HISTONE DEACETYLASE6 Controls Gene Expression Patterning and DNA Methylation-Independent Euchromatic Silencing

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To investigate the role of chromatin regulators in patterning gene expression, we employed a unique epigenetically controlled and highly tissue-specific green fluorescent protein reporter line in Arabidopsis (Arabidopsis thaliana). Using a combination of forward and reverse genetic approaches on this line, we show here that distinct epigenetic regulators are involved in silencing the transgene in different tissues. The forward genetic screen led to the identification of a novel HISTONE DEACETYLASE6 (HDA6) mutant allele (epigenetic control1, hda6–8). This allele differs from the previously reported alleles, as it did not affect DNA methylation and only had a very modest effect on the release of transposable elements and other heterochromatic transcripts. Overall, our data shows that HDA6 has at least two clearly separable activities in different genomic regions. In addition, we present an unexpected role for HDA6 in the control of DNA methylation at CG dinucleotides.

Epigenetic regulation of gene expression plays important roles in a number of processes, among which protection of genome stability by preventing activation of transposable elements (TEs) has been extensively studied (Zilberman, 2008; Bucher et al., 2012). However, the maintenance of tissue-specific transcriptional programs is also subjected to epigenetic control. Differentiation and specialization of cells is a process that is dependent on spatial and temporal regulation of gene expression. Several studies have shown that different types of chromatin modifications, such as DNA methylation, histone modifications, and structural conformations, are required for these processes (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Roudier et al., 2011).

The histone deacetylase HDA6 is one of the key epigenetic regulators in Arabidopsis (Arabidopsis thaliana).

It is involved in silencing TEs, ribosomal RNA genes, and transgenes (Aufsatz et al., 2002; Probst et al., 2004; Earley et al., 2010), and it is involved in developmental processes (Luo et al., 2012). HDA6 was shown to play a role in maintenance and de novo DNA methylation via its interaction with DNA METHYLTRANSFERASE1 (MET1) and the RNA-directed DNA methylation (RdDM) pathway (Aufsatz et al., 2002; Liu et al., 2012). Central components of the RdDM machinery are the plant-specific RNA polymerases IV and V in which NUCLEAR RNA POLYMERASE D1 (NRPD1) and NUCLEAR RNA POLYMERASE E1 (NRPE1) are the largest subunits, respectively (Herr et al., 2005; Kanno et al., 2006; Onodera et al., 2005; Pontier et al., 2005). MORPHEUS’ MOLECULE1 (MOM1) is also a plant-specific and still enigmatic protein that plays an important role in repressing transcription of intermediate heterochromatin (Habu et al., 2006). Notably, plants defective in MOM1 activity display no or only minor epigenetic changes at the released loci (Vaillant et al., 2006).

Genomewide studies of plants defective in these epigenetic pathways have provided valuable information about the localization of different chromatin marks and their general effect on transcription (Zhang et al., 2006, 2007; Reinders et al., 2008). While there are some reports proposing a tissue-specific distribution of these marks in plants (Costa and Shaw, 2006; Caro et al., 2007), the majority of studies have focused on germ cells (Hsieh et al., 2009; Slotkin et al., 2009; She et al., 2013). Currently, little is known about how different epigenetic marks influence tissue-specific gene expression patterns in sporophytic tissues.
In this study we first present a reporter line in which GFP expression is repressed by several different epigenetic silencing pathways. Using this line, we show here that defects in different epigenetic regulators can result in different GFP expression patterns, thus allowing an assessment of how different epigenetic regulators contribute to gene expression patterning. It therefore represents a powerful tool to characterize epigenetic mutations.

In the forward genetic mutant screen for modifiers of GFP expression, we identified epigenetic control1 (epic1), an interesting and, to our knowledge, novel allele of HDA6 that affects histone acetylation but not DNA methylation. The detailed analysis of this allele revealed that HDA6 has at least two independent euchromatic and heterochromatic functions. We postulate a new regulatory mechanism in which HDA6 may inhibit de novo DNA methylation in the CG sequence context.

Taken together, our data suggests that epigenetic transcriptional regulators play a yet underestimated role in fine-tuning tissue-specific gene expression patterns in plants.

