Development 128, 4847-4858 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV0383

Mutations in *LIKE HETEROCHROMATIN PROTEIN 1* affect flowering time and plant architecture in *Arabidopsis*

Valérie Gaudin[1,*], Marc Libault[1], Sylvie Pouteau[1], Trine Juul[1], Gengchun Zhao[1,‡], Delphine Lefebvre[1] and Olivier Grandjean[2]

[1]Laboratoire de Biologie Cellulaire, [2]Laboratoire de Génétique, INRA, route de St Cyr, 78026 Versailles cedex, France
[‡]Present address: Northeast Normal University, Changchun, China
*Author for correspondence (e-mail: valerie.gaudin@versailles.inra.fr)

Accepted 31 August 2001

SUMMARY

In plants, recent studies have demonstrated links between the regulation of developmental processes and chromatin dynamics and organisation. Analysis of new mutations affecting overall plant architecture, leaf development and flowering time in *Arabidopsis* has allowed us to clone and characterise *LHP1*, the *Drosophila* heterochromatin protein 1 (HP1) homologue. *LHP1* has the chromo and chromo shadow domains central to the function of animal proteins. Yeast two hybrid studies and in planta deletion experiments suggest similar modes of action in plants and animals via homodimer formation. In vivo localisation experiments revealed a specific subnuclear protein distribution in foci throughout the nucleus. Our data suggest that LHP1 may act as a main regulator of gene expression in plants, through formation of heterochromatin-like repressive complexes, to control developmental pathways involved in organ and cell size, and the vegetative to reproductive phase transition.

Key words: *Arabidopsis thaliana*, Chromatin, HP1, Flowering time, Leaf development

INTRODUCTION

Regulation of gene transcription is central to developmental processes. By establishing and maintaining specific patterns of transcription, various pathways are co-ordinated for correct development of the organism. Primary control occurs through interactions between specific regulatory DNA sequences and a large variety of transcription factors. A higher order of control occurs through regulation of chromatin states. Position effect variegation in *Drosophila* is a well known example of how gene expression can be affected by chromatin organisation (Lu and Eissenberg, 1998). Heterochromatin-associated protein 1 (HP1) of *Drosophila*, is one of the key components in this phenomenon, and is involved in the generation and maintenance of an inactive heterochromatin structure that silences gene expression (Eissenberg and Elgin, 2000). Cell differentiation or developmental processes such as embryogenesis, also require chromatin modifications to maintain repression of homeotic genes mediated by Polycomb-group proteins (Pirrotta, 1997; Müller and Leutz, 2001). Chromatin changes underlie regulatory mechanisms that modulate the accessibility of the transcriptional machinery to genetic information and *cis*-regulatory elements through ATP-dependent chromatin remodelling, nucleosome displacement or histone phosphorylation, methylation or acetylation (Flaus and Owen-Hughes, 2001; Marmorstein and Roth, 2001). A dynamic balance between open chromatin states and more condensed or heterochromatin-like states appears to be the basis of this level of regulation. Identifying the mode of action of these chromatin-associated multiprotein complexes and their components is therefore central to our understanding of developmental processes.

In plants, only a few links between the regulation of developmental processes and chromatin dynamics have been identified so far (Preuss, 1999; Meyer, 2000; Habu et al., 2001). The first example was the *curly leaf* (*clf*) mutation, which affects *Arabidopsis* flowering time, leaf morphology and flower development (Goodrich et al., 1997). *CLF* encodes a Polycomb-group protein that is homologous to the *Drosophila* gene *Enhancer of zeste* (*E[z]*). CLF acts by repressing genes such as the *AGAMOUS* homeotic gene involved in floral whorl identity. More recently, screens for developmental mutants allowed the identification of several plant homologues of chromatin-associated proteins. Two of them are Polycomb-group genes and are involved in the control of embryo and endosperm formation during seed development: *MEDEA/FIS1*, is homologous to *E[z]* (Grossniklaus et al., 1998; Kiyosue et al., 1999; Luo et al., 1999; Kinoshita et al., 1999), while *FIE/FIS3* is similar to *Extra sex combs* and encodes a WD Polycomb-group protein (Ohad et al., 1999) which interacts with MEDEA. *FASCIATA1* and *FASCIATA2* encode proteins homologous to two subunits of the chromatin assembly factor 1 (CAF1) (Kaya et al., 2001). Mutations in these genes perturb the organisation and function of root and shoot apical meristems causing stem fasciation, abnormal phyllotaxy and root modifications. *PICKLE/GYMNOS*, encoding a CHD3

family chromatin-remodelling factor and is involved in regulating the developmental transition from embryonic to vegetative phase by repressing the *LEC1* gene, an activator of embryo-specific genes (Eshed et al., 1999; Ogas et al., 1999). Finally, screens for mutations that affect transcriptionally silenced gene expression also identified *DDM1* (Jeddeloh et al., 1999) and *MOM* (Amedeo et al., 2000), which encode SWI2/SNF2-like chromatin remodelling factors. Here we investigate how a new chromatin-associated plant component controls major developmental changes in *Arabidopsis*.

