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Abstract

Polycomb group (PcG) proteins are essential to maintain gene expression patterns during development. Transcriptional repression by PcG proteins involves trimethylation of H3K27 (H3K27me3) by Polycomb Repressive Complex 2 (PRC2) in animals and plants. PRC1 binds to H3K27me3 and is required for transcriptional repression in animals, but in plants PRC1-like activities have remained elusive. One candidate protein that could be involved in PRC1-like functions in plants is LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), because LHP1 associates with genes marked by H3K27me3 in vivo and has a chromodomain that binds H3K27me3 in vitro. Here, we show that disruption of the chromodomain of Arabidopsis thaliana LHP1 abolishes H3K27me3 recognition, releases gene silencing and causes similar phenotypic alterations as transcriptional lhp1 null mutants. Therefore, binding to H3K27me3 is essential for LHP1 protein function.
The LHP1 chromodomain is essential for H3K27me3 binding and function during Arabidopsis development

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Abbreviations: CD, chromodomain; H3K27me3, trimethylation of histone H3 lysine 27; LHP1, LIKE HETEROCHROMATIN PROTEIN 1; PcG, Polycomb group
Polycomb group (PcG) proteins are essential to maintain gene expression patterns during development. Transcriptional repression by PcG proteins involves trimethylation of H3K27 (H3K27me3) by Polycomb Repressive Complex 2 (PRC2) in animals and plants. PRC1 binds to H3K27me3 and is required for transcriptional repression in animals, but in plants PRC1-like activities have remained elusive. One candidate protein that could be involved in PRC1-like functions in plants is LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), because LHP1 has a chromodomain that binds H3K27me3 in vitro. Here, we show that disruption of the chromodomain of LHP1 abolishes H3K27me3 recognition, releases gene silencing and causes similar phenotypic alterations as transcriptional lhp1 null mutants. Therefore, binding to H3K27me3 is essential for LHP1 protein function.
INTRODUCTION

Polycomb group (PcG) proteins maintain gene expression patterns during development in animals and plants by establishing a cellular memory system for transcriptional repression (Köhler and Villar, 2008). Although many functional details of PcG proteins remain unknown, current models suggest that repression involves trimethylation of histone H3 lysine 27 (H3K27me3) by Polycomb repressive complex 2 (PRC2). In insects and mammals, H3K27me3 assists in the recruitment of PRC1 (Schwartz and Pirrotta, 2007). Binding of PRC1 to H3K27me3 is mediated by the chromodomain (CD) of the PRC1 subunit Polycomb (Pc) (Fischle et al., 2003). Although the PcG system is present in plants and PRC2 homologs have similar functions, no clear plant PRC1 homologs have been identified (Köhler and Villar, 2008). Proteins that may have PRC1-like functions in plants include EMBRYONIC FLOWER 1, VERNALIZATION 1, LIKE HETEROCHROMATIN PROTEIN 1 (LHP1) and RAWUL-proteins (Aubert et al., 2001; Calonje et al., 2008; Mylne et al., 2006; Sanchez-Pulido et al., 2008).

Arabidopsis LHP1, also known as TERMINAL FLOWER 2 (TFL2), was originally identified as a homolog of metazoan HETEROCHROMATIN PROTEIN1 (HP1) (Gaudin et al., 2001; Kotake et al., 2003; Larsson et al., 1998; Takada and Goto, 2003). Similar to HP1, LHP1 contains a CD and a chromo shadow domain (Gaudin et al., 2001; Kotake et al., 2003). Unlike HP1, however, LHP1 is usually localized in euchromatin and is needed for maintenance of gene silencing in euchromatin but not in heterochromatin (Libault et al., 2005; Nakahigashi et al., 2005). Finally, LHP1 can bind to H3K27me3 \textit{in vitro} and associates with genes marked by H3K27me3 \textit{in vivo} (Turck et al., 2007; Zhang et al., 2007).
Together, the model has emerged that LHP1 binds to PcG target loci that have been trimethylated at H3K27 by PRC2 to establish persistent transcriptional repression. We tested this hypothesis using a LHP1 mutant with a defective CD. In agreement with predictions from structural homology-based modeling, the Trp129Cys mutation in the CD domain strongly reduced LHP1 binding to H3K27me3 \textit{in vitro}. Furthermore, recruitment to target genes and intra-nuclear localization of mutated LHP1 was greatly impaired \textit{in vivo}. Because the phenotype of this new \textit{lhp1} allele is very similar to an \textit{lhpl} null allele, we conclude that CD-mediated binding of LHP1 to H3K27me3 is essential for LHP1 function. These results support the model that LHP1 has a PRC1-like function in plants.