RESULTS

The Silex Reporter Line

We have previously identified a gene in Arabidopsis that was under complex epigenetic control. ARABIDOPSIS PUMILIO9 (APUM9), which encodes for an RNA-binding protein containing the conserved Pumilio homology domains, was synergistically repressed by two independent silencing mechanisms controlled by MOM1 and NRPE1 (Yokthongwattana et al., 2010). Notably, APUM9 carries a ROMANIAT5 copia-like retrotransposon 772 bp upstream of its predicted transcription start site (Fig. 1A). The complexity of the epigenetic regulation of this gene drew our attention, and to analyze it more easily, we obtained a GFP reporter line for the APUM9 promoter from a collection of GFP reporter lines of low-expressing genes in Arabidopsis (line AGRAC-60-1-1; Xiao et al., 2010). The transgene construct includes 1.5 kb of ROMANIAT5 located upstream of the promoter (Fig. 1B). The GFP reporter line showed no detectable GFP expression at juvenile stages. Later, strong GFP expression was observed in the valve margin of siliques (Fig. 1C). Because this line showed GFP expression in siliques, we termed the line silex (siliques expression). Classic mapping approaches, segregation analysis, and whole-genome sequencing data confirmed that the transgene was present as a single locus insertion on the upper arm of chromosome 3 (euchromatic region and second intron of AT3G07640, a gene coding for an unknown protein).

The silex Reporter Transgene Is Epigenetically Repressed

We showed previously that endogenous APUM9 transcription was released in mom1 mutant plants and to an even higher extent in the mom1 nrpe1 double mutant line, but not in nrpe1 plants. This synergistic effect of the two epigenetic regulators suggested that they might act independently of each other (Yokthongwattana et al., 2010). Introggressions of mom1 (mom1-2; Habu et al., 2006) and nrpe1 (nrpe1-2; Pontier et al., 2005) into the silex reporter line showed no visible GFP fluorescence, as opposed to the introgression of mom1 nrpe1, which led to GFP fluorescence in the veins that was visible on the abaxial side of juvenile leaves (Fig. 2A). Quantiﬁcation of GFP mRNA levels by quantitative real-time PCR corresponded well with the observed increase in GFP fluorescence. GFP transcript accumulation was highest in mom1 nrpe1 and showed weak activation of the transgene in nrpe1 (2-fold increase) and activation in mom1 plants (Fig. 2B). However, activation in mom1 plants was insufﬁcient to produce microscopically detectable GFP fluorescence (Fig. 2A). The reporter transgene therefore recapitulated the synergistic effect that the mom1 nrpe1 double mutant has on the APUM9 endogene. To further analyze the role of DNA methylation in repressing the transgene, we treated the silex line with 5-aza-2′-deoxycytidine, which efficiently reduces global DNA methylation (Doerfler, 1983). In treated seedlings, we observed a stochastic release of GFP silencing in cotyledons. Drug-induced release of GFP transcription was also conﬁrmed at the transcriptional level (Supplemental Fig. S1A).

Taken together, this data shows that the silex line adequately reports on APUM9 transcription and suggests that the transgene has acquired similar repressive epigenetic marks as the endogene.

HDA6 Silences Silex Transgene Expression in Young Leaves

To identify epigenetic factors that control APUM9 expression, we carried out an ethyl methanesulphonate (EMS) mutagenesis on seeds of the silex line and screened seedlings (M2 generation) for activation of GFP expression. Here we report on a mutant that released GFP expression in young leaves (Fig. 3A). Assuming that our mutant may be affected in epigenetic control of gene expression, we termed it epic1. The epic1 mutation was recessive and was backcrossed to the parental silex line twice prior to further analysis in all following experiments. Real-time PCR measurements of APUM9 and GFP transcript levels in epic1 seedlings confirmed that these targets were derepressed (Fig. 3B). Steady-state transcript levels of these two targets were also compared in dissected young leaves where the GFP fluorescence is present in epic1 and the corresponding tissue in silex (Supplemental Fig. S1B).

To identify the causal mutation in epic1, we performed whole-genome resequencing on a pool of DNA extracted from 10 GFP-positive F2 plants resulting from a backcross of epic1 into the silex line.

