out as described previously (Johnson *et al.*, 2002; Gendrel *et al.*, 2005). Chromatin extracts were prepared from young leaves treated by formaldehyde. The chromatin was sheared to an average length of 500–1500 bp by sonication and immunoprecipitated with the anti-acetyl-histone H3 K9 and K14 antibody (Catalogue no. 06–599, Upstate, Charlottesville, VA). The immunocomplexes were harvested with Protein A agarose and heated at 65 °C for 5 h to release DNA cross-linked to the immunoprecipitated proteins. The DNA cross-linked to the immunoprecipitated proteins was analysed by PCR. To assess non-specific binding, the immunoprecipitation reaction was also performed in the absence of antibody. The primers used to amplify *FLC* were FLC-B (5′-TGTAGGCACGACTTTGGTAACACC-3′ and 5′- GCAGAAAGAACCTCCACTCTACATC-3′), FLC-P (5′-CGACTTGAACCCAAACCTGAGGATCAAAT-3′ and 5′-AGAAGATAAAAGGGGGAACAAATGAAAAC-3′), FLC-CH (5′-CTGCGACCATGATAGATACATGAGA-3′ and 5′- TTCACTCAACAACATCGAGCACG-3′), FLC-V2 (5′- ATAGATTTGCCTCATATTTATGTGATTGT-3′ and 5′-TTCATTATAGATCCGTACCAAAGAGGTTG-3′), and FLC-U (5′-GCTGATAAGGGCGAGCGTTTG-3′ and 5′-AAGCCGTAGGCTTCTTCACTG-3′). The primer pair JP1595 (5′-CGTTTCGCTTTCCTTAGTGTTAGCT-3′) and JP1596 (5′-AGCGAACGGATCTAGAGACTCACCTTG-3′) was used to amplify *ACTIN* (Johnson *et al.*, 2002). 30 cycles of PCR (94 °C for 25 s, 59 °C for 35 s, 72 °C for 30 s) were performed as separate reactions for *FLC* and *ACTIN* amplification.

Measurement of chlorophyll content and photochemical efficiency

From about 15 DAE (days after leaf emergence) onwards, the sixth rosette leaf, which was fully grown, was chosen for chlorophyll extraction and photochemical efficiency of photosystem II (PSII) measurement. Chlorophyll was extracted from equal volumes of leaf discs by grinding the leaves in liquid nitrogen and dissolving in 80% acetone. Chlorophyll concentration per g fresh weight of leaf was calculated as described by Porra *et al.* (1989). The photochemical efficiency of PSII was deduced from the characteristics of chlorophyll fluorescence (Oh *et al.*, 1997) using a portable plant efficiency analyser (Hansatech Instruments, Morfolk, England). The ratio of maximum variable fluorescence to maximum yield of fluorescence (F_v/F_m), which corresponds to the potential quantum yield of the photochemical reactions of PSII, was used as the measurement of the photochemical efficiency of PSII (Oh *et al.*, 1997).

Results

Expression and localization of HDA6

The expression pattern of *HDA6:GUS* was analysed by fusing the 1.3 kb *HDA6* promoter fragment to a *GUS* reporter gene in *Arabidopsis* plants. In 2-week-old transgenic seedlings, *GUS* was strongly expressed in cotyledons and hypocotyls (Fig. 1A). In adult plants, *GUS* expression was detected in the mature leaves and stems. GUS activity was also detected in all parts of flowers except the stamens. However, GUS activity was not detected in the seeds (Fig. 1A). The expression pattern of *HDA6:GUS* is therefore different from that of *HDA19*:*GUS*, which was expressed in all of the organs analysed (Zhou *et al.*, 2005). Expression of *HDA6* and other RPD3-type *HDA*s was analysed further by using RT-PCR. As shown in Fig. 1B, *HDA6* and *HDA19* transcripts were detected in roots, leaves, stems, and flowers of *Arabidopsis*. By comparison, *HDA9* transcript could only be detected in flowers.

To investigate the cellular distribution of HDA6 protein, an *in vivo* targeting experiment was performed using green fluorescent protein (GFP). *HDA6-GFP* gene fusion driven by the 35S promoter was created and introduced into *Arabidopsis* using the *Agrobacterium*-mediated floral

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dip method (Clough and Bent, 1998). To confirm that the fusion protein entered the nucleus, the fluorescence of GFP was monitored at the cellular level. Protoplasts were isolated from seedlings of transgenic *Arabidopsis*, and localization of the fusion protein was determined by a fluorescence microscope. As shown in Fig. 1C, bright green fluorescence was observed only in the nuclei of *HDA6-GFP* transgenic plants. This indicates that HDA6-GFP fusion protein was localized in the nucleus of the *Arabidopsis* cells. This result is consistent with the observation of Earley *et al.* (2006), who demonstrated the nuclear localization of HDA6-Flag by immunolocalization. These results support the idea that HDA6 is involved in transcription regulation.