**MATERIALS AND METHODS**

**Plant material**

All mutants used (Supplemental Tab. S1) are in the Columbia (Col) wild-type accession of \textit{Arabidopsis thaliana}. A new \textit{lhp1} allele, \textit{lhp1-6}, was identified in the SALK T-DNA insertion mutant collection (SALK_011762). Details on growth conditions can be found in the supplement.

**In vitro transcription/translation and pull down assays**

\textit{LHP1} and \textit{lhp1-7} cDNAs were cloned into vector pRSET-A (Invitrogen) for \textit{in vitro} transcription/translation reactions (TNT® T7 Quick Coupled Transcription/Translation System, Promega, Madison, WI) supplemented with L-[\textsuperscript{35}S]methionine. Equal amounts of wild-type and mutant protein were incubated with H3K27 or H3K27me3 peptides (LATKAARKSAPATGGC) coupled to SulfoLink Coupling Gel (Pierce Perbio, Lausanne, Switzerland). Samples were resolved by
SDS-PAGE, exposed to a storage phosphor screen (Amersham Biosciences, Otelfingen, Switzerland) and visualized using a Molecular Imager FX Pro Plus System (BioRad, Reinach, Switzerland).

RNA isolation, RT-PCR and Real Time PCR

RNA isolation and RT-PCR was performed as previously described (Hennig et al., 2003). For qPCR analysis, the Universal ProbeLibrary system (Roche Diagnostics, Rotkreuz, Switzerland) was used on a 7500 Fast Real-Time PCR instrument (Applied Biosystems, Lincoln, CA). Further details are given in supplemental methods online and in Supplemental Tables S2 and S3.

Immuno-localisation, Chromatin Immunoprecipitation, sequence alignments, phylogenetic analysis and homology modelling

Experimental and computational details can be found in the supplemental methods online.

RESULTS

A LHP1 mutant protein with a defective chromodomain

The new lhp1-7 allele was discovered in a suppressor screen of the late flowering msil-tap1 transgenic line (Bouveret et al., 2006). Because of a newly created splice site, the processed lhp1-7 transcript has an insertion of nine additional nucleotides. This results in three additional amino acids (Cys-Glu-Arg) in the CD adjacent the conserved tryptophan 129, which is changed into a cysteine (see Supplemental Methods online and Supplemental Fig. S1). The wild-type splice
variant was not detected in \textit{lhp1-7} (Supplemental Fig. S2), suggesting that \textit{lhp1-7} produces no or very little wild-type protein.

Homology-based modelling revealed that similar to HP1 and Pc the CD of LHP1 has the potential to form a binding cage containing three aromatic residues (Fig. 1A). Because one of the three aromatic residues, tryptophan 129, was changed to a cysteine in the CD of \textit{lhp1-7}, it is likely that this protein cannot form the typical cage and will be called LHP1-CD* (Fig. 1B). To distinguish between the \textit{lhp1-6} null allele and the \textit{lhp1-7} mutant, we will refer to these alleles as \textit{lhp1-6 (null)} and \textit{lhp1-7 (CD*)}.

We tested whether binding to H3K27me3 was affected by the \textit{lhp1-7 (CD*)} mutation. Similar to previously reported results, wild-type LHP1 bound strongly to the H3K27me3 peptide \textit{in vitro}, but LHP1-CD* binding to H3K27me3 was significantly reduced and similar to unmethylated H3K27 (Fig. 1C). The reduced binding affinity to H3K27me3 \textit{in vitro} suggests that LHP1-CD* could have compromised activity \textit{in vivo}.

\textbf{The LHP1 chromodomain is required for correct sub-nuclear localization and binding to target genes}

To analyze the \textit{in vivo} activity of LHP1-CD*, we introduced LHP1-GFP and LHP1-CD*-GFP fusion proteins into \textit{lhp1-7(CD*)}. We found several lines in which the LHP1-GFP fusion protein could complement \textit{lhp1-7(CD*)}, demonstrating that LHP1-GFP is fully functional (Fig. 2A, B). In contrast, the LHP1-CD*-GFP fusion protein was expressed (Fig. 2G, H) but unable to complement the mutant, suggesting that LHP1-CD*-GFP cannot substitute for wild-type LHP1.

Microscopic inspection of the LHP1-GFP and LHP1-CD*-GFP lines revealed that both wild-type and the mutant fusion proteins were targeted to the nucleus. The
LHP1-GFP fusion protein showed a speckled pattern throughout the nucleus in most lines (Fig. 2C-F), similar to published data (Libault et al., 2005). In contrast, the mutant LHP1-CD* was more uniformly distributed in the nucleus, often with additional strong accumulation in the nucleolus (Fig. 2G-K). Accumulation of mutant LHP1 versions in the nucleolus has been reported before (Libault et al., 2005; Zemach et al., 2006), but the relevance of this abnormal targeting is unknown.