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This allowed us to map the mutation to HDA6 (Supplemental Fig. S1C). To confirm that a mutation in HDA6 could release GFP expression in young leaves, we introgressed the hda6-6 (auxin gene expression1-5 [axe1-5]) mutant allele of HDA6 (Murfett et al., 2001) into silex. This resulted in the same GFP expression pattern as we had previously observed in epic1 (Fig. 3C). Because we validated epic1 to be mutated in HDA6, we termed this allele hda6-8 (for a map depicting the HDA6 mutant alleles used in this study, see Supplemental Fig. S1D). Predictions of the secondary structure of HDA6 suggested that the hda6-8 mutation did not cause a change in the secondary structure of the protein (Rice et al., 2000). However, we found that the hda6-8 allele was of interest because it was mutated in a highly conserved amino acid of the previously described Essential SAS Family Acetyltransferase1-Reduced Potassium Dependency3 (Rpd3; E-R) motif. Notably, this motif is present in both histone acetylases and histone deacetylases (Adachi et al., 2002; Fig. 3D). Phenotypically, hda6-8 did not differ from hda6-6 or hda6-7 (RNA-mediated transcriptional silencing1 [rts1]) and also showed delayed flowering as was previously reported for hda6-6 (Yu et al., 2011).

To identify in which tissues HDA6 was expressed, we created an HDA6pro:GUS reporter line. GUS staining was observed in young developing leaves, corresponding well with the release of GFP expression we had observed in hda6-8 (Fig. 3E). We consistently observed the same GUS expression pattern in two independent transformation experiments. Because HDA6 is required for histone deacetylation, we tested if the chromatin at the promoters of the APUM9 endogene and the silex transgene were hyperacetylated in hda6-8 plants. We observed a strong increase in H4-tetra-acetylations at these loci (Fig. 3F).

To confirm that a mutation in HDA6 could release GFP expression in young leaves, we introgressed the hda6-6 (auxin gene expression1-5 [axe1-5]) mutant allele of HDA6 (Murfett et al., 2001) into silex. This resulted in the same GFP expression pattern as we had previously observed in epic1 (Fig. 3C). We then rescued epic1 by transformation with HDA6pro:HDA6 and thereby confirmed that the causal mutation was located in HDA6 (Fig. 3C). Because we validated epic1 to be mutated in HDA6, we termed this allele hda6-8 (for a map depicting the HDA6 mutant alleles used in this study, see Supplemental Fig. S1D). Predictions of the secondary structure of HDA6 suggested that the hda6-8 mutation did not cause a change in the secondary structure of the protein (Rice et al., 2000). However, we found that the hda6-8 allele was of interest because it was mutated in a highly conserved amino acid of the previously described Essential SAS Family Acetyltransferase1-Reduced Potassium Dependency3 (Rpd3; E-R) motif. Notably, this motif is present in both histone acetylases and histone deacetylases (Adachi et al., 2002; Fig. 3D). Phenotypically, hda6-8 did not differ from hda6-6 or hda6-7 (RNA-mediated transcriptional silencing1 [rts1]) and also showed delayed flowering as was previously reported for hda6-6 (Yu et al., 2011).

hda6-8 Affects Histone Acetylation But Not DNA Methylation

Because HDA6 has been reported to play a role in the maintenance of DNA methylation (Probst et al., 2004), we assessed DNA methylation levels at the transgene locus using bisulfite sequencing. However, we did not detect significant changes in DNA methylation in any of the sequence contexts in hda6-8 (Fig. 3G).

We then compared transcription of known target genes controlled by HDA6 in hda6-8 and the well-characterized hda6-7 (rts1-1) null mutant allele of
HDA6 (Aufsatz et al., 2002; Pontvianne et al., 2012). We found that silencing of targets controlled by RdDM such as solo LTR (Huettel et al., 2006) and AT4G04293 (AtIS112A; Numae et al., 2009; Yokthongwattana et al., 2010) was released in both hda6-7 and hda6-8; however, hda6-8 tended to have a weaker effect (Fig. 4A). A clear difference between hda6-8 and hda6-7 was observed when we assessed transcription of the HDA6 targets AT5G41660 and AT3G4470 (Fig. 4A; To et al., 2011). In contrast to hda6-7, hda6-8 showed no release of transcriptional suppression of these two targets.

