Different Domains Control the Localization and Mobility of LIKE HETEROCHROMATIN PROTEIN1 in *Arabidopsis* Nuclei [W]

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Plants possess a single gene for the structurally related HETEROCHROMATIN PROTEIN1 (HP1), termed LIKE-HP1 (LHP1). We investigated the subnuclear localization, binding properties, and dynamics of LHP1 proteins in *Arabidopsis thaliana* cells. Transient expression assays showed that tomato (*Solanum lycopersicum*) LHP1 fused to green fluorescent protein (GFP; Sl LHP1-GFP) and *Arabidopsis* LHP1 (At LHP1-GFP) localized to heterochromatic chromocenters and showed punctuated distribution within the nucleus; tomato but not *Arabidopsis* LHP1 was also localized within the nucleolus. Mutations of aromatic cage residues that recognize methyl K9 of histone H3 abolished their punctuated distribution and localization to chromocenters. Sl LHP1-GFP plants displayed cell type–dependent subnuclear localization. The diverse localization pattern of tomato LHP1 did not require the chromo shadow domain (CSD), whereas the chromodomain alone was insufficient for localization to chromocenters; a nucleolar localization signal was identified within the hinge region. Fluorescence recovery after photobleaching showed that Sl LHP1 is a highly mobile protein whose localization and retention are controlled by distinct domains; retention at the nucleolus and chromocenters is conferred by the CSD. Our results imply that LHP1 recruitment to chromatin is mediated, at least in part, through interaction with methyl K9 and that LHP1 controls different nuclear processes via transient binding to its nuclear sites.

INTRODUCTION

The basic structural unit of chromatin, the nucleosome, is made up of DNA wrapped around histone octamers containing two copies of each of the four core histone proteins, H2A, H2B, H3, and H4 (reviewed in Kornberg and Lorch, 1999). This basic structure is further organized into higher order chromatin structure by the aid of multiple proteins or protein complexes (reviewed in Grewal and Moazed, 2003). Changes in chromatin structure are influenced by DNA modifications (e.g., cytosine methylation) as well as by posttranslational modifications of histone N-terminal tails. Within histone tails, there are specific amino acids (Arg, Lys, Ser) that undergo a number of posttranslational modifications, including acetylation, methylation, and phosphorylation (Wolffe, 1992), thus generating a code for the recruitment of proteins or protein complexes that affect chromatin structure and gene expression (reviewed by Jenuwein and Allis, 2001; Zhang and Reinberg, 2001; Grewal and Moazed, 2003). The effect of chromatin structure on gene expression is well exemplified by the phenomenon of

position effect variegation in *Drosophila*. Position effect variegation occurs when euchromatic genes are brought into close proximity with heterochromatic regions, resulting in a variegated expression pattern (Dorer and Henikoff, 1994; Wallrath and Elgin, 1995). One of the best-studied modifiers of position effect variegation in *Drosophila* is the *Su(var)2-5* gene encoding HETEROCHROMATIN PROTEIN1 (HP1) (Elgin, 1996), a chromodomain (CD) protein implicated in heterochromatin formation and gene silencing (Cavalli and Paro, 1998). HP1-like proteins are composed of two related functional domains: an N-terminal CD and a C-terminal chromo shadow domain (CSD) (Aasland and Stewart, 1995). It has been suggested that HP1 proteins function as chromatin organizers, participating in the assembly of multiprotein complexes that promote the silencing of euchromatic genes and the expression of heterochromatic genes interspersed in heterochromatic domains (Eissenberg and Elgin, 2000). The discovery of histone methyltransferase activity associated with human SUV39H1, fission yeast Clr4 (Rea et al., 2000; Nakayama et al., 2001), and *Arabidopsis thaliana* KYP/SUVH4 (Jackson et al., 2002) sheds light on the molecular events leading to HP1-induced heterochromatinization. These enzymes specifically methylate histone H3 at Lys-9 residues, generating a code for the recruitment of HP1 proteins (Bannister et al., 2001; Lachner et al., 2001; Jackson et al., 2002). However, recruitment of HP1 to chromatin may not be exclusively dependent on H3-K9 methylation (Li et al., 2002; Meehan et al., 2003), and other HP1-interacting proteins, such as the retinoblastoma protein, may also be involved (Williams and Grafi, 2000; Nielsen et al., 2001; Li et al., 2002).

In humans, the three members of the HP1 family (HP1α, HP1β, and HP1γ) display different patterns of subnuclear localization:

HP1α and HP1β tend to be associated with condensed chromatin, whereas HP1γ is associated with euchromatic regions (Minc et al., 1999). In *Drosophila*, there are five members of the HP1 family showing different expression patterns. Whereas HP1A, HP1B, and HP1C are expressed in adult *Drosophila* tissues, HP1D/Rhino and HP1E are expressed mainly in ovaries and testes, respectively (Vermaak et al., 2005). These HP1 proteins also display diverse nuclear localization: HP1A is associated with heterochromatin, HP1B is found both on heterochromatin and euchromatin, HP1C is restricted to euchromatin (Smothers and Henikoff, 2001), and HP1D/Rhino is localized within the heterochromatic domain in tissue culture cells, although its localization does not overlap that of H3-K9 methylation (Vermaak et al., 2005). Recent studies using fluorescence recovery after photobleaching (FRAP) revealed that despite their role in maintaining stable heterochromatin configuration, HP1 proteins are not static but rather highly mobile in living cells (Cheutin et al., 2003, 2004; Festenstein et al., 2003).

Plants possess a single gene for HP1, LIKE HETEROCHROMATIN PROTEIN1 (LHP1) (Gaudin et al., 2001), having a greater molecular mass (~45 kD) than its animal counterparts. In tobacco (*Nicotiana tabacum*) cells, *Arabidopsis* LHP1 fused to green fluorescent protein (GFP) was restricted to the nucleus, showing punctuated distribution (Gaudin et al., 2001; Libault et al., 2005), or colocalized with heterochromatic sites enriched with histone H3 methylated at K9 (Yu et al., 2004); truncated *Arabidopsis* LHP1 containing the hinge region was localized to the nucleolus in tobacco BY2 cells (Libault et al., 2005). The *Arabidopsis* LHP1/TFL2 protein localized to multiple subnuclear foci, excluding chromocenters (Kotake et al., 2003; Nakahigashi et al., 2005) in transgenic *Arabidopsis* plants and was capable, at least in part, of complementing the *Arabidopsis lhp1* mutant phenotype (Gaudin et al., 2001) as well as yeast *swi6⁻* mutants (Kotake et al., 2003). The finding that *Arabidopsis* LHP1 binds histone H3 methylated at Lys-9 (Jackson et al., 2002), a histone modification associated with chromocenters in *Arabidopsis* cells (Soppe et al., 2002; Jasencakova et al., 2003; Jackson et al., 2004), suggests a possible role for *Arabidopsis* LHP1 in controlling heterochromatin structure. To gain better understanding of how LHP1 controls chromatin structure, we studied the subnuclear localization and binding properties of *Arabidopsis* (At LHP1) and tomato (*Solanum lycopersicum*) (Sl LHP1) LHP1 fused to GFP in *Arabidopsis* nuclei. *Arabidopsis* cells were selected for this study because of their high degree of resolution in analyzing subnuclear localization and the ease with which one can distinguish chromocenters in their nuclei. Our results demonstrate multiple subnuclear localization sites for At LHP1 and Sl LHP1 in *Arabidopsis*, which only partly coincide with H3-K9 methylation. Sl LHP1 was found to be a highly mobile protein whose localization and retention at specific nuclear sites are controlled by distinct protein domains.