We have analysed new mutations at the *LHP1* locus that affect *Arabidopsis* plant architecture, flowering time and leaf development. The *LHP1* gene is the *Arabidopsis* homologue of the *Drosophila* heterochromatin protein 1, HP1. LHP1 contains both the chromo and chromo shadow domains shown to be critical for the function of animal proteins. Despite sequence divergence between chromo shadow domains in the plant and animal kingdoms, we showed that the plant chromo shadow domain is important for the protein function and mediates its dimerisation, suggesting similar modes of action in plants and animals. In vivo localisation experiments have shown a specific subnuclear distribution in foci throughout the nucleus. Our study reveals new links between the regulation of developmental processes and chromatin dynamics and organisation, through the plant heterochromatin-like protein LHP1.

MATERIALS AND METHODS

Plant materials

The two mutants, *lhp1-1* and *lhp1-2*, were isolated from the Versailles T-DNA insertion collection of *A. thaliana* (L.) Heynh., Wassilewskija (WS) ecotype. The Versailles collection was obtained by T-DNA insertion mutagenesis (Bechtold et al., 1993) using the pGKB5 vector (Bouchez et al., 1993). For flowering time analyses, plants were grown in Fitotron^{TM} (Sanyo Gallenkamp, ref. SGC066.PFX.F) growth chambers, with 220 μmol/m^2/second white light and 2 μmol/m^2/second tungsten light, at 20°C constant, 70% humidity, under short-day (SD; 8 hours light/16 hours dark), or long-day (LD; 16 hours light/8 hours dark) conditions. *Nicotiana tabacum* plants, doubled-haploid cultivar XHFD8 (Bourgin, 1978), were grown in the greenhouse as described previously (Chupeau et al., 1974).

Cloning of the *LHP1* gene

Genomic DNA from *lhp1-1* plants was isolated as described previously (Doyle and Doyle, 1990). Standard procedures were followed for all molecular protocols (Sambrook et al., 1989). In Southern blot analyses, probes were derived from the pGKB5 plasmid (Bouchez et al., 1993), corresponding to the T-DNA RB (right border), LB (left border) and an internal fragment bearing the kanamycin resistance gene. By using the kanamycin plasmid rescue technique, a 1.6 kb fragment, corresponding to the *lhp1-1* genomic sequence adjacent to the RB of the T-DNA was cloned into the pResc38 vector (Bouchez et al., 1996). A 307 bp fragment corresponding to the T-DNA left-border::plant DNA junction was isolated by PCR. The two clones were sequenced.

Complementation of the *lhp1-1* mutant

The Col-0 genomic P1 clone MVA3 (81701 bp; accession number AB006706) bearing the region of the *lhp1-1* T-DNA insertion, was kindly provided by the Kazusa DNA Research Institute (Japan). An 11 kb *Sal*I restriction fragment, corresponding to the 5192-16149 MVA3 region, was subcloned into the binary vector pCambia1300 (carrying the hygromycin resistance gene) to give the pCaS plasmid. After digestion of the pCaS plasmid with *Eco*RI, a 15.3 kb fragment was isolated corresponding to the pCambia1300 vector and the 9790-16149 MVA3 region and religated. The resulting plasmid was named pCaES. The pCaSSP plasmid was constructed by cloning the *Sna*BI/*Spe*I fragment corresponding to 8623-14191 MVA3 region, in the *Sma*I site of pCambia1300. *Arabidopsis* in planta tranformations (Bechtold et al., 1993) were performed using Silwet co-polymer L-77 (OSI) at 0.005 (v/v)% in a 5% (w/v) sucrose, 10 mM $MgCl_2$ solution. The three plasmids described above were transformed into the *lhp1-1* mutant for complementation experiments. Transgenic plants were selected on plates containing hygromycin and transferred to soil in the greenhouse. After self-seed set and segregation analyses on hygromycin selection medium, 8 independent homozygous transgenic plants were selected and analysed for each of the three constructs.

Isolation of *LHP1* cDNA

A cDNA library was kindly provided by Lacroute and Minet (Minet et al., 1992). It was constructed in the pFL61 vector using poly(A)$^+$ RNA isolated from young seedlings (2-leaf stage) of *A. thaliana*, Landsberg *erecta* ecotype. About 6×10^5 colonies of the library were screened with a 1.8 kb genomic probe derived by PCR from the MVA3 P1 clone with the two primers mav5 (5′CGATTGTACTTGAGAT-GTTGCT3′) and mav8 (5′GGAGGTGGAAGTGGAGAGTC3′). These primers were designed based on the genomic region to amplify the putative gene according to gene prediction programs. Six positive clones were obtained after two steps of purification and analysed using restriction enzymes. The clone pFLcbx5, bearing the longest cDNA (1841 bp), was sequenced and analysed.