To test if histone acetylation levels at these HDA6 targets were affected in hda6-8, we performed chromatin immunoprecipitation (ChIP; Jaskiewicz et al., 2011) of histone H4 Lys residues associated with tetra-acetylation (K5, K8, K12, and K16 on H4). Confirming previous reports (To et al., 2011), we observed a strong increase of H4 tetra-acetylation in the hda6-7 null mutant at all tested targets (Fig. 4B). Compared with the silex reporter line, hda6-8 also showed significantly increased H4 tetra-acetylation levels at solo LTR and AT4G04293. In the case of AT5G41660 and AT3G4470, we only observed a very modest increase of H4 tetra-acetylation in hda6-8.

It has been reported that release of silencing of certain targets in HDA6-defective plants was coinciding with loss of DNA methylation in the CG context (Earley et al., 2010; To et al., 2011). One such target that has previously been described is AT5G41660. Confirming these previous reports, we found loss of CG methylation at AT5G41660 in hda6-7, but we did not observe loss of DNA methylation in hda6-8 (Fig. 4C). Other tested HDA6 targets did not show loss of DNA methylation in either hda6-7 or hda6-8 (Fig. 4C). Furthermore, DNA methylation levels in the CHH sequence context were assessed at selected loci we found to be released in hda6-8 (solo LTR, AT4G04293, and MULE). None of these targets lost CHH methylation (Supplemental Fig. S1E).

**Transcriptional Release of Heterochromatic Regions Differ in hda6-6 and hda6-8**

To globally compare the genomic regions that were transcriptionally activated in hda6-6 and hda6-8, we generated a transcriptome of hda6-8 using tiling arrays and compared it with the previously reported hda6-6 transcriptome (To et al., 2011). It was shown previously that the hda6-6 and hda6-7 mutant alleles efficiently release transcription of TEs (Probst et al., 2004; To et al., 2011; Blevins et al., 2014). To obtain an overview of the chromosomal distribution of the
up-regulated transcripts in hda6-6 and hda6-8, they were plotted onto the five Arabidopsis chromosomes (Fig. 5A). Notably, on chromosomes 4 and 5, a high number of up-regulated transcripts can be observed in heterochromatic regions (represented as TE-rich regions here) in hda6-6, while it is not the case in hda6-8. In accordance with this observation, hda6-8 released transcription of very few TEs compared with hda6-6 (Fig. 5B).

To confirm the tiling array data obtained for hda6-8, seven targets were chosen for validation by real-time PCR (Supplemental Fig. S1F).
A Highly Conserved Amino Acid in HDA6 Prevents de Novo CG Methylation

We then wanted to assess if HDA6 could play a role in de novo DNA methylation by testing if DNA methylation that was lost in hda6-7 could be recovered by restoring HDA6 activity. hda6-7 plants were transformed with wild-type HDA6 or with the mutant hda6-8 allele (hda6-7,HDA6 and hda6-7,hda6-8, respectively). Transformation of hda6-7 with HDA6 did not restore CG methylation at AT5G41660, similarly to the Epistatic HDA6-RdDM Target7 (ETR7) gene that was previously described (Blevins et al., 2014). However, we unexpectedly observed acquisition of de novo DNA methylation at AT5G41660 in hda6-7 plants rescued with hda6-8 (Fig. 5C). The same results were obtained with two independent transformants for each line presented here. All plants were genotyped for the presence of the transgene and the hda6-7 mutation. This data suggests that the highly conserved E-R motif of HDA6 is involved in repressing de novo DNA methylation activity at certain targets. As an additional control, we included AT2G34655 (ETR15 in Blevins et al., 2014), which is up-regulated in hda6 mutants but where CG methylation is not lost (Fig. 5C).

DISCUSSION

Numerous factors repressing transcription of silenced chromatin in Arabidopsis have been described (for review, see Furner and Matzke, 2011; Eun et al., 2012; Matzke and Mosher, 2014). Intriguingly, many of these factors only affect certain subsets of silenced TEs and genes (Vaillant et al., 2006; Slotkin, 2010; Yokthongwattana et al., 2010), suggesting that each factor has a distinct target specificity. However, how these factors contribute to gene expression patterning has so far not been investigated in detail.
To explore this systematically, we used the epigenetically controlled and highly tissue-specific silex reporter line.