RESULTS

Sl LHP1-GFP Complemented the *lhp1* Mutant

The Sl LHP1 gene encodes 399 amino acids with a calculated molecular mass of ~44 kD. The protein contains a CD (amino

acids 93 to 142) and a CSD (amino acids 339 to 396), which are characteristic features of the HP1 protein family. Sl LHP1 shares high amino acid sequence similarity with *Arabidopsis* LHP1/TFL2, particularly within the CD and CSD regions (Kotake et al., 2003).

We examined the ability of Sl LHP1 to complement the *lhp1* mutant (Gaudin et al., 2001). This mutant is a T-DNA insertion in the promoter region of the *LHP1* gene, which leads to a significant reduction in *LHP1* transcription and consequently to altered leaf morphology (small, curly leaves) and early flowering; *lhp1/tfl2* mutants also display terminal flowers (Larsson et al., 1998) and alteration in glucosinolate levels, defense-related secondary metabolites (Kim et al., 2004). Sl LHP1 fused to GFP under the control of the 35S promoter was introduced into *lhp1*, and several plants showing phenotypic complementation of *lhp1* coupled with expression of Sl LHP1-GFP were isolated. Analysis of T2 progeny revealed cosegregation of the wild-type phenotype with the GFP signal; GFP-positive, phenotypically wild-type progeny were cosegregated in a Mendelian manner (3:1, wild type:*lhp1*). In these plants, the Sl LHP1-GFP transgene underwent strong silencing in subsequent generations, reverting to the phenotype conferred by *lhp1*. Together, our results support functional similarities between the *Arabidopsis* and tomato LHP1 proteins.

Sl LHP1 Binds in Vitro Histone H3 Methylated at Lys-9

H3-K9 methylation is a key epigenetic mark controlling chromatin structure, at least in part through the recruitment of HP1 proteins. Glutathione *S*-transferase (GST) pull-down assays have previously shown the capability of *Arabidopsis* LHP1 to bind K9-methylated histone H3 (Jackson et al., 2002). We analyzed the ability of Sl LHP1 to bind methylated H3-K9 isolated from tobacco leaves. Primarily, Sl LHP1 was predicted to bind methylated H3-K9 through the CD, because the aromatic cage residues that recognize methyl K9 (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002) are conserved in Sl LHP1 (see Figure 3B below). GST pull-down assays were performed with glutathione–Sepharose containing GST alone, GST-Sl LHP1, GST-CD, and GST-CSD. As shown in Figure 1A, the tobacco leaf acid-soluble fraction contained three polypeptides that reacted with anti-H3 (lane 1). GST-Sl LHP1 (lane 3) bound two of the three histone H3 polypeptides, an interaction that required the CD (lane 5) but not the CSD (lane 4). In addition, histone H3 methylated in vitro by SUV39H1-H320R (Rea et al., 2000) was bound by GST-Sl LHP1 but not by GST alone (Figure 1B).

Sl LHP1-GFP and At LHP1-GFP Are Localized at H3-K9 Methylated Chromocenters

Based on the ability of Sl LHP1 to bind in vitro to H3 methylated at K9, we next investigated whether its subnuclear localization coincides with that of methylated H3-K9. For subnuclear localization study of Sl LHP1, we selected *Arabidopsis* cells because of their high degree of resolution in analyzing subnuclear localization and the ease with which one can distinguish chromocenters in *Arabidopsis* nuclei; there are 10 chromocenters, most of which are positioned at the nuclear periphery (Fransz et al., 2003). Transient expression into *Arabidopsis* protoplasts
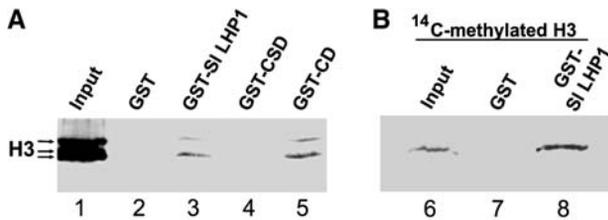
Figure 1. Sl LHP1 Binds in Vitro K9-Methylated Histone H3 in a CD-Dependent Manner.

(A) In vitro binding of Sl LHP1 to histone H3 prepared from tobacco leaves. GST pull-down assays were performed with the indicated GST fusion proteins. GST-CD contains the CD and GST-CSD contains the CSD. Bound proteins were resolved by 18% SDS-PAGE and immunoblotted with anti-H3 (lanes 1 to 5). Input indicates 20% of the input histones.

(B) Sl LHP1 protein binds histone H3 that was methylated at Lys-9 by SUV39H1 HMTase. The acid-soluble fraction from tobacco leaves was subjected to methylation by GST-SUV39H1 (H320R). Methylated histones were collected and used in GST pull-down assays with GST alone or GST-Sl LHP1 (lanes 5 to 8). Input indicates 35% of the input methylated H3.

showed a diversified distribution of Sl LHP1-GFP in *Arabidopsis* nuclei: it was localized to several (7 to 10) large spots at the nuclear periphery, which are likely to be chromocenters enriched with dimethylated H3-K9. However, Sl LHP1-GFP was also dispersed in a speckle-like pattern within the nucleus and was found within the nucleolus (Figure 2A), a nuclear compartment engaged in the transcription of rRNA genes. Hence, the subnuclear localization of Sl LHP1 in *Arabidopsis* is not limited to sites known to be methylated at H3-K9. Notably, the distribution of Sl LHP1 at chromocenters was uneven, as it tends to occupy the pericentromeric region, leaving the center as a black hole (Figure 2A, arrows in panels 3 and 4). Likewise, Sl LHP1 was localized at specific nucleolar compartments, excluding sphere-shaped sites, which are reminiscent of fibrillar centers (Thiry et al., 2000). For comparison, we transiently expressed At LHP1 in *Arabidopsis* cells and found that its subnuclear localization generally resembled that of Sl LHP1 except for its absence from the nucleolus. Cells expressing At LHP1-GFP displayed three types of localization: punctuated, chromocentric, or both (Figure 2B). Localization at chromocenters was further demonstrated using a confocal microscope equipped with a laser diode system capable of 4′,6-diamidino-2-phenylindole (DAPI) detection. As shown in Figure 3A, both Sl LHP1-GFP and At LHP1-GFP were colocalized to the intensely DAPI-stained chromocenters.

Mutations of aromatic cage residues (W114G/W117G in Sl LHP1; W132G in At LHP1) (Figure 3B) that recognize methyl K9 (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002) abolished the punctuated distribution within the nucleus as well as the localization to chromocenters of both At LHP1 and Sl LHP1 (Figure 3C). In these assays, the mutated form of Sl LHP1 was localized almost exclusively to the nucleolus, whereas that of At LHP1 was evenly dispersed within the nucleus (Figure 3C), implying that localization at these subnuclear domains is mediated through interaction with methylated K9 of histone H3.

Sl LHP1 Displays Cell Type–Dependent Subnuclear Localization in Transgenic Plants and Is Associated with Centromeric Repeats

Sl LHP1-GFP in the *lhp1* mutant background was localized exclusively to the nucleus, showing different subnuclear distribution patterns in different cell types (Figure 4A). In guard cells, Sl LHP1-GFP was localized to fewer large spots at the nuclear periphery (chromocenters) and dispersed within the nucleus in a speckle-like manner; no localization was detected within the nucleolus. In trichomes, having large endoreduplicated nuclei (Melaragno et al., 1993), Sl LHP1-GFP was localized within the nucleolus and dispersed unevenly within the nucleus; localization to chromocenters could not be verified microscopically. In mesophyll cells, Sl LHP1-GFP was dispersed mainly in a speckle-like manner throughout the nucleus. These localization patterns indicate the dynamic nature of Sl LHP1 subnuclear distribution and its cell type dependence.