Dimerisation experiments in the yeast two hybrid system

The full-length coding region of *LHP1* (bp 146-1480), the N-terminal region (bp 146-727), the C-terminal region (bp 629-1480) and the chromo shadow domain (CSD; bp 1277-1480) were cloned into the pAS2-1 vector (carrying the *TRP1* gene; Clontech) containing the GAL4 DNA-binding domain (BD). These fragments were also cloned into the pACT2 vector (carrying the *LEU2* gene; Clontech) containing the GAL4 activation domain (AD). The control plasmids pTD1-1 and pVA3-1 (Clontech) encode the interacting proteins, tumor suppressor p53 and SV40 large T-antigen (Iwabuchi et al., 1993), fused with the BD and AD, respectively. Interaction of the encoded fusion proteins was investigated by co-transforming appropriate plasmids into the yeast reporter strain pJ69-4A (*MAT***a** *trp*1-90 *leu*2-3,112 *ura*3-52 *his*3-200 gal4Δ *gal*80Δ *LYS*2::GAL1-*HIS*3 *GAL*2-*ADE*2 *met*2::*GAL*7-*lac*Z) (James et al., 1996). Transformed yeast cells were plated onto medium lacking leucine and tryptophan and grown at 28°C for 4 days to select for the presence of both plasmids. Colonies were then transferred to medium lacking leucine, tryptophan and histidine or to rich YPD medium lacking adenine to select for interactions. Yeast colonies were grown on nitrocellulose filters placed on selective medium lacking leucine and tryptophan to perform the β-galactosidase assay. After 3 days of growth at 28°C, the filters were lifted and placed in a solution of 6.25% (v/v) $CHCl_3$ and 0.1% (w/v) SDS for 5 minutes to lyse the yeast cells. The filters were incubated in Z-buffer (60 mM Na_2HPO_4, 20 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgCl_2$, pH 7.0) with 0.9% (v/v) β-mercaptoethanol and 0.1% (w/v) X-gal.

Transcription analysis

Total RNAs were prepared from various tissues using the TRIzol reagent (Life Technologies) according to the supplier's instructions. 5 μg of total RNAs were used for each reverse transcription reaction, using dT primers in a 20 μl reaction mix containing 3 mM MgCl2, 75 mM KCl, 50 mM Tris-HCl pH 8.3, 375 ng of dT primers, 1 mM of dNTPs, 10 mM DTT, 200 U of M-MLV reverse transcriptase (Gibco-BRL), and incubating 2 hours at 37°C, in the presence of 10 U of ribonuclease inhibitor (Gibco-BRL). 0.5 μl of the cDNA samples were used for PCR amplification. For *LHP1* cDNA amplification, the

primers mav5 (5′CGATTGTACTTGAGATGTTGCT3′, located in the sixth exon) and mav8 (5′GGAGGTGGAAGTGGAGAGTCG3′, located in the first exon) were used. Amplification from cDNA template gives rise to a 1.1 kb PCR product whereas samples with contaminating genomic DNA result in a secondary product of 1.8 kb. Since the reaction is performed in non limiting conditions, this has no consequence for the interpretation of the results. The primers CO_{50} (5′CTCCTCGGCTTCGATTTCTC3′) and CO_{51} (5′CATTAACCA-TAACGCATACATTTC3′, this spans the *CO* intron, the position of which is indicated by the hyphen) were used for specific *CO* cDNA amplification (Putterill et al., 1995). The primers apt1 (5′TCC-CAGAATCGCTAAGATTGC3′, located 152 bp upstream of the start codon) and apt2 (5′CCTTTCC-CTTAAGCTCTG3′, spanning the fourth intron, the position of which is indicated by the hyphen) were used to amplify the *APT1* cDNA, encoding adenine phosphorybosyl transferase (Moffat et al., 1994). PCR reactions were performed as follows: 4 minutes at 94°C; 35 cycles (45 seconds at 94°C, 1 minute at 58°C (*LHP1/CO*) or 52°C (*APT1*), 1 minute 30 seconds at 72°C); 10 minutes at 72°C.