**Epigenetic Regulation of Gene Expression Patterning**

The silex reporter line showed GFP expression in the valve margin of siliques. This was unexpected, as transcription of the *APUM9* gene is under tight epigenetic repression implemented by MOM1 and NRPE1 (Yokthongwattana et al., 2010). This suggests that both MOM1 and NRPE1 may be inactive in this tissue. Interestingly, this observation fits well with the developmental relaxation of TE silencing that has been proposed (Martínez and Slotkin, 2012). In Arabidopsis, this has been extensively studied in reproductive and...
gametophytic tissues, but little is so far known concerning sporophytic tissues. The presented GFP reporter line now allows addressing this issue as well.

Introgression of the individual mom1 and nrpe1 mutations into the silex line showed no visible release of GFP expression, but we could detect some GFP mRNAs in mom1 plants. Interestingly, the mom1 nrpe1 double mutant released GFP expression at the veins, which was only visible on the abaxial side of the leaves. Taking into consideration previous reports on the global effect of mom1 in transgene activation in the 6b5 reporter line, this phenotype was unexpected (Vaucheret and Fagard, 2001). The vein-specific activation of the transgene in mom1 nrpe1 plants, and the stochastic activation upon 5-aza-2′-deoxycytidine treatment, indicated that several epigenetic regulators might influence the expression pattern of the silex transgene. The forward genetic screen confirmed these observations because we recovered epic1, a mutant releasing GFP expression in the shoot apex of seedlings. This mutant depicted a distinct GFP expression pattern compared with mom1 nrpe1 (Fig. 3A).

We mapped the epic1 mutation to HDA6 (and we thus renamed this allele hda6-8), a well-characterized histone deacetylase that has been recovered in several mutant screens designed to identify factors involved in transcriptional gene silencing (Murfett et al., 2001; Aufsatz et al., 2002; Probst et al., 2004). The GFP expression pattern corresponded well with the specific transcription of HDA6 in the shoot apex (Fig. 3E; Winter et al., 2007) and to the pattern of release of GUS silencing initially described in the HDA6 mutant axe1-3 (Murfett et al., 2001). This suggests that the main activity of HDA6 takes place in the shoot apex, where it repressed the GFP transgene of the silex line. This is well in line with a recent report that showed that expression of meristem-specific silencing factors is important to repress TEs (Yadav et al., 2009; Baubec et al., 2014). Notably, as leaves expand and mature, GFP fluorescence is lost. It is thus possible that other epigenetic regulators, such as the histone-deacetylase HDA19 that is more ubiquitously expressed in seedlings (Zhou et al., 2005), take over the role of silencing the transgene in these tissues. Another possibility that cannot be excluded is posttranscriptional repression of the transgene that might occur as the leaf ages.

A Highly Conserved Domain in HDA6 Is Required for Its Target Specificity

Because hda6-8 solely carried a single amino acid substitution, we wanted to compare its effect on the release of known HDA6 targets with an HDA6 null mutant, hda6-7 (rts1-1; Aufsatz et al., 2002). We found that hda6-8 strongly released RdDM-dependent HDA6 loci (solo LTR and AT4G04293) but not the RdDM-independent HDA6 targets (AT5G41660 and AT3G44070). The effect on transcription was also reflected by changes in epigenetic marks because the RdDM-independent targets did not gain histone H4 acetylation in hda6-8 (Fig. 4B). Interestingly, we also found that at AT5G41660, DNA methylation was lost in the CG context in hda6-7 but not in hda6-8 (Fig. 4C). This finding that CG methylation was not reduced in hda6-8 indicates that recruitment of MET1 by HDA6 was not affected in hda6-8 plants (To et al., 2011; Liu et al., 2012). Detailed analysis revealed that the mutation in hda6-8 was located in the highly conserved E-R motif that is present in histone acetylases and deacetylases (Adachi et al., 2002; Fig. 3D). This observation, combined with the finding that only a subset of HDA6 targets was affected in hda6-8, suggests that the mutated amino acid in the E-R motif may play an important role in targeting HDA6 to specific loci. More specifically, our data supports observations made in yeast (Saccharomyces cerevisiae) that the first amino acid of the E-R motif is not required for the enzymatic activity of histone deacetylases (Adachi et al., 2002), because hda6-8 retained its histone deacetylase activity at the RdDM-independent targets. The E-R motif may therefore be involved in recognition of specific DNA sequence contexts and/or chromatin modifications, thereby defining the target specificity of HDA6. Alternatively, the mutation in the E-R motif may influence protein-protein interactions that are required for proper targeting of HDA6 to specific regions or to specific histone residues and modifications in the genome. In yeast, RPD3, the ortholog of Arabidopsis HDA6, has been shown to be part of a large protein complex that includes transcriptional repressors such as Switch Independent3 and Unscheduled Meiotic gene Expression1 (Grzenda et al., 2009). And there is evidence that plant HDAs also operate in multiprotein complexes containing histone-binding proteins, corepressors, and associated proteins (Gu et al., 2011; Perrella et al., 2013; López-González et al., 2014). It is thus possible that the hda6-8 mutation in the E-R motif affects the interaction of HDA6 with the complex.