Altered in vitro binding and sub-nuclear distribution of LHP1-CD* could also affect binding to individual target loci. We used the GFP fusion lines to test binding of LHP1 to AGAMOUS (AG) and SEPALATA 3 (SEP3), which are well-established PcG and LHP1 targets (Nakahigashi et al., 2005; Zhang et al., 2007). After chromatin immunoprecipitation we found that LHP1-GFP, but not LHP1-CD*-GFP, bound efficiently to both loci (Fig. 2L). Together, these results show that LHP1-CD* lost specificity for H3K27me3 in vitro and that LHP1-CD*-GFP cannot bind to at least some LHP1 targets in vivo, which may explain its altered sub-nuclear localization.

**Development is altered in lhp1-7(CD*) mutants**

We compared the lhp1-7(CD*) mutant to wild-type and lhp1-6(null) mutant plants to establish which aspects of LHP1 function depend on CD binding to H3K27me3. Analysis of flowering time revealed that both lhp1-7(CD*) and lhp1-6(null) plants flowered at similar times but much earlier than wild-type under long and short day conditions (Fig. 3A and Supplemental Fig. S3A-C). Early flowering was characterized by shortened juvenile and adult phases concomitant with strong FT upregulation (Fig. 3B and Supplemental Fig. S3D). Epidermal cells of lhp1 mutant rosette leaves were much smaller, although they maintained the characteristic jigsaw like shape (Fig. 3C and Supplemental Fig. S4A-C). Leaf cell number and expansion
were reduced in both lhp1 alleles, causing a strongly decreased rosette leaf size (Supplemental Fig. S4D, E).

Arabidopsis LHP1 was initially identified genetically for its terminal flower phenotype (Larsson et al., 1998). Both lhp1-6(null) and lhp1-7(CD*) have the terminal flower phenotype, but lhp1-7(CD*) formed the terminal flower later than lhp1-6(null) (Fig. 3D). Consistently, primary stem growth ceased much earlier in lhp1 mutants than in wild-type plants, but later in lhp1-7(CD*) than in lhp1-6(null). In both lhp1 alleles, not only duration of primary stem growth but also growth rates were reduced (Supplemental Fig. S4F-H). Together, lhp1-7(CD*) is phenotypically similar to lhp1-6(null) during early plant development, but has a slightly milder phenotype late in development.

**Silencing of PcG target genes is lost in lhp1-7(CD*) mutants**

Flowers produced late during lhp1-6 and lhp1-7(CD*) development often have supernumerary, missing or deformed organs (Fig. 4A-C), which may be caused by deregulation of floral homeotic genes. AG and SEP3 were ectopically expressed in lhp1-6(null) and lhp1-7(CD*) rosette leaves (Fig. 4D). Similarly, MEDEA and AGL19, two PcG targets (Katz et al., 2004; Schönrock et al., 2006), were de-repressed in both lhp1 alleles (Fig. 4D and data not shown). The observation that there was no reactivation of transposons or pseudogenes (At4g03760, MU1, TA2) or of targets of the RNA-dependent DNA-methylation pathway (IG/LINE, IG2, IG5, RPL18) (Fig. 4E and data not shown) confirmed that loss of LHP1 does not affect silencing in heterochromatin (Libault et al., 2005; Nakahigashi et al., 2005).

Together, our results show that similar to lhp1-6(null) major developmental regulatory genes (e.g., FT, AG and SEP3) are not repressed in lhp1-7(CD*) at times
when they should be silent. Thus, we conclude that specific binding of LHP1 to H3K27me3 is essential to maintain repression of PcG target genes.

**DISCUSSION**

In animals, PRC2 complexes set H3K27me3 marks, which assist to recruit PRC1 to mediate stable silencing (Schwartz and Pirrotta, 2007). Homologues of PRC2 but not of PRC1 protein complex subunits have been identified in plants. Plant LHP1 proteins are similar to metazoan HP1 and could have PRC1 functions. Phylogenetic analysis suggests that these two protein subfamilies have strongly diverged (Supplemental Fig. S5). We found LHP1 homologues in genomes of higher plants and mosses but not in the genomes of chlorophyte algae *Volvox carteri* and *Chlamydomonas reinhardtii*, suggesting that the presence of LHP1 is linked to multicellular development in the plant kingdom. Because chromatin immunoprecipitation has shown that LHP1 binding overlaps with H3K27me3 and LHP1 can bind H3K27me3 *in vitro*, it was suggested that the CD-protein LHP1 is a PRC1 equivalent of plants (Turck et al., 2007; Zhang et al., 2007).