Localization of Sl LHP1 at chromocenters was verified by immunolabeling/fluorescence in situ hybridization (FISH) assay.

Figure 2. Subnuclear Localization of LHP1-GFP Proteins in *Arabidopsis* Cells.

(A) Sequential confocal optical sections (slices of 0.4 μm; panels 1 to 8) showing the subnuclear localization of Sl LHP1 fused to GFP. Section 9 is an extended view of slices 1 to 8. Note the uneven distribution of the GFP signal within the nucleolus and the exclusion of Sl LHP1 from the center of the chromocenters (arrows in panels 3 and 4). ChC, chromocenters; nuc, nucleolus. Bars = 5 μm.

(B) Transient expression of At LHP1-GFP in *Arabidopsis* protoplasts showing different types of subnuclear localization. Note that At LHP1-GFP is absent from the nucleolus. Bars = 5 μm.
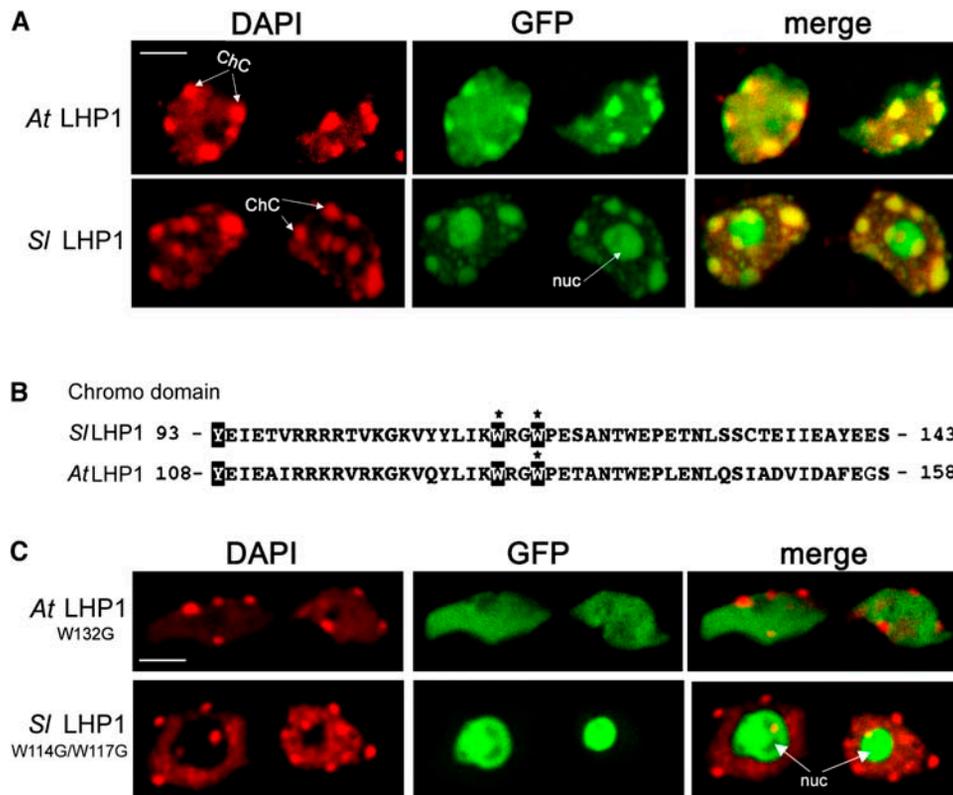
Figure 3. Localization of Sl LHP1-GFP and At LHP1-GFP at Chromocenters and Their Punctuated Distribution Require the Aromatic Cage Residues That Recognize Methyl K9.

(A) *Arabidopsis* protoplasts transiently expressing the indicated LHP1-GFP proteins were stained with DAPI and inspected with a confocal microscope equipped with a laser diode system for DAPI detection. ChC, chromocenters; nuc, nucleolus. Bar = 2 μm.

(B) Amino acid sequences of the CDs of Sl LHP1 and At LHP1. The aromatic cage residues that recognize methyl K9 are highlighted. Asterisks indicate Trp (W) residues converted to Gly (G).

(C) *Arabidopsis* protoplasts transiently expressing the mutated forms of LHP1 proteins, At LHP1(W132G)-GFP and Sl LHP1(W114G/W117G)-GFP, were stained with DAPI and inspected with a confocal microscope. Note that the mutated form of At LHP1 is evenly dispersed within the nucleus, whereas that of Sl LHP1 is localized to the nucleolus. DAPI is pseudocolored red. Bar = 2 μm.

Fixed nuclei were first immunolabeled with anti-GFP, followed by FISH with tetramethylrhodamine-5-dUTP–labeled centromeric repeats (CEN180). A small fraction of nuclei showed that Sl LHP1-GFP was closely associated with CEN180 (Figure 4B), confirming localization at chromocenters.

Localization of Sl LHP1-GFP Is Not Affected in Mutants Displaying Decreased DNA and Histone Methylation

To examine the importance of DNA and histone H3 methylation for LHP1 subnuclear localization, we transiently expressed Sl LHP1-GFP and At LHP1-GFP in several mutants: *ddm1-2*, a mutant in the SWI2/SNF2 chromatin-remodeling gene (Kakutani et al., 1996; Jeddeloh et al., 1999); *met1-1*, a mutant in the DNA Methyltransferase1 (*MET1*) gene (Kankel et al., 2003); *kyp-2*, a mutant in the Kryptonite H3-K9 methyltransferase (*KYP*) gene (Jackson et al., 2002); and *suvh2*, a mutant in a histone methyltransferase (*SUVH2*) gene whose mutation caused a strong reduction in histone methylation (Naumann et al., 2005). Al-

though all of these mutants have reduced H3-K9 methylation (Figure 5A) (Soppe et al., 2002; Jasencakova et al., 2003; Jackson et al., 2004), *ddm1-2* and *met1-1* also display significant reductions in DNA methylation (Kakutani et al., 1996; Kankel et al., 2003). In all mutants examined, the subnuclear localization of Sl LHP1-GFP (Figure 5B) and At LHP1-GFP (data not shown) was similar to that of wild-type cells. Similarly, mutations in the SET-domain genes *MEDEA* and *CURLY LEAF* (Goodrich et al., 1997) did not affect the localization of Sl LHP1-GFP to the various nuclear domains (data not shown).

Nuclear and Subnuclear Localization Properties of Sl LHP1

To determine the Sl LHP1 protein region(s) responsible for its nuclear and subnuclear localization, we divided the protein into several portions, each fused with GFP at its C terminus (see scheme in Figure 6A). Using PSORT II software, three putative nuclear localization signals (NLS1 to NLS3) were identified. NLS1 is located at the CD (RRRR, 99 to 102), and the other two are
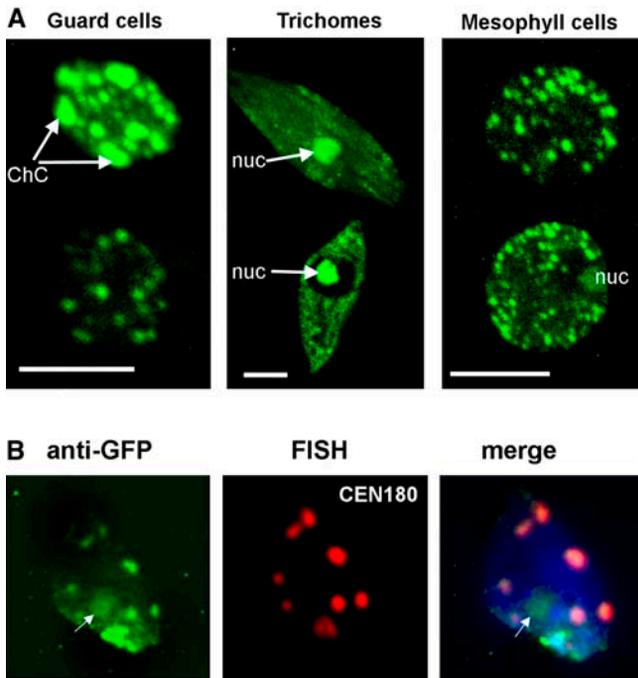
Figure 4. Subnuclear Localization of Sl LHP1-GFP in Transgenic *Arabidopsis lhp1* Plants.