HDA6 Has Distinct Activities in Euchromatin and Heterochromatin

The strong mutant alleles of HDA6 have been shown to release silencing of heterochromatic transcripts (Probst et al., 2004; To et al., 2011; Liu et al., 2012; Blevins et al., 2014). A striking difference we observed in the hda6-8 transcriptome was that it had only very little effect on TEs and heterochromatic transcripts (Fig. 5, A and B). This suggests that the amino acid change in the highly conserved E-R domain only plays a role in silencing euchromatic genes and that it has little to no role in heterochromatin silencing, probably because this mutation does not affect the interaction of HDA6 with MET1 (To et al., 2011; Liu et al., 2012). This observation is intriguing because it suggests that, depending on the chromosomal location, HDA6 may interact with different
proteins to silence genes or, conversely, that interacting proteins define HDA6 target specificity.

It has been documented that epigenetic changes caused by defects in met1-3 and decrease in DNA methylation can cumulate over generations of inbreeding (Kakutani et al., 1996; Mathieu et al., 2007). We analyzed hda6-8 directly after the second backcross, thus excluding potential inbreeding effects as they might have accumulated in hda6-6 and hda6-7. These mutants have been discovered more than a decade ago and likely have been inbred over multiple generations since their initial discovery (Murfett et al., 2001; Aufsatz et al., 2002). We thus cannot exclude that some of the differences between the mutant alleles that we observe may also be caused by such inbreeding effects.

Interestingly, hda6-8 efficiently released the GFP transgene in silico, even thought it had acquired heterochromatic properties. This observation might be due to the insertion of the transgene in euchromatin, and it may thus be under a less repressive state than bona fide heterochromatic regions.

The HDA6 E-R Motif Represses de Novo DNA Methylation

HDA6 is implicated in silencing diverse endogenous targets, either via RdDM (Aufsatz et al., 2002) and in interaction with MET1 (To et al., 2011; Liu et al., 2012). An interesting group of targets has been described previously (Blevins et al., 2014). The described group E targets lose DNA methylation in hda6-6; however, this methylation is not recovered in hda6-6 plants rescued with HDA6, showing that HDA6 is required to maintain DNA methylation. We found the same to be true at the AT5G16660 locus. However, when we complemented hda6-7 with the hda6-8 allele of HDA6, we reproducibly found that CG methylation could be restored. This observation is in line with experiments that were carried out in animal cells, where inhibition of histone deacetylases leads to an increase in CG methylation at certain targets (Jia et al., 2015). It suggests that even though DNA methylation is lost in hda6-7 plants and not restored in the complementation assay, an epigenetic memory, either in the form of histone modifications or small RNAs, is still present, allowing DNA methylation to come back under certain circumstances. Notably, the mutation in hda6-8 is located within the C-terminal region of HDA6 that has been shown to interact with MET1 (Liu et al., 2012). It is therefore possible that the E-R motif is involved in regulating MET1 activity. It is currently unclear how hda6-8 may target de novo methylation, and it will be interesting to investigate this activity in more detail in the near future.

CONCLUSION

The processes of cell specialization and differentiation depend on a complex network of highly controlled expression of gene sets in space and time. Chromatin modifications and epigenetic regulation of gene expression play important roles in the regulation of cell fate. However, our understanding about the mechanisms involved in these processes is currently limited. The results presented here open the door to tissue- and cell-type-specific analysis of epigenetic regulation of gene expression. It will be very interesting to further investigate these aspects, because it may answer one of the most fundamental questions in molecular biology: how do genes know when and where to be expressed?