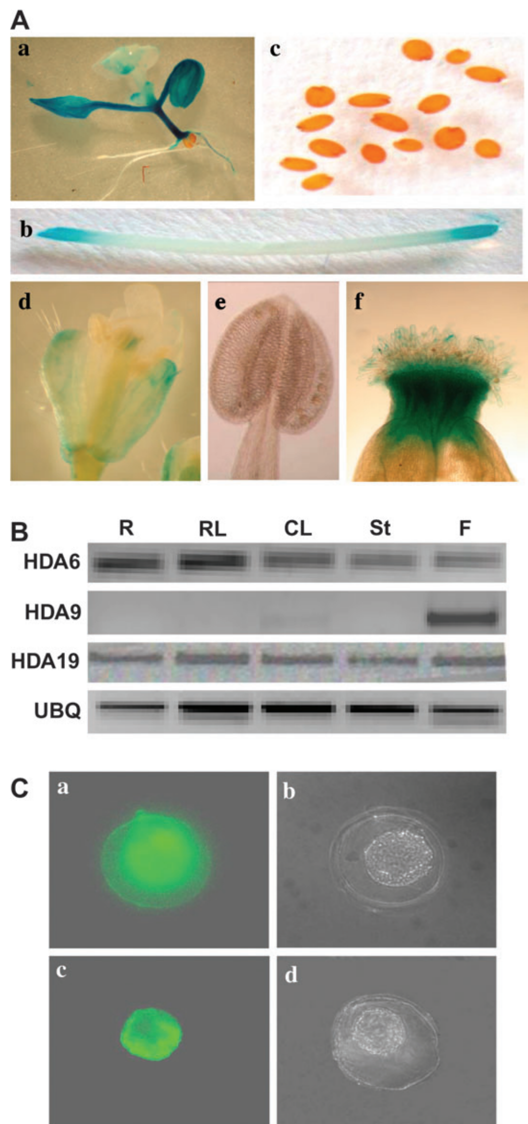

Fig. 1. *HDA6* expression and HDA6 localization. (A) GUS staining in the *HDA6:GUS* plants from a seedling (a), stem (b), seeds (c), flower (d), stamen (e), and stigma (f). (B) RT-PCR analysis of expression of *HDA6, HDA9*,and *HDA19* genes in *Arabidopsis*. Total RNA for RT-PCR analysis was isolated from roots (R), rosette leaves (RL), cauline leaves (CL), stems (St), and flowers (F) of *A. thaliana*. *Ubiquitin* (*UBQ*) was shown as an internal control. (C) Subcellular localization of HDA6-GFP. Protoplasts were isolated from the leaves of *35S*:*GFP* (a, b) and *35S*:*HDA6-GFP* (c, d) transgenic *Arabidopsis* plants. GFP fluorescence was examined by fluorescence microscopy under UV light (a, c) and white light (b, d).

HDA6 affected JA-responsive gene expression

To investigate the function of HDA6 further, two *HDA6*-RNAi lines, CS24038 and CS24039 (ecotype Wassilewskija [Ws]), generated by expressing a transgene that encodes double-stranded *HDA6* RNA (Plant Chromatin Database: http://chromdb.org) were analysed. RT-PCR analysis indicated that *HDA6* transcript levels in the *HDA6*-RNAi plants were significantly reduced relative to non-transgenic control plants (Fig. 2A). CS24039 plants had greater reduction in *HDA6* expression compared with CS24038 plants. The expression of other *RPD3*-class *HDAC*s, *HDA19* and *HDA9*, was not affected in the *HDA6*-RNAi plants.

The levels of acetylated histone H3 in *HDA6*-RNAi plants were analysed by protein gel blot analysis. As shown in Fig. 2B, there were obvious increased levels of acetylated H3 in two *HDA6*-RNAi lines compared with the Ws wild type, suggesting that the *HDA6* transcript level affects histone acetylation levels globally. Similarly, increased level of acetylated H3 was also observed in the *HDA6* mutant, *axe1-5* [ecotype Columbia (Col)], when compared with the Col wild type (Fig. 2B). *axe1-5 is* a splice site mutant that has a base change at an intron splice site resulting in two *HDA6* transcripts with altered lengths (Murfett *et al.*, 2001). Changed levels of acetylated H3 in