(A) Leaf tissue inspected with a confocal microscope showing distinctive subnuclear localization of Sl LHP1-GFP in the indicated cell types. ChC, chromocenter; nuc, nucleolus. Bars = 5 μm.
(B) Immunolabeling/FISH analysis of nuclei from transgenic *lhp1* expressing Sl LHP1-GFP showing that this protein is associated with the centromeric 180-bp repeats (CEN180). Arrows indicate the nucleolus.

located at the hinge region: a bipartite NLS2 (RKRKFGATQTHP-MIKQQRR, 150 to 170) and NLS3 (KKRK, 281 to 284). Transient expression into *Arabidopsis* protoplasts showed (Figure 6B) that the localization of Sl LHP1(1–306), containing all NLSs but lacking the CSD, was indistinguishable from that of the intact protein (1 to 399). The C-terminal part (202 to 399), containing NLS3 and the CSD, was evenly localized within the nucleus, excluding the nucleolus. Further analysis showed that the N-terminal region containing NLS1 (1 to 103) was dispersed both in the cytoplasm and within the nucleus in a pattern similar to that displayed by GFP alone (data not shown), suggesting that the putative NLS1 is nonfunctional. This was further supported by the behavior of the protein region containing the NLS1-CD (90 to 145), in which the GFP signal showed a distribution pattern similar to that of the N terminus (1 to 103). These results suggest that neither the CD alone nor the CSD has site-specific nuclear targeting properties. The protein portion containing the CD and NLS2 (90 to 171) was localized to the nucleus, showing a preference for the nucleolus and, to some extent, to chromocenters, but it failed to show the speckle-like distribution within the nucleus. Finally, we showed that the region encompassing residues 141 to 399 retained the nucleolus-targeting localization property and was also localized at fewer, tiny, yet unidentified nuclear bodies. Together, our results suggest that the protein

portion between amino acids 141 and 171, corresponding to the bipartite NLS2, possesses the nucleolar localization signal (NoLS). To verify this prediction, we next fused this portion (141 to 171) with GFP, and after transformation, we analyzed its subnuclear distribution. The results clearly showed (Figure 6B) that this protein portion targeted the GFP signal almost exclusively to the nucleolus, indicating its function as a NoLS.

The CSD Stabilizes the Association of Sl LHP1 at the Nucleolus and Chromocenters

We next sought to identify the protein region(s) responsible for Sl LHP1 retention within the nucleolus and chromocenters using FRAP (Misteli, 2001). In this assay, physical interaction of Sl LHP1 with nuclear proteins would lead to stable association and, consequently, to slower FRAP (Misteli, 2001). The finding that Sl LHP1(141–399) containing the CSD, but not other Sl LHP1 truncated proteins, retained the specific intranucleolar distribution pattern as the full-length protein (Figure 7A) suggests that the CSD stabilizes the association of the protein within the nucleolus. *Arabidopsis* protoplasts transiently expressing full-length Sl LHP1 or its derivatives, namely Sl LHP1(141–171), Sl LHP1(141–399), and Sl LHP1(1–306), were bleached by high-powered laser pulses in a rectangular area of the nucleolus. Fluorescence recovery in the bleached area was monitored during a 40- to 50-s postbleaching period by sequential imaging scans. Sl LHP1(141–171) behaved similarly to GFP alone (Kruhlak et al., 2000); that is, it infiltrated the bleached area so fast that recovery was nearly complete during the time required to capture a single frame (∼170 ms) (Figures 7B and 7C). Generally, FRAP analysis showed that, like animal HP1 proteins (Cheutin et al., 2003; Festenstein et al., 2003), Sl LHP1 is a highly mobile protein that binds transiently to its chromosomal sites. The Sl LHP1 derivatives showed different rates of fluorescence recovery in the nucleolus; Sl LHP1(1–306) lacking the CSD displayed a significantly faster mobility (50% recovery time of 1.07 s) than either the full-length Sl LHP1 or Sl LHP1(141–399) (50% recovery times of 10.62 and 9.75 s, respectively) (Figures 7B and 7C). This finding suggests that the CSD functions in the positioning and stabilization of Sl LHP1 within the nucleolus. Likewise, we found that the CSD contributes to the stabilization of the Sl LHP1 association with chromocenters. FRAP at chromocenters was faster when the CSD was omitted [Sl LHP1(1–306)] compared with full-length Sl LHP1, displaying 50% recovery times of 1.43 and 8.53 s, respectively (Figures 8A and 8B; see Supplemental Videos 1 and 2 online). Together, our results suggest that the stable association of Sl LHP1 with chromocenters and within the nucleolus is largely dependent on the CSD.

DISCUSSION

The multiple subnuclear localization sites of Sl LHP1 in *Arabidopsis* cells suggest, on the one hand, different mechanisms through which LHP1 is recruited to chromatin, and on the other hand, diverse nuclear processes in which LHP1 proteins might be involved. In contrast with transient assays, in stably transformed plants, Sl LHP1-GFP displayed cell type–dependent subnuclear localization. The cell type dependence of nuclear organization has been reported in plants (Fang et al., 2004; Fang
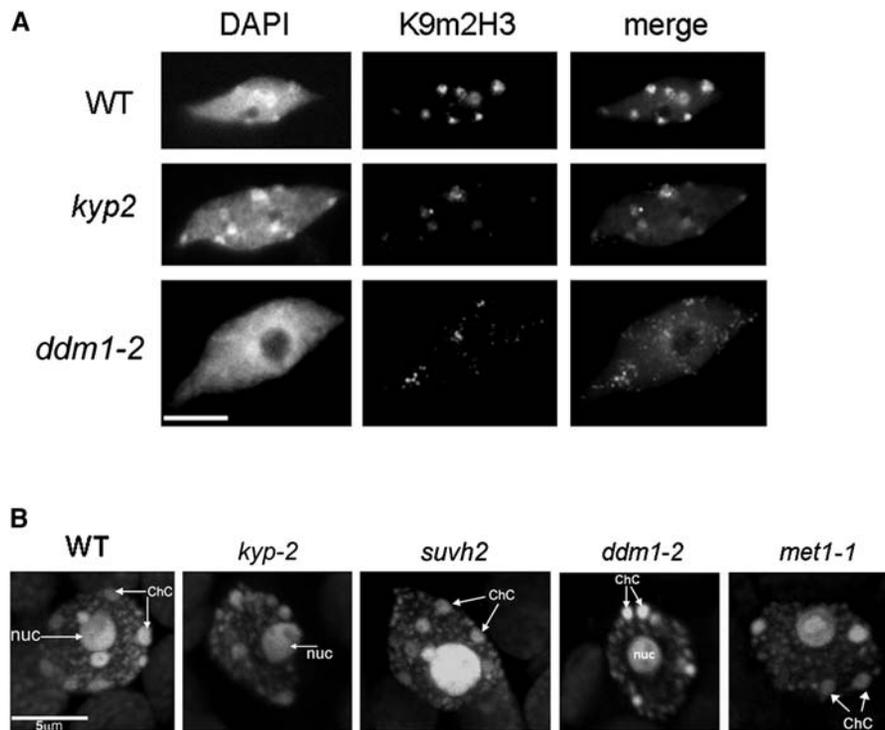
Figure 5. Localization of Sl LHP1 at Chromocenters Is Not Affected in Mutants Displaying Reduced H3-K9 Methylation.