Construction of the GFP-LHP1 protein fusions

A PCR fragment corresponding to the *LHP1* full-length coding sequence was amplified with primers N-termCD (5′GA**AGATCT**T-CCATGGCAATGAAAGGGGCAAGTGTT3′) and C-termCD (5′TC-**AGATCT**CCATGGAAGGCGTTCGATTGTACTT3′) bearing *Bgl*II (bold) and *Nco*I (underlined) restriction sites, using the pFLcbx5 plasmid as template. The PCR fragment was digested with *Nco*I and inserted at the *Nco*I restriction site of the pAVA121 vector harboring the red-shifted S65T GFP protein driven by the constitutive 35S CaMV promoter (von Arnim et al., 1998). In the resulting pAVA-NF construct, LHP1 is fused to the N-terminal end of the GFP. The pAVA-BF construct bearing LHP1 fused to the C-terminal end of the GFP was obtained by *Bgl*II digestion of the PCR fragment and ligation into the pAVA121 vector, at the *Bgl*II site. Sequencing was performed to verify the sequence of the PCR fragments and the translational fusions. A vector bearing GFP(S65T) fused to the 38 amino acids of the C-terminal region of the VirD2 protein from *Agrobacterium tumefaciens* was kindly provided by H. Mireau (INRA, Versailles). The VirD2 region contains a bipartite nuclear localisation signal (NLS) shown to be functional in plants (Tinland et al., 1992; Howard et al., 1992; Citovsky et al., 1994). A *Not*I fragment corresponding to the GFP/VirD2-NLS fusion was subcloned into the pLBR19 vector, at the *Sma*I site downstream of the 35S CaMV promoter, to give plasmid p35S/GFP-NLSV.

Protoplast transient expression assay

Mesophyll protoplasts were prepared from tobacco plants, electroporated with 50 μg of supercoiled plasmid purified on CsCl gradient and then cultured in the dark, in To medium, as described (Chupeau et al., 1974; Guerche et al., 1987). Protoplasts were observed 48 hours after electroporation using a LEICA TCS-NT confocal microscope (Leica, Heidelberg, Germany) equipped with an argon/krypton laser (Omnichrome, Chino, CA) and AOTF for excitation. The GFP(S65T) protein fusion is excited at 488 nm (maximun absorption at 479 nm) and GFP emission occurs at 507 nm. The GFP and chlorophyll fluorescences were analysed with the BP530/30 and LP590 filters, respectively. Series of optical sections at a pinhole of approximately 50 μm and at 1 μm interval steps were made for maximum projection using PL FLUOTAR 40×1 or PL APO 63×1.32 lenses. Resolutions of 512×512 or 1024×1024 pixels were used. Representative images were chosen to illustrate the observations performed on several cells (an average of 30) for each construct in at least three independent electroporation experiments.

Low-temperature scanning electron microscopy and image analysis

Fresh leaf samples were rapidly frozen at –210°C in subcooled nitrogen using a Cryotrans CP1500 (Oxford). After cryofixation, the samples were rapidly transferred to the cooled specimen chamber of a 525M Philips SEM microscope. Specimen coating was performed by diode sputter coating with gold in a low-pressure atmosphere of argon inert gas (Jeffree and Read, 1991). Cell sizes were measured using the image analysis software Optimas 6.0™ (Imasys, Suresnes, France) and the average surface of 20-30 individual cells was calculated.

RESULTS

Arabidopsis lhp1 mutants have altered flowering time, leaf development and plant architecture

The *lhp1-1* and *lhp1-2* mutants were identified as early flowering mutants of *Arabidopsis thaliana*, ecotype Wassilewskija, from a screen of the Versailles collection of T-DNA insertion mutants (Bechtold et al., 1993). The two mutants had very similar pleiotropic phenotypes, showing abnormal flowering time, organ development and plant architecture (Fig. 1). Allelism tests showed that they were affected at the same *LHP1* locus. They were both inherited as recessive nuclear mutations.

Flowering time can be measured by the number of rosette leaves produced before the reproductive switch and by the number of days from sowing to bolting. *Arabidopsis* wild-type