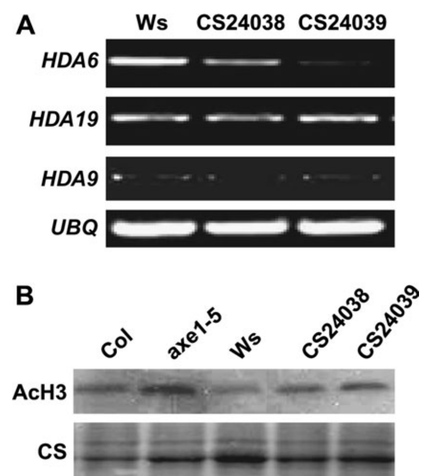

Fig. 2. Expression of *HDA6* and levels of acetylated H3 in *HDA6*-RNAi lines. (A) RT-PCR analysis of *HDA6, HDA19, and HDA9* expression. Total RNA for RT-PCR analysis was isolated from leaf tissues of Ws wild-type and *HDA6*-RNAi lines (CS24038 and CS24039). *Ubiquitin* (*UBQ*) was shown as an internal control. (B) Protein gel blot analysis detecting acetylated H3 (AcH3) (top panel) using α-AcH3 antibodies on protein extracts from Col wild-type, *axe1-5*, Ws wild-type, and *HDA6*-RNAi lines (CS24038 and CS24039). Bottom panel, Coomassie staining (CS) shows equal protein loading.

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axe1-5 and *HDA6*-RNAi plants support the observation that HDA6 has a HDAC activity (Earley *et al*., 2006).

The interaction of HDA6 with COI1, an F-box protein that was required for JA-mediated plant defence responses, has been demonstrated (Devoto *et al*, 2002), suggesting a possible role of HDA6 in JA response. To investigate further the involvement of HDA6 in the JA-responsive pathway, semi-quantitative RT-PCR was conducted to examine transcript levels of JA-responsive genes, *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* (Lorenzo *et al*., 2003, 2004), in the *axe1-5* and *HDA6*-RNAi plants. As shown in Fig. 3A, expression of *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* was down-regulated in *axe1-5* and *HDA6*-RNAi plants. Expression of *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* can be induced by JA in Col wild-type plants (Fig. 3B). By comparison, expression of these JA-responsive genes can not be induced by JA in *axe1-5* plants (Fig. 3B). Down-regulation of *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* in *axe1-5* and *HDA6*-RNAi plants suggests that *HDA6* is required for expression of the JA-responsive genes.

axe1-5 and HDA6-RNAi plants displayed increased leaf longevity

JA was found to be involved in leaf senescence in *Arabidopsis* (He *et al*., 2002). To investigate whether *HDA6* is required for leaf senescence, leaf longevity of the *axe1-5* and *HDA6*-RNAi plants was examined. The phenotype of individual leaves grown under the long-day (LD, 16 h light and 8 h dark) condition was followed from the formation of a visually recognizable leaf primordium (1 mm in size) and the leaf was considered dead when the entire leaf turned yellow (Grbi and Bleecker, 1995). As shown in Fig. 4A, the leaves of *axe1-5* and *HDA6*-RNAi plants turned yellow much slower and showed increased leaf longevity when compared with their wild-type counterparts.

Leaf longevity was also assessed by measuring typical senescence-associated physiological markers, such as chlorophyll contents and photochemical efficiency of PSII (Fan *et al*., 1997; Oh *et al*., 1997). Chlorophyll contents decline at the onset of senescence and it is considered as an important indicator of the rate of senescence (Nam, 1997). Chlorophyll contents were measured from 15 DAE (days after leaf emergence), which was also the day of the 6th rosette leaf that was fully grown. At 45 DAE, the leaves of Ws and Col wild type lost 65–75% of their chlorophylls, whereas these of *axe1-5* and *HDA6*-RNAi just lost 15–35% (Fig. 4B). Delayed senescence of the mutants was also defined as delay in the decrease in photosynthetic activity (Fig. 4C). The PSII efficiency has also been shown to be an effective indicator of leaf senescence in plants (Lu and Zhang, 1998*a*, *b*). It was demonstrated that, during senescence, the PSII efficiency declines rapidly, leading to a loss of photosynthetic capabilities of the leaves and leading to the eventual death of the leaves. This analysis indicated that *axe1-5* and *HDA6*-RNAi leaves consistently showed later development of senescence-associated changes. These results suggest that decreased expression of *HDA6* causes increased leaf longevity in *Arabidopsis*.