(A) Immunofluorescence assays showing reduced H3-K9 methylation in *kyp-2* and *ddm1-2* mutants compared with wild-type plants. Bar = 5 μm.
(B) Localization of Sl LHP1-GFP in *kyp-2*, *suvh2*, *ddm1-2*, and *met1-1* is indistinguishable from that of wild-type plants. ChC, chromocenters; nuc, nucleolus.

and Spector, 2005) and animals (Parada et al., 2004). Notably, in most cells of transgenic plants, Sl LHP1-GFP was excluded from chromocenters, showing punctuated distribution consistent with previous reports (Gaudin et al., 2001; Libault et al., 2005; Nakahigashi et al., 2005). Thus, the differential subnuclear localization in transgenic plants may reflect cell type–dependent requirements for the regulation of diverse nuclear processes mediated by LHP1 proteins.

We showed that both Sl LHP1 and At LHP1 are localized to heterochromatic chromocenters enriched in methylated H3-K9 as well as dispersed, in a speckle-like pattern, within the nucleus. Sl LHP1, but not At LHP1, was also localized within the nucleolus, a site engaged in the transcription and assembly of rRNAs (Olson et al., 2002). We determined the region that targets Sl LHP1 to the nucleolus (amino acids 141 to 171) and found it to overlap with the bipartite NLS2 motif (150-RRRKRKFGATQTHPMIKQQRR-170). Interestingly, a bipartite NLS is also found in the hinge region of At LHP1 (166-RKRKRKYAGPHSQMKKKQRL-185), yet it appeared nonfunctional in nucleolar localization. A recent report, however, showed that a truncated At LHP1 containing the hinge region is targeted almost exclusively to the nucleolus in tobacco BY-2 cells (Libault et al., 2005), suggesting that other regions, most likely the N-terminal region containing the CD, render At LHP1 incapable of entering the nucleolus. Localization to chromocenters was primarily deduced from the association of LHP1-GFP with a few large spots positioned at the nuclear periphery, which are indicative of chromocenters (Fransz et al.,

2003). Within chromocenters, LHP1 appears to be associated with pericentromeric regions, as inferred from its unique localization pattern within these domains, where it was excluded from the center (Figure 2A). This chromocentric localization was further confirmed by the association of Sl LHP1-GFP with the intensely DAPI-stained chromocenters and by FISH/immunolabeling assays showing that Sl LHP1-GFP is closely associated with the centromeric 180-bp repeats. Localization at heterochromatic chromocenters is consistent with the finding that in tobacco cells, At LHP1 colocalizes with heterochromatic sites enriched with histone H3 methylated at K9 (Yu et al., 2004). Also, the *Drosophila* HP1 protein was reported to be localized to chromocenters in *Arabidopsis* nuclei (Naumann et al., 2005).

The findings that Sl LHP1 and At LHP1 bind in vitro to histone H3 methylated at K9 (Figure 1) (Jackson et al., 2002) and that both are localized in vivo at H3-K9–enriched chromocenters (Figure 2) suggest that this histone modification is involved in the recruitment of LHP1 to chromocenters. This is further supported by the finding that mutation of aromatic cage residues that recognize methyl K9 (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002) abolished localization to chromocenters as well as the punctuated distribution within the nucleus (Figure 3C). Indeed, in fission yeast, recruitment of Swi6, a homolog of *Drosophila* HP1, to heterochromatic regions requires H3-K9 methylation (Nakayama et al., 2001). However, the finding that Sl LHP1 as well as At LHP1 (data not shown) subnuclear localization was unaffected in *Arabidopsis* mutants displaying
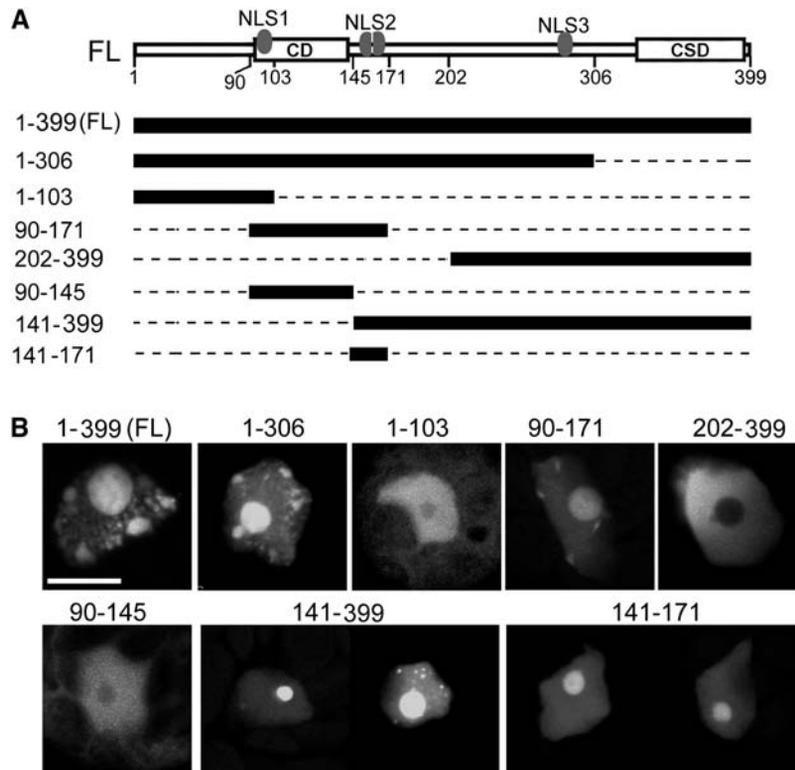
Figure 6. Subnuclear Localization of Various Sl LHP1 Domains.

(A) Scheme of Sl LHP1 and its derivatives fused to GFP. FL, full-length; NLS, nuclear localization signal.

(B) The indicated constructs were transiently expressed in *Arabidopsis* protoplasts and inspected with a confocal microscope. Note that neither the CSD (202 to 399) nor the CD (90 to 145) has site-specific subnuclear targeting properties and that the nucleolar targeting signal is located between amino acids 141 and 171. Bar = 5 μm.

a significant reduction in H3-K9 methylation raises some doubt about the role of H3-K9 methylation in LHP1 recruitment to chromocenters. Alternatively, one may postulate that the residual H3-K9 methylation present at chromocenters of these mutants could suffice to recruit LHP1. Nevertheless, H3-K9 methylation appears to be dispensable for the localization of LHP1 at other subnuclear domains. This is consistent with the finding that recruitment of animal HP1 to chromatin is not always dependent on histone methylation (Li et al., 2002), whereas the binding of *Xenopus* HP1 to native chromatin in vitro requires the hinge region, which is incapable of binding histone H3 (Meehan et al., 2003). It is likely that additional factors capable of interacting with HP1, such as the retinoblastoma protein (Williams and Grafi, 2000; Nielsen et al., 2001), as well as many other nuclear proteins (Li et al., 2002) function in recruiting the protein to specific chromatin subdomains. The role played by LHP1 at heterochromatic chromocenters is not clear. Genetic analysis showed that LHP1 plays no role in the DNA methylation of various studied loci, including CEN180, *Ta3* retrotransposon, and the *PAI* gene, or in transposon silencing (Lindroth et al., 2001; Malagnac et al., 2002). Also, in *lhp1/tfl2*, no effect could be detected on chromocenter organization, as revealed by DAPI staining and FISH with CEN180 (Libault et al., 2005), or on the expression of genes within heterochromatin (Nakahigashi et al.,

2005). Thus, it is possible that LHP1 functions in heterochromatin redundantly with other proteins and/or that LHP1 plays an as yet unknown role at these heterochromatic sites.