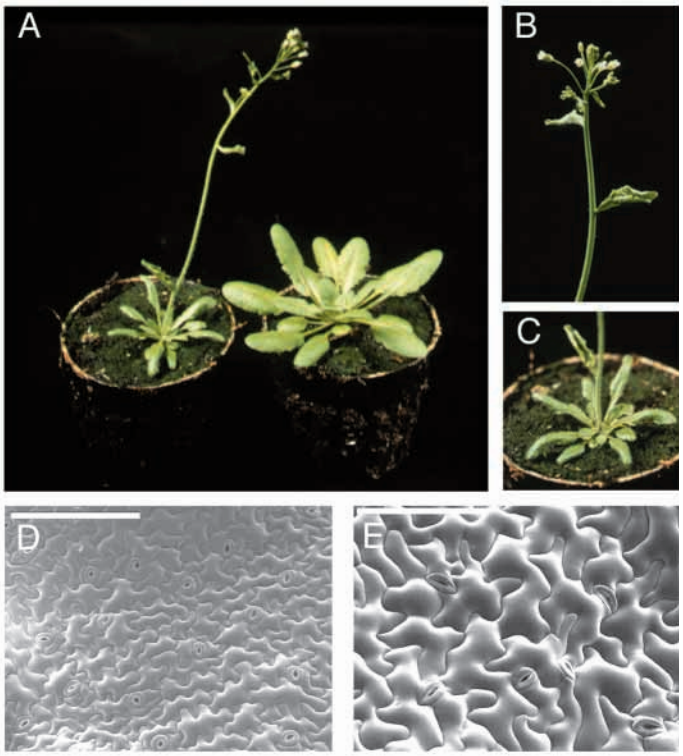

Fig. 1. *Arabidopsis lhp1* mutant phenotype. (A) Six-week-old wild-type *Arabidopsis* plant (right) and the *lhp1-1* mutant (left) under SD. (B) Close-up view of an *lhp1-1* inflorescence showing small and upwardly curled cauline leaves, and normal inflorescences and flowers. (C) Close-up view of an *lhp1-1* rosette with small, narrow leaves downwardly curled along the longitudinal axis of the leaf. (D,E) Scanning electron micrographs of the upper epidermis of a third rosette leaf from 33-day-old *lhp1-1* (D) and wild-type (E) plants. Scale bars, 100 μm.

Table 1. *Arabidopsis lhp1* mutants are early flowering plants with reduced cell elongation

	Flowering time		Rosette leaves		Cauline leaves		Epidermal cell size (μm^2)		
	SD	LD	SD	LD	SD	LD	18d	33d	Size increase
WS	47.4±2.4	22.4±0.7	31.6±1.3	10.5±0.7	9.1±0.7	4.1±0.3	1806±527	5388±2443	×3
hpl1-1	23.3±1.6	17.7±0.7	9.3±0.6	7.3±0.5	3.6±0.8	3.4±0.4	510±133	682±160	×1.3
hpl1-2	22.5±0.7	17.2±0.4	7.6±0.5	7.0±0.7	3.6±0.8	2.6±0.3	–	–	–

For flowering time analyses, 10 plants were grown under SD or LD conditions in growth cabinets. Flowering time is expressed as the number of days from sowing to the appearance of a 0.2 cm stem. The numbers of rosette and cauline leaves were recorded.

Upper epidermal cell sizes were measured on the third rosette leaf of 18- and 33-day-old plants, grown in LD. Values are ±standard deviation.

plants are sensitive to photoperiod and flower much later under short-day (SD) than under long-day (LD) conditions (Redei, 1962; Koornneef et al., 1991; Simon and Coupland, 1996). We showed that the two *Arabidopsis lhp1* mutants flower much earlier under SD and LD compared to wild type, both in terms of the number of days to bolting and number of rosette leaves (Table 1). Under SD conditions, the two mutants flowered 5 days later than in LD, showing that they were still responding to environmental conditions but with a reduced sensitivity to photoperiod.

Mutations in *LHP1* strongly affected leaf morphology: leaf blade expansion was reduced, giving rise to small, narrow and curled leaves. Strikingly, the orientation of the curling changed with floral transition: rosette leaves were curled downwards, whereas cauline leaves were curled upwards. The *curly leaf* (*clf*) mutant (Goodrich et al., 1997) shares the same curling phenotype with *lhp1* for cauline leaves, but has the opposite curling for rosette leaves (being curled upwards in *clf*). Both *clf* and *lhp1* are early flowering mutants. However, *lhp1* did not show the homeotic transformations of floral organs observed in *clf*, as inflorescence and floral organisation were normal. Nevertheless, *lhp1* inflorescence stems were shorter (more than 50% reduction in length for the main stem in LD) with a reduction of internode length. Although *lhp1* mutant plants were fertile, they produced smaller (about 30% reduction in length, in LD) and fewer siliques.

The *lhp1* mutants showed a strong decrease in overall plant size with reduced stem, leaf, flower and silique sizes. To investigate the origin of size reduction, scanning electron microscopy of the upper epidermis of rosette leaves was used to measure cell dimensions. Leaves from 18- or 33-day-old wild-type or mutant plants grown under LD conditions were analysed. The upper epidermal leaf cell size was approximately 8 times smaller in the mutant than in 33-day-old wild-type plants (Table 1). Between 18 and 33 days, cell size increased slowly in the mutant compared to wild-type plants (e.g., 1.3 fold in *lhp1*, 3 fold in WS). These results show that a reduction of cell expansion in the mutant contributed to the reduced cell size. Similar analyses in *clf* mutants also revealed a reduction in cell elongation during leaf expansion (Kim et al., 1998). As yet, we cannot rule out that a defect in cell division may also be involved in the changes in organ and plant size. Modifications of cell elongation and cell division might explain the curled leaf morphology, but the origin of the curling change in the *lhp1* mutant remains unclear.