Leaf senescence is accompanied by the decreased expression of genes related to photosynthesis and protein synthesis genes (*PAG*s) (Bate *et al*., 1991) and increased expression of senescence-associated genes (*SAG*s) (Nam, 1997). The expression patterns were examined of two *SAG* genes, *SAG12* and *SEN4*, which have been shown to be up-regulated during senescence (Gan and Amasino, 1997; Park *et al*, 1998), in the *axe1-5* and *HDA6*-RNAi plants. As shown in Fig. 5, the expression of *SAG12* and *SEN4* was down-regulated in the *axe1-5* and *HDA6*-RNAi plants when compared with the wild-type. By comparison, the expression of *RPS17*, a *PAG* gene that encodes the chloroplast ribosomal protein S17 (Woo *et al*., 2002), was up-regulated in *axe1-5* and *HDA6*-RNAi plants (Fig. 5). These results support the idea that HDA6 is required for *SAG*s expression and therefore it is involved in senescence progression. In the absence of *HDA6*, *PAG* genes are up-regulated, which leads to a higher rate of photosynthesis, resulting in higher PSII efficiencies and higher chlorophyll contents in *axe1-5* and *HDA6*-RNAi plants.

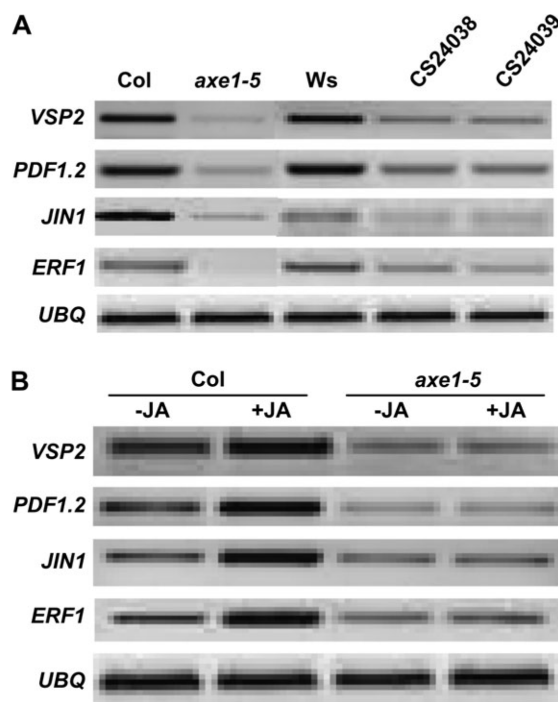

Fig. 3. JA-responsive gene expression in *axe1-5* and *HDA6*-RNAi plants. (A) Expression of *PDF1.2*, *VSP2*, *JIN1*, and *ERF1* in Col wild-type, *axe1-5*, Ws wild-type, and *HDA6*-RNAi lines (CS24038 and CS24039) plants. Total RNA for RT-PCR analysis was isolated from leaf tissues. *Ubiquitin* (*UBQ*) was shown as an internal control. (B) Induction of *VSP2*, *PDF1.2*, *JIN1*, and *ERF1* expression by JA in Col wild-type and *axe1-5* plants. Total RNA for RT-PCR analysis was isolated from leaf tissues with (+) or without (–) 0.1 mM JA treatment for 12 h. *Ubiquitin* (*UBQ*) was shown as an internal control.

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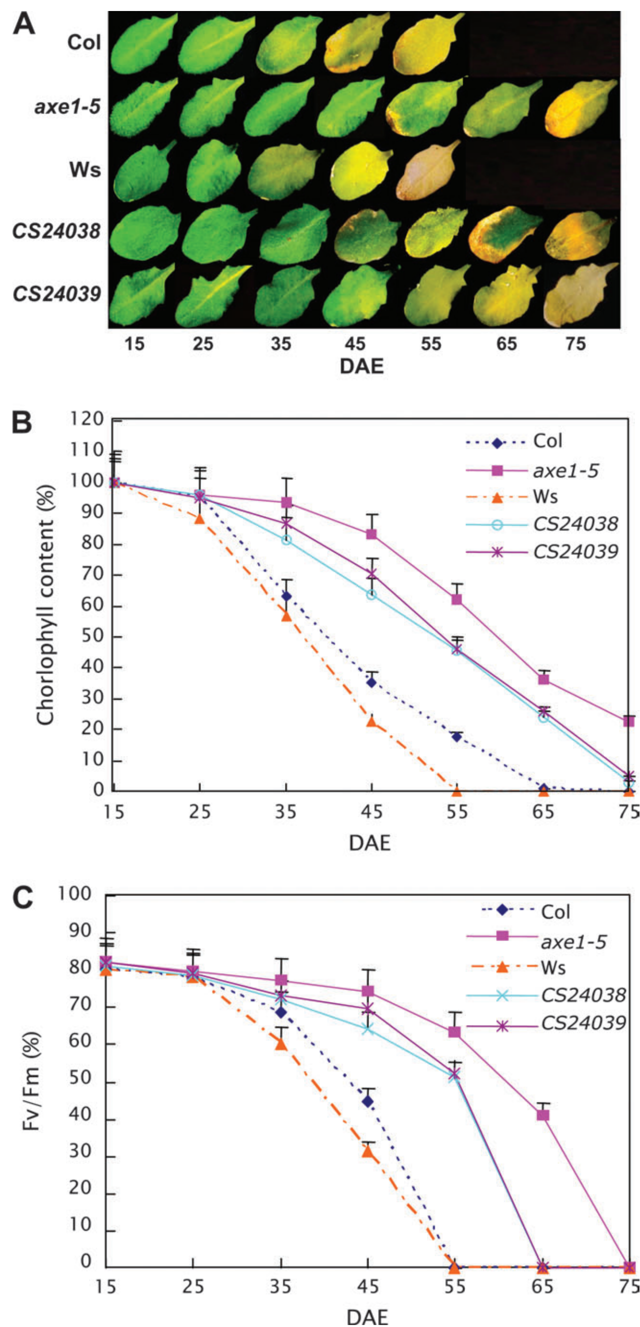