MATERIALS AND METHODS

Plant Material, Mutagenesis, and Mapping

All plants used in this study come from the Columbia accession. The silex reporter line was obtained from a collection created by The Institute for Genomic Research (J. Craig Venter Institute, line AGRAc-60-1; Xia et al., 2010). Mutants used in this work were mom1-2 (Habu et al., 2006), nrp1-1-2 (formerly nrp1b-2; Ponstier et al., 2005), met1-3 (Saze et al., 2003), axl1-5 (Murfett et al., 2001), and rts1 (Aufsatz et al., 2002). EMS mutagenesis was carried out as described previously (Weigel and Glazebrook, 2002), and plants were grown in Sanyo MLR-350 chambers at 24°C with 16 h of light.

Causal EMS mutations were mapped by whole-genome sequencing combined with classical mapping by crossing the mutants with the Wassilewskija accession. Reads were mapped against the reference genome and single nucleotide polymorphisms called in Geneious (Biomatters). Using R, single nucleotide polymorphisms were filtered for EMS mutations (G→A/T) and zygosity called based on the variant frequency provided by Geneious (≥80% homozygous mutation, ≥45%, and ≤55% heterozygous mutation). Plots were then created by calculating the ratio of the number of homozygous and heterozygous mutations and in a 500-kb window (Supplemental Fig. SIC).

Transgenic Lines

Promoter and rescue constructs were all cloned into the pCAMBIA1304 plasmid. The HDA6 promoter (including 1,057 bp upstream of the transcription start site) and the full-length HDA6 gene including the promoter (4,044 bp) were PCR amplified from genomic DNA of wild-type (rescue and promoter constructs) or hda6-8 plants (rescue constructs) using the primers 702/972 and 702/701 (for primer sequences, see Supplemental Table S1), respectively. The obtained PCR products were cloned into pGEM-T Easy (Promega), sequenced, and then cloned into pCAMBIA1304 using the restriction enzymes indicated in the primer list in Supplemental Table S1.

DNA Methylation Analysis

For methylation-sensitive PCR, genomic DNA from fresh leaf tissue was isolated using the DNeasy Plant Mini Kit (Qiagen). Fifty nanograms of DNA was then digested with Ddel and Hps1 restriction endonucleases (New England Biolabs) overnight. It was then PCR amplified using specific primers for the promoter regions of target genes. Sequences of primers used in this study are listed in Supplemental Table S1.

Bisulfite analyses were carried out as previously described (Yokthongwattana et al., 2010) with the following modification: to be able to differentiate between the endogenous and transgenic ROMANIAT5 LTR, genomic DNA was digested with SspI (New England Biolabs) and religated prior to bisulfite treatment. This resulted in an inverse PCR-like approach allowing specific amplification of the LTR present in the transgene.

Real-Time PCR and Transcriptome Analysis

Total RNA from 100 mg of fresh leaf tissue of Arabidopsis (Arabidopsis thaliana) was isolated with innuPREP Plant RNA Kit (Analytik Jena). Five hundred nanograms of RNA was used for complementary DNA
synthesis (Script cDNA Synthesis Kit, Bio-Rad). Expression of target genes was measured by quantitative PCR in a Light-Cycler 480 (Roche) using SYBR Green I Master Mix. Primer sequences are presented in Supplemental Table S1. Steady-state mRNA levels were calculated with the Light-Cycler 480 software (Roche) using ACTIN2 (ACT2) for normalization.

Transcription profiling on the silex reporter line and epic1 was carried out on RNA extracted from leaves of 17-d-old plants as described previously on one biological replicate (Yokthongwattana et al., 2010). The raw data has been submitted to the Gene Expression Omnibus repository as study number GSE65640.

ChIP

ChIP experiments were performed on chromatin extracted from leaves of 3-week-old plants as described by Jaskiewicz et al. (2011) using anti-H4 tetra-acetylation antibody (06-866) from Millipore (To et al., 2011). Relative histone acetylation was calculated using the comparative threshold cycle method (Schmittgen and Livak, 2008) by normalizing against input and ACT2.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Characterization of epic1 and the silex reporter line.
Supplemental Table S1. Primers used in this study.
Supplemental Table S2. Up-regulated genes identified in hda6-8.

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LITERATURE CITED


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