Cloning of *LHP1*

Linkage analyses between the mutant phenotype and kanamycin resistance conferred by the T-DNA suggested that only the *lhp1-1* mutant was tagged (no recombinant was found in the progeny of 100 *lhp1-1* segregating individuals). Further analyses were therefore focused on *lhp1-1*. Southern blot experiments revealed one simple insertion of a full length T-DNA in the *lhp1-1* genome. A 1.6 kb genomic fragment adjacent to the right border (RB) of the T-DNA was isolated and mapped to the P1 clone MVA3, which is located on the top of chromosome 5 (*Arabidopsis* sequencing program,

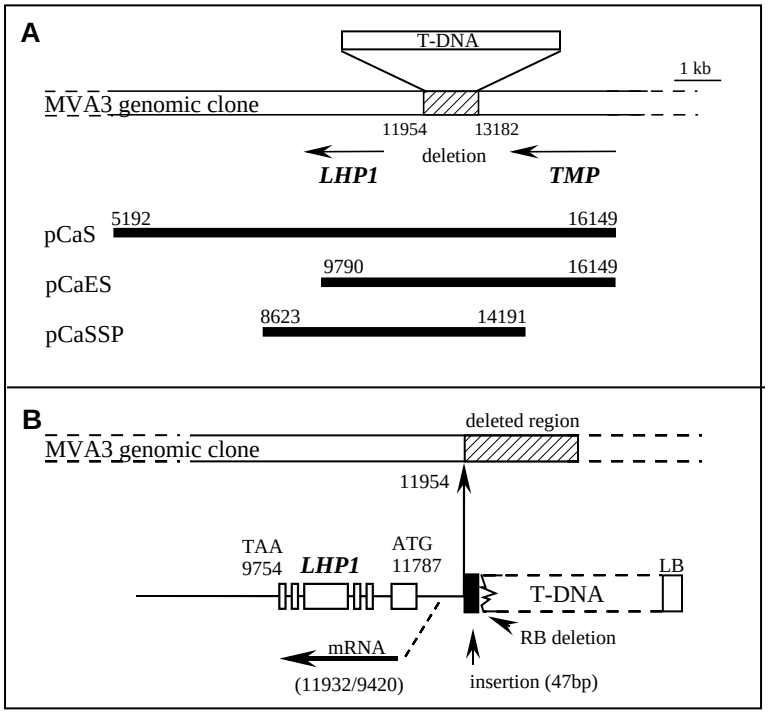

Fig. 2. Structure of the *lhp1-1* locus. (A) Localisation of the 1.2 kb deletion on the genomic MVA3 P1 clone which accompanied the T-DNA region insertion in *lhp1-1*. Database searches indicated two putative genes in this region, *LHP1* and *TMP*. The plasmids, pCaS, pCaES and pCaSSP, were used for complementation experiments. (B) Detail of the T-DNA insertion in *lhp1-1*. Insertion occurs at position 11954, 22 bp upstream of the 5′ end of the isolated cDNA (11932). At the T-DNA right-border::genomic DNA junction, the integration was accompanied by deletions of the 24 bp RB repeat and the 21 bp adjacent to it. A small 47 bp insertion with no identified homology was also detected. The T-DNA LB was better conserved than the RB as commonly observed during T-DNA integration. Numbers refer to the sequence of the MVA3 P1 clone (accession number AB006706).

Kazusa DNA Research Institute Database). This region is enriched in other mapped flowering time QTLs or mutants such as *FLC*, *TFL2*, *CO*, *FY*, *EMF1* (Levy and Dean, 1998). The cloning of a 307 bp fragment corresponding to the left-border T-DNA::plant genomic DNA junction showed that the T-DNA insertion induced a deletion of 1.2 kb in the *lhp1-1* allele (Fig. 2).

The T-DNA integration/deletion occurred between two putative genes organised in direct tandem and separated by 1.4 kb, named *LIKE HETEROCHROMATIN PROTEIN 1* gene (*LHP1*) and *TRANSMEMBRANE PROTEIN* gene (*TMP*) (Fig. 2). Searches in databases revealed the existence of a *Brassica rapa* EST (BNAF1113E) corresponding to *LHP1* and an *A. thaliana* EST corresponding to *TMP*. To determine the molecular structure of this region bearing two putative functional genes, complementation experiments and cDNA library screens were undertaken. For phenotype restoration, binary vectors bearing different fragments of the region of interest were constructed based on gene prediction analysis and used to transform mutants (Fig. 2A). Transformants obtained with pCaS and pCaSSP were completely restored with overall plant size, leaf morphology and flowering time similar to the wild type. In contrast, pCaES did not complement the mutant phenotype. cDNA library screens allowed the isolation of *LHP1* full-length cDNA (1841 bp) and *TMP* full-length cDNA (2146 bp) (data not shown). Analyses of the precise localisation of the two genes and of the different constructs indicated that none of the binary vectors contained a full-length copy of the *TMP* gene with its minimal regulatory elements. The pCaES vector, which does not allow restoration of the phenotype, bears a truncated *LHP1*, whereas the complete *LHP1* gene is present in pCaS and pCaSSP. Therefore, the *LHP1* gene is involved in the mutant phenotype. The sequencing of the *lhp1-2* allele confirmed that result. A deletion of a T in the fourth exon (at position 775 based on the cDNA sequence) was the only change observed in the sequence of the *lhp1-2* genomic region spanning from 48 bp upstream of the 5′ end of the cDNA to 11 bp downstream of the 3′ end. The deletion created a new restriction site whose presence was confirmed by digestion and hybridisation experiments (data not shown). The mutation generated a stop codon 3 amino acids downstream of the deletion and a possible truncated LHP1 protein (210 aa).