Fig. 4. Age-dependent senescence symptoms in *axe1-5* and *HAD6*-RNAi plants. (A) The age-dependent senescence phenotype of Col wild-type (Col), *axe1-5*, Ws wild-type (Ws), and *HAD6*-RNAi (CS24038 and CS24039) leaves grown under the long-day (LD, 16 h light and 8 h dark) conditions. Photographs show representative leaves at each time point. Pictures were taken every 10 d from 15 DAE (days after leaf emergence). (B, C) Chlorophyll content (B) and photochemical efficiency of PSII (C). Data were collected every 10 d from 15 DAE, when the 6th rosette leaves were just fully grown. F_v/F_m, maximum quantum yield of PSII electron transport (maximum variable fluorescence/maximum yield of fluorescence). Error bars indicate SE ($n \geq 15$).

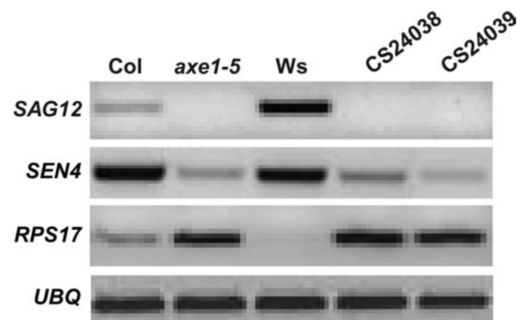

Fig. 5. Expression of *SAG12, SEN4*, and *RPS17* in *axe1-5* and *HDA6*-RNAi plants. RT-PCR analysis of *SAG12, SEN4*, and *RPS17* expression in Col wild-type (Col), *axe1-5*, Ws wild-type (Ws), and *HDA6*-RNAi (CS24038 and CS24039) plants. Total RNA for RT-PCR analysis was isolated from leaf tissues. *Ubiquitin* (*UBQ*) was shown as an internal control.

axe1-5 *and* HDA6*-RNAi plants displyed delayed flowering*

axe1-5 and *HDA6*-RNAi mutants displayed later flowering phenotypes, as measured by the days to bolting and the rosette leaf numbers at flowering (Fig. 6). Col, Ws, and *HDA6* mutants were grown in long-day (LD, 16 h light and 8 h dark) and short-day (SD, 8 h light and 16 h dark) conditions, and the flowering time was compared. The flowering of *axe1-5* and *HDA6*-RNAi plants was greatly delayed in SD as well as in LD in terms of both the days to flowering and the rosette leaf numbers at flowering initiation (Fig. 6). *axe1-5* plants did not flower even at 104 d after germination in SD, when some rosette leaves showed senescence. The delay in flowering time of *axe1-5* and *HDA6*-RNAi plants was completely corrected by 45 d of vernalization at 4 °C (Fig. 6B). These observations suggest that HDA6 is involved in the autonomous pathway of flowering.

The delayed flowering of the *axe1-5* and *HDA6*-RNAi plants prompted us to analyse whether the expression of *FLC*, a transcription factor that controls the transition from vegetative to reproductive development, was affected. RT-PCR analysis indicated that expression of *FLC* was increased in *axe1-5* and *HDA6*-RNAi plants compared with the wild type (Fig. 7). In addition to FLC, other MADS-box transcriptional factors, such as MAF1, MAF2, MAF4, and MAF5, are closely related to FLC and also function as repressors of flowering in *Arabidopsis* (Scortecci *et al*., 2001; Ratcliffe *et al*., 2003). It was found that expression of *MAF4* and *MAF5* was also increased in *axe1-5* and *HDA6*-RNAi plants (Fig. 7).