Although the interaction of HP1 with K9-methylated H3 was shown to be mediated in vitro by the CD (Bannister et al., 2001; Lachner et al., 2001; Fass et al., 2002), this domain may not be sufficient to direct the protein to methylated H3-K9–enriched chromocenters. The *Drosophila* HP1a CD as well as the CSD, each fused to GFP, showed uniform distribution throughout the nucleus, whereas the hinge region was targeted mainly to heterochromatin-rich chromocenters (Smothers and Henikoff, 2001). This pattern is similar to that of Sl LHP1 CD and CSD, in which each fused to GFP displayed even distribution within the nucleus, excluding chromocenters and the nucleolus. Our analysis shows that although the hinge region is important for nuclear and nucleolar localization of Sl LHP1, it is not sufficient for localization at numerous sites within the nucleus or for localization at chromocenters. It appears that both the hinge region and the CD cooperate structurally to generate binding surfaces that enable the association of LHP1 proteins with various subnuclear sites.

Similar to that for Sl LHP1, punctuated distribution was reported for At LHP1/TFL2-GFP transiently expressed in tobacco cells (Gaudin et al., 2001) and in transgenic *Arabidopsis* plants (Kotake et al., 2003). Recent evidence suggests that HP1
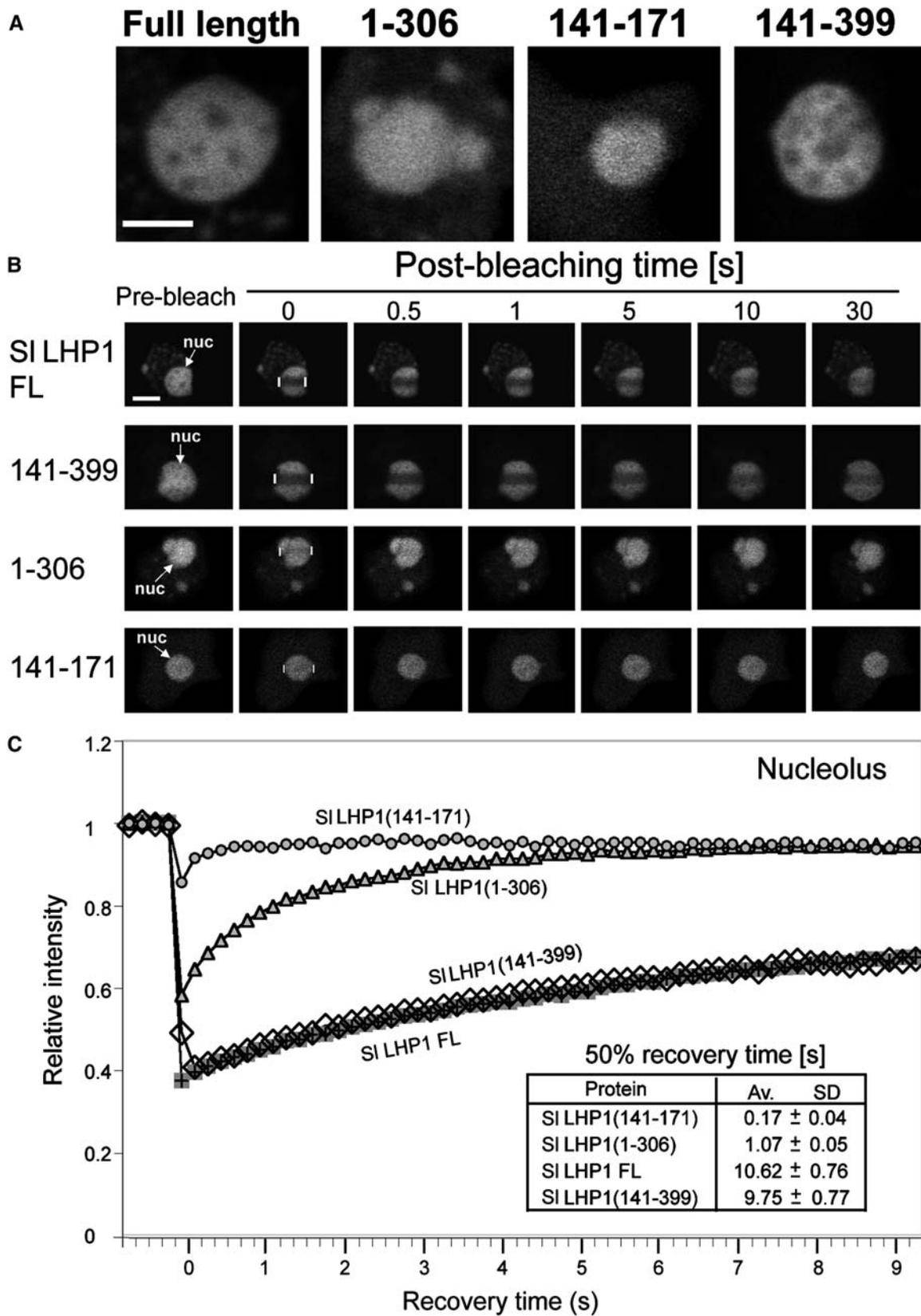
Figure 7. FRAP Analysis Showing That Nucleolar Retention Is Conferred by the CSD.
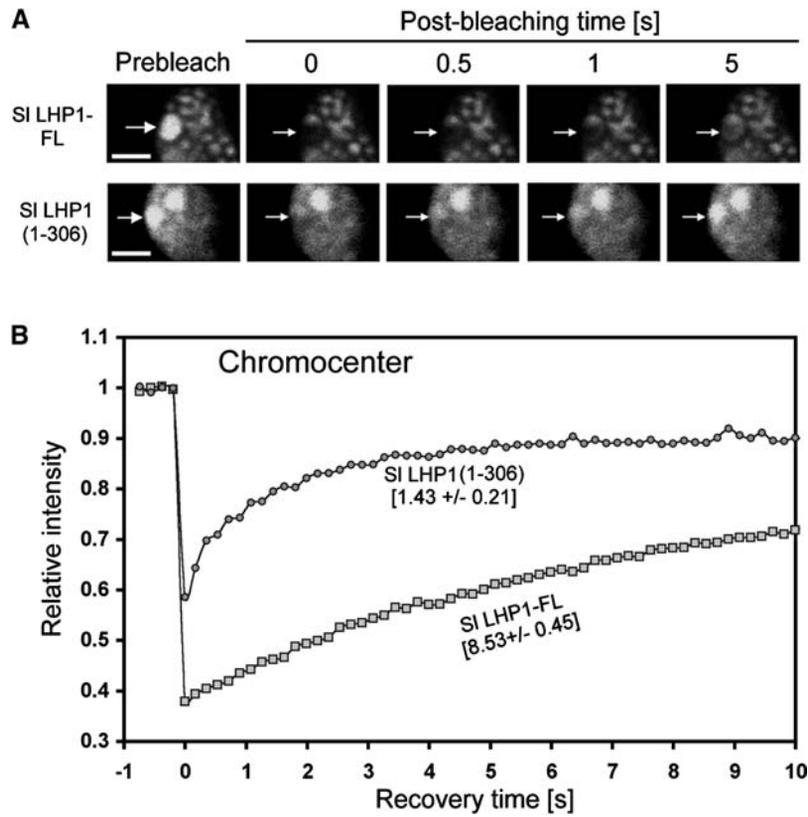
Figure 8. The CSD Stabilizes the Association of SI LHP1 with Chromocenters.