With its chromo and chromo shadow domains, the *Arabidopsis* LHP1 protein belongs to the HP1 family

Sequence analyses of the *LHP1* cDNA revealed that *LHP1* has 6 exons and encodes a 445 amino acid (aa) protein with regions homologous to HETEROCHROMATIN PROTEIN 1 (HP1) from *Drosophila*, and therefore named *LHP1*, for Like-HP1 protein. LHP1 has the two characteristic HP1 motifs, the chromo (**chr**omatin **o**rganisation **mo**difier) domain (CD) and chromo shadow (CSD) domains (Paro and Hogness, 1991; Aasland and Stewart, 1995) located in the amino and carboxy-terminal regions of LHP1, respectively. These domains are separated by a long hinge region (219 aa) (Fig. 3). The LHP1 protein has an acidic region close to its N-terminus similar to HP1. LHP1 also possesses five K-R/K-X-R/K classical nuclear localisation signals (NLS) (Fig. 3) (Dingwall and Laskey, 1991). Four NLS are located in the N-terminal end of the protein, two of them are separated by 10 aa and form a characteristic bipartite motif. One single motif is present in the C-terminal end.

Southern blot experiments and database searches revealed that *LHP1* is unique in the *Arabidopsis* genome. However, *Arabidopsis* proteins with chromo domains, but no chromo shadow domain, have been identified. These include a DNA methyltransferase homologue (Henikoff and Comai, 1998), the chloroplastic CAO protein involved in the light-harvesting chlorophyll a/b binding protein complex of photosystem II (Klimyuk et al., 1999) and PICKLE, a CHD3 chromatin-remodelling factor (Ogas et al., 1999). Searches among other plant species identified two possible orthologues: the carrot *DcCB1* gene (Kiyosue et al., 1998) originally described as a Polycomb-group gene and the previously mentioned *B. rapa* EST, whose sequencing was completed and referred to as *BrLHP1* (Fig. 4). Protein sequence comparisons revealed