Chromatin immunoprecipitation (ChIP) analysis was carried out using the anti-acetyl-histone H3 antibody to determine if the acetylation level of *FLC* was affected in *HDA6* mutants. A 295 base pair region (FLC-CH) of the first intron of *FLC* was shown to be hyperacetylated in the *fld* and *fve* mutants of the autonomous flowering pathway (He *et al*., 2003). Therefore the histone acetylation status

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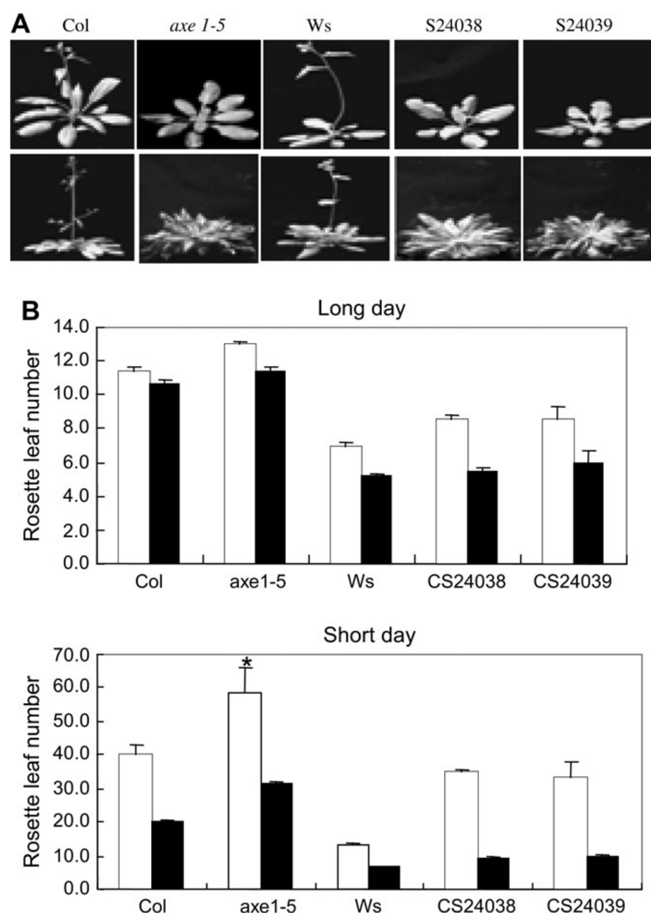

Fig. 6. Delayed flowering of *axe1-5* and *HDA6*-RNAi plants. (A) Plants were grown under LD (top panel) or SD (bottom panel) without vernalization. Col wild-type and *axe1-5* plants were grown under LD and SD for 31 d and 87 d, respectively; whereas Ws wild-type and *HDA6*-RNAi (CS24038 and CS24039) plants were grown under LD and SD for 24 d and 64 d, respectively. (B) Rosette leaf number ±SE at bolting of Col, *axe1-5*, Ws, and two *HDA6*-RNAi lines (CS24038 and CS24039) grown under LD or SD conditions without vernalization (white columns) or after vernalization for 45 d (black columns). Values are the mean of 10–20 plants per genotype. (Asterisk) Under SD without vernalization, *axe1-5* plants did not flower 104 d after germination and the rosette leaf number of *axe1-5* was counted at 104 d.

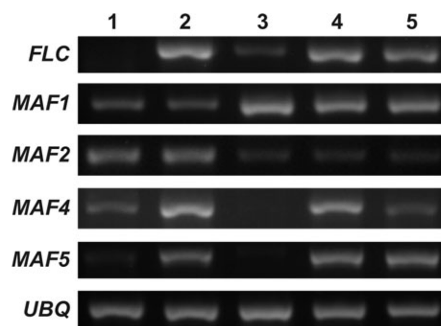

Fig. 7. Expression of *FLC* in *axe1-5* and *HDA6*-RNAi plants. RT-PCR analysis of *FLC*, *MAF1*, *MAF2*, *MAF4*, and *MAF5* expression in Col (1), *axe1-5* (2), Ws (3), CS24038 (4), and CS24039 (5) plants. Total RNA for RT-PCR analysis was isolated from leaf tissues. *Ubiquitin* (*UBQ*) was shown as an internal control.