Three aromatic residues form the binding cavity for methylated lysines of H3 in the CD of animal HP1 and Pc (Fischle et al., 2003; Jacobs and Khorasanizadeh, 2002; Min et al., 2003; Nielsen et al., 2002). Based on protein homology modeling, the CD of plant LHP1 forms a similar binding pocket. Therefore we suggest that the novel *lhp1* allele *lhp1-7(CD*) has a defective binding pocket for the quaternary ammonium group because the preference of LHP1 for H3K27me3 over H3K27 was lost for LHP1-CD*. Energy calculations using CHARMM (Brooks et al., 1983) and the CHARMm (Momany and Rone, 1992) force field are in qualitative agreement with the relative affinities measured by the pull-down assay. A quantitative agreement is not expected because of approximations inherent to the force field and the
qualitative nature of the pull-down assays. A LHP1-CD*-GFP fusion did not efficiently bind to target gene chromatin and had lost its correct sub-nuclear distribution, suggesting that CD-mediated binding to H3K27me3 is essential for LHP1 targeting in vivo. In contrast, the CD might not be sufficient and necessary for targeting of animal HP1 in vivo (Cowell et al., 2002; Dialynas et al., 2006; Meehan et al., 2003).

Mutations in Arabidopsis LHP1 strongly affect development (Gaudin et al., 2001; Larsson et al., 1998). The phenotype of the lhp1-7(CD*) allele was very similar to that of an lhp1 null allele, suggesting that LHP1 function requires an intact CD. Because only LHP1-GFP but not LHP1-CD*-GFP could rescue lhp1 mutants, LHP1-CD* has no or strongly reduced biological activity. Residual binding of LHP1-CD* to H3K27me3 could explain the phenotypic differences between lhp-7(CD*) and lhp1-6(null) plants.

Loss of LHP1 or PRC2 share many similar developmental and molecular effects. Our experimental results, supported by homology modeling and previous reports, have revealed that LHP1 contributes to PRC1-like functions in plants and that CD-mediated binding to H3K27me3 is required for this activity.

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FIGURE LEGENDS

Figure 1. *lhp1-7(CD*) encodes a LHP1 mutant protein with a defective chromodomain. (A) Structural model of the LHP1 CD based on homology modelling using the coordinates of the Drosophila Pc CD complexed with an H3K27me3 peptide (Fischle et al., 2003). (B) Structural model of the LHP1-CD* CD, which is encoded by *lhp1-6(CD*)*. The position of the trimethylated lysine side chain in (A) and (B) was derived from the template crystal structure. (C) Peptide-binding pull-down assay for wild-type LHP1 and LHP1-CD* (left) and quantification (right).

Figure 2. Altered sub-nuclear localization of LHP1-CD*--GFP. (A) Wild-type (Col, left) and *lhp1-7(CD*)* (right) after five weeks of growth under long day photoperiod. (B) LHP1 35S::LHP1-GFP (left) and *lhp1-7(CD*)* 35S::LHP1-GFP (right) plants. (C-K) 35S::LHP1-GFP (C-F) and 35S::lhp1-7(CD*)-GFP plants (G-K) were used to analyze protein localization in leaf nuclei. Protein localization detected by confocal laser scanning microscopy of GFP-fluorescence (C, G) or by immunolocalization (D, H). (E, I) DAPI-staining of the nuclei in D and H; merged images of D and E (F) and of H and I (K). (L) ChIP assays for binding of LHP1-GFP and LHP1-CD*-GFP to the AG and SEP3 loci. Top: Genomic structure of AG and SEP3 loci. Black lines represent introns, narrow bars 3’ and 5’ UTRs and wide bars represent coding exons. Black lines represent regions probed by qPCR. Values are recovery as percent of input; IgG served as negative control.

Figure 3. Altered development in *lhp1* mutants. (A) Rosette leaves produced until bolting in long days (LD). (B) FT expression at zeitgeber (ZG) = 4h in 12 days old
seedlings in LD. (C) Cell size in the adaxial epidermis of the first and second rosette leaves. (D) The total number of reproductive organs (siliques, flowers and flower buds) on the primary shoot of five weeks old plants from LD. For all panels: White, grey, and dark-grey bars represent wild-type, lhp1-6(null) and lhp1-7(CD*), respectively. Values are mean ± S.E. (n ≥ 7 (A), n=4 (B), n ≥ 244 (C), n ≥ 8 (D)).

Figure 4. Loss of silencing at PcG targets and maintenance of silencing at heterochromatic loci in lhp1 mutants. (A-C) Flowers of wild-type Col (A), lhp1-6(null) (B) and lhp1-7(CD*) (C) produced late during development. (D) Expression of PcG targets in seedlings at ZG=5h after 16 days in LD. (E) Expression of heterochromatic loci in rosette leaves at ZG=5h after 25 days in LD. RNA from ddm1-2 was used as positive control.
Fig. 2
Fig. 3
Fig. 4