(A) Selected images were taken at various times after a chromocenter (arrows) was photobleached, showing the fluorescence recovery of the full-length (FL) and truncated (1 to 306) SI LHP1-GFP proteins. Bars = 2 μm.

(B) Kinetics of fluorescence recovery of the SI LHP1-GFP proteins. The average 50% recovery time for each protein and the SD are given in brackets.

localization within euchromatin may be related, at least in part, to its involvement in the positive regulation of gene expression (Piacentini et al., 2003; Cryderman et al., 2005). This suggestion gains further support by the localization of SI LHP1-GFP within the nucleolus, a site known to be engaged in the transcription of rRNA. Likewise, nucleolar localization has been reported for the mouse HP1-like protein M31 (Wreggett et al., 1994) and for human HP1 proteins (Andersen et al., 2002), yet the significance of this localization has not been elucidated. Notably, SI LHP1 occupies distinct domains within the nucleolus and appears to be excluded from fibrillar center–like domains, which are sites of rRNA transcription (Thiry et al., 2000), suggesting that it may play a role in the assembly of rRNAs. Alternatively, the nucleolus may function to store excess SI LHP1-GFP generated in these experiments and/or inactive dephosphorylated forms of SI LHP1 (Zhao et al., 2001). Considering that the nucleolus has

also been implicated in diverse cellular processes, including cell cycle regulation, aging, and telomerase activity (Pederson, 1998; Visintin and Amon, 2000; Olson et al., 2002), it is possible that SI LHP1 is targeted to the nucleolus to perform an as yet unknown function.

FRAP analysis showed that, similar to animal HP1 proteins (Cheutin et al., 2003, 2004; Festenstein et al., 2003), SI LHP1-GFP is a highly mobile protein that transiently interacts with its nuclear sites. The half-fluorescence recovery time, representing the transport of unbleached SI LHP1-GFP molecules into the photobleached area, is in the range of 10 s. Thus, plant HP1 proteins appear to perform their diverse nuclear functions in a dynamic manner, displaying a stop-and-go mode of mobility (Misteli, 2001). The FRAP assay showed that SI LHP1 subnuclear targeting and retention are controlled by different domains. Although targeting to the nucleolus is mediated by the NLS2

Figure 7. (continued).

(A) Distribution of SI LHP1 truncated proteins within the nucleolus. Note that the unique, uneven distribution pattern within the nucleolus is retained by SI LHP1(141–399), whereas other SI LHP1 truncated proteins show diffused patterns. Bar = 2 μm.

(B) Selected images were taken at various times after a band across the nucleolus was photobleached (bar at top), showing fluorescence recovery within the nucleolus of the indicated SI LHP1-GFP proteins. Bar = 2 μm.

(C) Kinetics of fluorescence recovery of the various SI LHP1-GFP proteins. The average (Av.) 50% recovery time for each protein and the SD are given.

region (141 to 171), retention is conferred by the CSD. Likewise, the CSD, but not the CD, was found to stabilize the association of Sl LHP1 with chromocenters. Consistent with our results, FRAP assays have demonstrated that deletion of the CSD weakens the association of HP1 with heterochromatin in both mammalian and yeast cells (Cheutin et al., 2003, 2004). Hence, although the CD and NoLS contributes to LHP1 subnuclear localization, the CSD stabilizes the LHP1 association with chromatin. The CSD is required for the dimerization of HP1 proteins, and this dimer configuration is thought to be the functional form of HP1 that mediates heterochromatin compaction (Ye et al., 1997; Wang et al., 2000). It has also been demonstrated that dimerization of the At LHP1 protein requires the CSD (Gaudin et al., 2001). Indeed, structural analysis revealed that dimerization of HP1 proteins occurs through the CSD, resulting in the formation of a putative protein–protein interaction pit (Brasher et al., 2000; Cowieson et al., 2000; Thiru et al., 2004). This pit may target HP1 proteins to particular chromosomal sites through interaction with multiple proteins (reviewed in Li et al., 2002; Singh and Georgatos, 2002), many of which contain a conserved pentapeptide with the core sequence PXVXL (Brasher et al., 2000; Smothers and Henikoff, 2000; Thiru et al., 2004). Whether Sl LHP1 retention at various nuclear sites is regulated by PXVXL-containing proteins or by a different mechanism(s) needs to be elucidated.

METHODS

Construction of Sl LHP1 and At LHP1 Plasmids

Tomato (*Solanum lycopersicum*) cDNA encoding LHP1 protein (clone identifier cLEC33C22 in pBluescript KS+; pBs-C22), designated Sl LHP1, was obtained from the BAC/EST Resource Center at Clemson University and fully sequenced (GenBank accession number AF428244). To generate pGEX-Sl LHP1, the Sl LHP1 DNA fragment was amplified by PCR using as a template the pBs-C22 and primers LeHP1-S (5′-CACAGGATCCA-GAATGAAAGAAGGGAAAAGG-3′) and LeHP1-AS (5′-GAGAGAATTC-TCGAGGCAGGCAACTCATTCAGTCGGATGG-3′). The PCR product was digested with *Bam*HI and *Eco*RI and subcloned into the same sites of pGEX-2T. The DNA fragment corresponding to the CD of Sl LHP1 was amplified by PCR using pGEX-Sl LHP1 as a template (5′-GAGACCC-GGGAGATCTATGAAAGAAGGGAAAAGGAAAAGCTCAAGG-3′) flanked with *Sma*I and *Bgl*II as a sense primer and with LHP1CD-AS flanked with the *Eco*RI site (5′-GAGAGAATTCTCATATCCTAACCTTCACAGCTGG-CCC-3′). Similarly, the DNA fragment corresponding to the CSD was amplified by PCR using pGEX-Sl LHP1 as a template, the above-mentioned LeHP1-AS as an antisense primer, and the sense primer Sl LHP1CSD-S flanked with the *Bam*HI site (5′-GAGAGGATCCGAG-GAGCCTACACCTTCACCC-3′). The CD PCR product was digested with *Bgl*II and *Eco*RI, the CSD product was digested with *Bam*HI and *Eco*RI, and each fragment was subcloned into *Bam*HI and *Eco*RI sites of pGEX-2T. To generate Sl LHP1 full length and its derivatives fused to GFP, we amplified the various fragments by PCR followed by digestion with *Bam*HI and *Sma*I and subcloning into *Bgl*II and *Sma*I sites of the plasmid pUC19-35S-GFP (a gift from A. Levitan and A. Danon) downstream from the 35S promoter and in frame with GFP. In all PCR procedures, we used pGEX-Sl LHP1 as a template and the following primers: for Sl LHP1(1–399), 1-S (5′-GAGAGGATCCATGAAAGAAGG-GAAAAGG-3′) and 399-AS (5′-GAGACCCGGGTTCAGTCGGATGGTATC-GCATA-3′); for Sl LHP1(1–306), 1-S and 306-AS (5′-CTCTCCCGG-GAGCATCTTGGGTATCATCCTTC-3′); for Sl LHP1(202–399), 202-S (5′-GAGAGGATCCATGGCTACAGATCTTGTGGACAG-3′) and 399-AS;

for Sl LHP1(90–171), 90-S (5′-GAGAGGATCCATGGAAGGTTTTTACGA-GATTG-3′) and 171-AS (5′-CTCTCCCGGGGAAACGCCGCTGCTGCTTT-ATC-3′); for Sl LHP1(1–103), 1-S and 103-AS (5′-CTCTCCCGGGAGA-TCTAGTTCTCCTTCTCCTAAC-3′); for Sl LHP1(90–145), 90-S and 145-AS (5′-TCTCCCCGGGCTTAAGCTCTCTTCATATGC-3′); for Sl LHP1(141–399), 141-S (5′-GAGAGGATCCATGGAAGAGAGCTTGAAGT-CAG-3′) and 399-AS; and for Sl LHP1(141–171), 141-S and 171-AS.