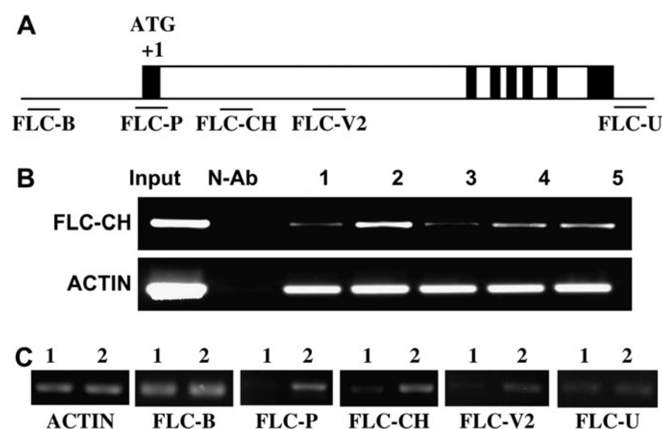

Fig. 8. Acetylation status of *FLC* in *axe1-5* and *HDA6*-RNAi plants. (A) Relative positions of amplified *FLC* regions used in the ChIP assay. The black boxes correspond to exons, white boxes to introns and single lines to 5′ or 3′ UTR. (B) ChIP analyses of the acetylation status of histone H3 in *FLC* chromatin in the Col wild-type (1), *axe1-5* (2), Ws (3), CS24038 (4), and CS24039 (5) plants. The input is Col chromatin before immunoprecipitation; N-Ab, control sample lacking antibody; *ACTIN* served as an internal control of the ChIP analysis. (C) ChIP analyses of the acetylation status of histone H3 in *FLC* chromatin in the Col wild-type (1) and *axe1-5* (2) plants. Five regions were examined in *FLC* chromatin. *ACTIN* served as an internal control of the ChIP analysis.

of this region was analysed in *FLC*. As shown in Fig. 8B, *FLC* displayed hyperacetylation of histone H3 in the *axe1-5* and *HDA6*-RNAi plants compared with the wild type, suggesting that HDA6 is required to deacetylate *FLC* chromatin and repress *FLC* expression. The histone acetylation status of other regions of *FLC* was also analysed in the *axe1-5* mutant. There was increased acetylation of histone H3 in the 1st exon and 1st intron of *FLC*, but not in the promoter and the 3′ UTR regions (Fig. 8C).

Discussion

The *HDA6* mutant, *axe1,* was isolated based on deregulated expression of auxin-responsive transgenes (Murfett *et al*., 2001). Since then, other mutant alleles of *HDA6*, *rts1* and *sil1*, were also isolated based on their effects on specific transgene expression (Aufsatz *et al*., 2002; Probst *et al*., 2004). It was shown that mutations in *HDA6* result in loss of transcriptional silencing from several repetitive transgenic and endogenous templates. In the *axe1-5* mutant, significant hyperacetylation is identified in the nucleolus organizer regions that contain the rDNA repeats (Probst *et al*., 2004). It was suggested that HDA6 might play a role in regulating activity of rRNA genes (Earley *et al*., 2006), and this control might be functionally linked to silencing of other repetitive templates and to its role in RNA-directed DNA methylation (Probst *et al*., 2004).

This study indicates that HDA6 is involved in JA response, senescence, and flowering. It has been observed

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that the JA-responsive genes, *PDF1.2*, *JIN1*, *ERF1*, and *VSP2*, were down-regulated in *axe1-5* and *HDA6*-RNAi plants, suggesting that *HDA6* is required for the JA-responsive pathway. The finding that *HDA6* expression can be induced by JA also supports a role for HDA6 in the JA-response pathway (Zhou *et al.*, 2005). Our previous studies indicate that HDA19 plays an important role in the JA-signalling pathway (Zhou *et al.*, 2005). It would be interesting to know whether HDA6 and HDA19 have redundant function in the JA-response pathway. Further study by analysing *HDA6* and *HDA19* double mutants will be useful to address this question. *HDA6* mutants displayed delayed leaf senescence compared with wild-type plants. The delayed leaf senescence in *axe1-5* and *HDA6*-RNAi plants was demonstrated by measuring changes in typical senescence-associated physiological markers such as chlorophyll content and photochemical efficiency (Fan *et al.*, 1997; Oh *et al.*, 1997). Expression of two *SAG* genes, *SAG12* and *SEN4*, was decreased in *axe1-5* and *HDA6*-RNAi plants when compared with the wild type. By comparison, expression of a *PAG* gene, *RPS17*, was up-regulated in *axe1-5* and *HDA6*-RNAi plants. These results suggest that HDA6 is required for *SAG*s expression and is involved in senescence progression.

Although histone deacetylases have often been associated with the repression of gene expression, recent studies in yeast and animal cells indicate that histone deacetylation can also be required as a transcriptional activation signal. Deletion of yeast histone deacetylases Rpd3, or Hda1 results in decreased transcription of a number of genes and an increase rather than a loss of silencing, pointing to an alternative role of HDACs as transcriptional activators (Rundlett *et al.*, 1996; Bernstein *et al.*, 2000). In addition, SIN3, a major component of the HDAC-containing transcriptional repressor complex, can function as both a transcriptional corepressor and a transcriptional coactivator (Nawaz *et al.*, 1994). Furthermore, Mouse Histone Deacetylase 1 (HDAC1) can act as a negative regulator as well as a positive regulator of transcription (Zupkovitz *et al.*, 2006). These studies indicate that gene regulation by acetylation is more dynamic and HDACs may also function as activators (Nusinzon and Horvath, 2005). An alternative explanation for the repression of gene activity in *axe1-5* and *HDA6* RNAi plants could be that HDA6 may target transcription repressors.