To generate At LHP1 fused with GFP, the At LHP1 coding region was amplified by RT-PCR using total RNA prepared from *Arabidopsis thaliana* (Columbia) cauline leaves and the following primers: At LHP1SalI-S (5′-AAGTCGACAATGAAAGGGGCAAGTGG-3′) and At LHP1BglII-AS (5′-CCAGATCTGTTAAGGCGTTCGATTG-3′). The PCR product was cloned into a pGEM T-easy vector (Promega). The At LHP1 fragment was reamplified by PCR using another set of primers: AtHp1-S (5′-GAGA-GATCTATGAAAGGGGCAAGTGGTG-3′) and Athp1-as (5′-CCCGGGA-GGCGTTCGATTGTACTTG-3′), and the PCR product was digested with *Bgl*II and *Sma*I and subcloned into the same sites of pUC19-35S-GFP in-frame with GFP. pUC35S-Sl LHP1W114G/W117G-GFP was constructed by PCR using as a template pUC35S-Sl LHP1-GFP and the following two sets of primers: 1S and 145-AS, and a mutagenic primer, SlLHP1-Bclww-S (5′-TATTTGATCAAAgGGCGTGGCgGGCCGG-AGTCG-3′) and 399-AS. The PCR products were digested with *Bam*HI/ *Bcl*I and *Bcl*I/*Sma*I, respectively, and ligated into *Bgl*II/*Sma*I sites of pUC35S-GFP.

To generate pUC35S-At LHP1W132G-GFP, two PCR fragments were first generated using pUC35S-At LHP1-GFP as a template, two mutagenic oligonucleotides, At LHP1w-AS (5′-GTTTCAGGCCcTCCGCGC-CATTT-3′) and At LHP1w-S (5′-AAATGGCGCGGAgGGCCTGAAAC-3′), and two primers flanking the At LHP1 coding region, AtHp1-S and Athp1-as. The resulting two PCR fragments were mixed and subjected to PCR using AtHp1-S and Athp1-as as primers. The amplified DNA fragment was digested with *Bgl*II and *Sma*I and ligated to the same sites of pUC35S-GFP. All constructs were sequenced to ensure in-frame fusion with the GFP and used for protoplast transformation experiments. To generate transgenic plants, the 35S-Sl LHP1-GFP fragment was excised out using *Eco*RI and subcloned into the same site of the binary vector pPZP-111 to generate pPZP-35S-Sl LHP1-GFP, followed by transformation into *Agrobacterium tumefaciens* and into *Arabidopsis lhp1* mutant plants (Gaudin et al., 2001).

GST Pull-Down Assays

GST fusion proteins were expressed and purified by glutathione–Sepharose essentially as described in the manufacturer's protocol (GST gene fusion system; Amersham Pharmacia Biotech), except for the use of NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40) instead of PBS. In vitro binding of Sl LHP1 to histone H3 was determined by the GST pull-down assay with glutathione–Sepharose containing various GST fusion proteins, as indicated in the figures, and 2% trichloroacetic acid–soluble fraction from tobacco (*Nicotiana tabacum*) cells (Zhao and Grafi, 2000), followed by immunoblotting with anti-H3 (Upstate Biotechnology). In certain experiments, GST pull-down assays were performed with calf thymus histones (Roche) or with histone H3 labeled with histone methyltransferase [GST-SUV39H1(H320R), kindly provided by T. Jenuwein] in a reaction containing 300 nCi of *S*-adenosyl-[methyl-^{14}C]L-Met (25 μCi/mL; Amersham) as the methyl donor essentially as described (Rea et al., 2000).

Plant Material and Protoplast Transformation

Seeds of *Arabidopsis* mutants for *ddm1-2* and *met1* were kindly provided by E. Richards, for *kyp-2* by S. Jacobsen, for *suvh2* by G. Reuter, and for *clf* by J. Goodrich. The methylation mutants *ddm1-2* and *met1-1* were

verified by DNA gel blot analysis for the digestibility of the centromeric repeats (CEN180), the Athila retroelement, and the 18S rDNA by the methylation-sensitive *Hpa*II enzyme (Kakutani et al., 1996; Kankel et al., 2003) and by the redistribution of methyl CpG binding domain proteins when transiently expressed in these mutant cells (Zemach et al., 2005). *Arabidopsis* wild-type ecotypes Columbia and Wassilewskija as well as *Arabidopsis* mutants were grown under short-day conditions at 20°C. Rosette leaves from 4- to 6-week-old plants were used for the isolation of protoplasts and transformation as described (http://genetics.mgh. harvard.edu/sheenweb/protocols_reg.html). After 24 h, protoplasts were stained with DAPI and inspected with a laser confocal microscope (Olympus Fluoview FV500) equipped with a laser diode system (LD405; Olympus) to detect the DAPI signal. Images were obtained using an excitation wavelength of 405/488 nm; images for DAPI, GFP, and chlorophyll signals were collected through 415/435-nm, 505/525-nm, and 630-nm filters, respectively.

Immunofluorescence and FISH

Nuclei were isolated from leaves as described previously (Fass et al., 2002) and fixed in 4% paraformaldehyde dissolved in PBS for 15 min at room temperature, followed by washing twice with PBS. Nuclei were spread on slides, air-dried, permeabilized in cold acetone (100%) for 7 min at −20°C, and washed twice with PBS. Slides were blocked with 2% BSA in PBS for 2 h at room temperature, followed by overnight incubation at 4°C with 100 μL of primary antibody mixture containing 2 μg of anti-GFP (Roche) in 2% BSA. Slides were washed three times, 5 min each, in PBS, followed by 2 h of incubation at room temperature with secondary antibody (goat anti-rabbit IgG tagged with fluorescein isothiocyanate). Slides were washed as described above, stained with DAPI, mounted with Vectashield (Vector Laboratories), and inspected with a fluorescence microscope (Olympus) equipped with a charge-coupled device camera (Imago; Photonics). For FISH assays, slides washed as described above were fixed with 1% paraformaldehyde for 5 min, denatured with formamide, and probed with the 180-bp repeats (CEN180) labeled with tetramethylrhodamine-5-dUTP (Roche) as described (Avivi et al., 2004). Images were pseudocolored and merged using TILL Vision version 3.3 software. All images were processed using Adobe Photoshop software.

FRAP

FRAP experiments were performed using a laser confocal microscope (Olympus IX70, Fluoview FV500). Enhanced GFP images were obtained using an excitation wavelength of 488 nm, and signals were collected through a 505/525-nm filter. In the FRAP experiments, images of 196 × 96 pixels were collected at maximum speed of ∼170 ms using 1% laser power for 2 s before the bleach and up to 50 s afterwards The bleach pulse was by 100% laser power for 0.1 s in a rectangular area (∼1 μm²) of the nucleus. The relative fluorescence intensity was normalized to the nonbleached signal after subtraction of the background signal. Values are averages from at least five cells from three independent experiments. Fluorescence recovery curves were performed using Excel software (Microsoft). We determined the 50% recovery time in each experiment as the time required to achieve half-fluorescence recovery between the maximum intensity before photobleaching (marked as 1.0) and the initial relative intensity of the photobleached area. Statistical significance was determined by *t* test.

Accession Number

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF428244.

Supplemental Data

The following materials are available in the online version of this article.

   Supplemental Video 1. FRAP of Sl LHP1-GFP at the Chromocenter of an *Arabidopsis* Nucleus.

   Supplemental Video 2. FRAP of Sl LHP1(1−306)-GFP at the Chromocenter of an *Arabidopsis* Nucleus Showing Increased Mobility in the Absence of the CSD.

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