The interaction of HDA6 with COI1, an F-box protein that was required for JA-mediated plant defence responses, has been demonstrated (Devoto *et al.*, 2002). However, the function and biological significance of this interaction is unknown. F-box proteins interact with SKP1 and cullin proteins to form E3 ubiquitin ligases known as the SCF complexes that selectively recruit regulatory proteins targeted for ubiquitination (Vierstra, 2003). Co-immunoprecipitation experiments confirmed the interaction of COI1 with SKP1-like proteins and HDA6 *in planta*. Regulation of HDAC activities by ubiquitination has been demonstrated in mammalian cells (Gaughan *et al.*, 2005). It was proposed that COI1 may form a functional E3-type ubiquitin ligase in plants to regulate expression of JA-responsive genes, possibly by targeted ubiquitination of a histone deacetylase (Devoto and Turner, 2003). In addition to its role in targeting proteins for proteolytic degradation by the proteasome, ubiquitination can also regulate protein location, activity, and interaction with binding partners (Schnell and Hicke, 2003; Caron *et al.*, 2005). Further analysis is required to reveal whether HDA6 is regulated by ubiquitination and the biological function of this modification.

Plant flowering is regulated by both environmental and endogenous cues. Molecular genetic studies on the mechanism of flowering in *Arabidopsis*, a quantitative long-day plant, have revealed four major flowering pathways: the photoperiod, autonomous, vernalization, and gibberellin pathways (Mouradov *et al.*, 2002; Boss *et al.*, 2004; Henderson and Dean, 2004). A central player in the autonomous and vernalization flowering pathways is FLC, which blocks flowering by inhibiting genes required to switch the meristem from vegetative to flower development. *axe1-5* and *HDA6*-RNAi plants flowered later than the wild-type plants in both long-day and short-day photoperiods and flowered rapidly after exposure to a prolonged period of cold (vernalization), which is characteristic of autonomous-pathway mutants (He *et al.*, 2003). The genes in the autonomous pathway, such as FVE and FLD, promote flowering by suppressing FLC. More recent studies indicated that histone acetylation is involved in plant flowering (He *et al.*, 2003; Ausin *et al.*, 2004; Kim *et al.*, 2004). Mutations in FLD and FVE, two proteins that were found in histone deacetylase complexes, result in hyperacetylation of histones in *FLC* chromatin, up-regulation of *FLC* expression, and delayed flowering, indicating that the autonomous pathway regulates flowering in part by histone deacetylation (He *et al.*, 2003; Ausin *et al.*, 2004; Kim *et al.*, 2004). It was found that *FLC* was up-regulated and hyperacetylated in the *axe1-5* and *HDA6*-RNAi plants, suggesting that HDA6 is required to deacetylate *FLC* chromatin and repress its expression. FVE and FLD may therefore act with HDA6 to repress *FLC* expression.

In summary, our studies indicate that, in addition to its role in transgene expression, DNA methylation, and regulating activity of rRNA genes (Murfett *et al.*, 2001; Aufsatz *et al.*, 2002; Probst *et al.*, 2004; Earley *et al.*, 2006), HDA6 is involved in many aspects of plant development. In particular, HDA6 is required for JA response, senescence, and flowering in *Arabidopsis*. The role for JA in leaf senescence in *Arabidopsis* was demonstrated based on the fact that exogenous application of JA induces leaf senescence, and this induction requires an intact JA signalling pathway (He *et al.*, 2002). In

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addition, it was shown that the endogenous JA level in senescing leaves increased to nearly 500% of that in non-senescent counterpart leaves. Thus HDA6 may be involved in leaf senescence via the JA-signalling pathway. *Arabidopsis* mutants that affect both senescence and flowering time were isolated previously (Barth *et al*., 2006). For example, the *Arabidopsis* ascorbic acid-deficient mutant *vtc1* exhibits a delayed senescence and flowering phenotype (Pavet *et al*., 2005). By comparison, mutation of the matrix metalloproteinase At2-*MMP* that is important for the degradation and remodelling of the extracellular matrix causes late flowering but early senescence in *Arabidopsis* (Golldack *et al*., 2002). These results suggest that flowering and senescence could be regulated independently. It remains to be determined whether HDA6 is independently involved in these processes. Analysis of the genetic interaction between the *HDA6* mutation and mutations affecting JA signalling, senescence or flowering is required to dissect further the role of HDA6 in these pathways.

Acknowledgements

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