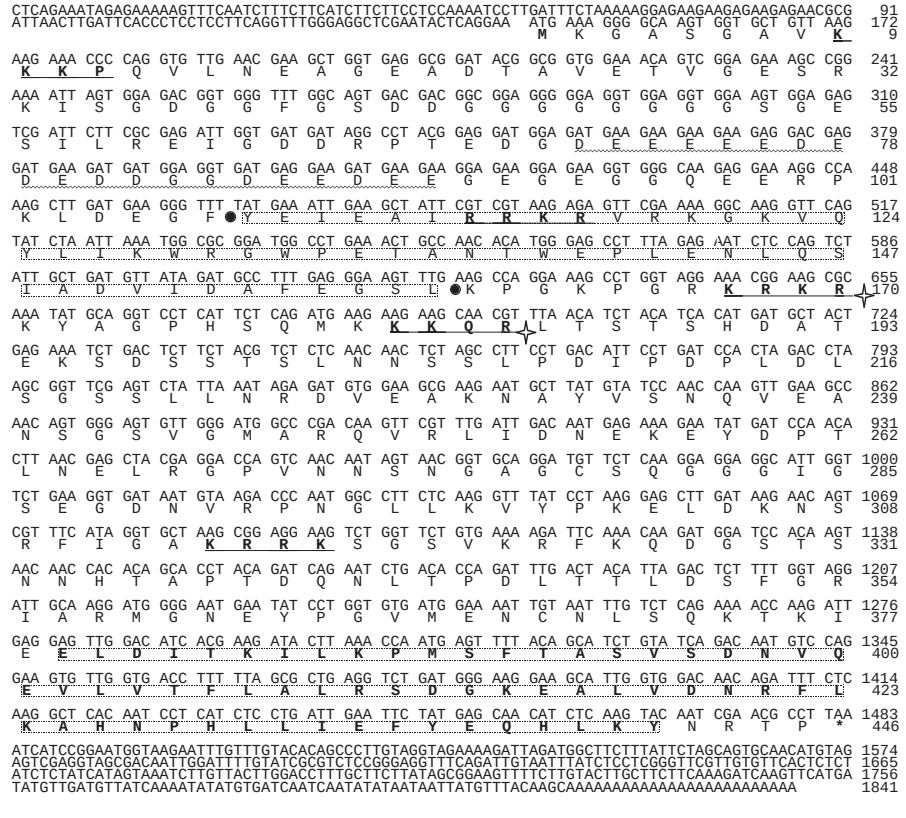

Fig. 3. Sequence of *LHP1*(GenBank accession no. AF387639). Nuclear localisation signals (NLS) are in bold and underlined. The bipartite NLS is marked with stars. The N-terminal acidic domain is indicated by a wavy underline. The chromo domain (108-159 aa) is boxed and framed with two dots. The chromo shadow domain (379-441 aa) is in bold and boxed. The sequence is highly conserved between two different ecotypes, L*er* and Col-0, with only one substitution from E22 (Col-0) to D (L*er*), and a change at nt 96 from C (Col-0) to A (L*er*).

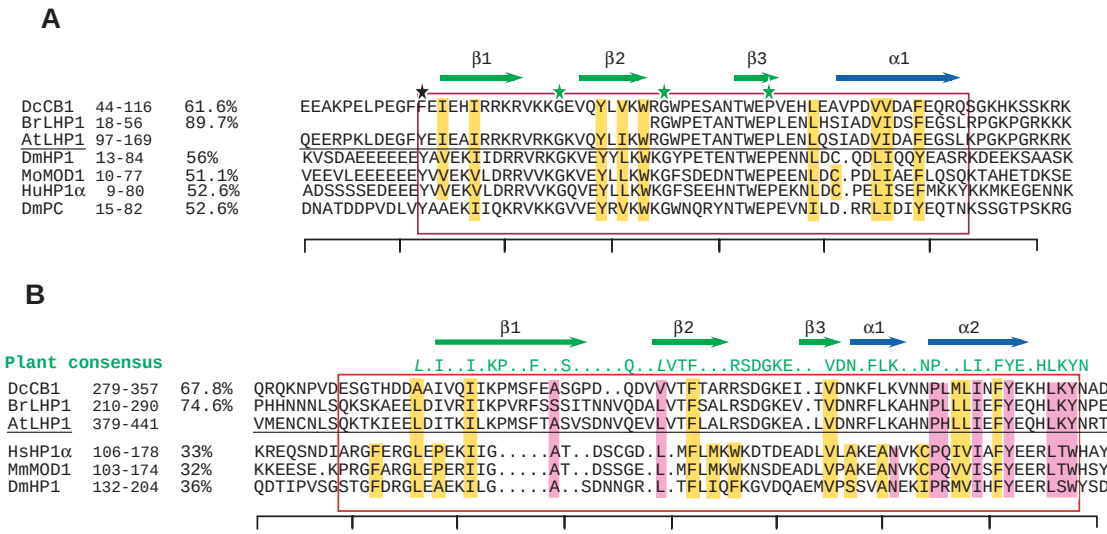

Fig. 4. Sequence comparisons of the chromo (A) and chromo shadow domains (B). *Arabidopsis* AtLHP1 (present work), *B. rapa* BrLHP1 (the EST sequence is likely truncated at the 5′ end), *Daucus carota* DcCB1 (D83719), *Drosophila melanogaster* HP1 (DmHP1; AAA28620) and Polycomb (DmPC; A38565), *Homo sapiens* HP1α (HuHP1α, P45973) and *Mus musculus* HP1 (MmMOD1; P23197). The positions of the regions used for alignments and identity scores between AtLHP1 and different CD/CSD are indicated. Based on NMR studies, β-sheet and α-helix secondary structures are indicated above the alignments (Ball et al., 1997; Brasher et al., 2000). The residues that form the hydrophobic core are highlighted in yellow. (A) Residues glycine 34 and 44 and proline 54, playing a crucial role in the tertiary CD structure, are indicated (green star). A black star indicates the position of the Y24F mutation in DmHP1. (B) Residues involved in dimerisation (with the main contacts being at MmMOD1 I161, Y164 and L168) are highlighted in purple.

42.4% identity with DcCB1 (392 aa) and 77.7% with BrLHP1 (290 aa, 5′ end truncated EST) reflecting phylogenetic distances between plant families and suggesting a global conservation of the protein in the plant kingdom.

Two chromo domains diversely conserved between plants and animals

Sequence comparisons were made among plant and animal HP1-like proteins in order to distinguish some general features of chromo and chromo shadow domains in different phyla (Fig. 4). The chromo domain is remarkably conserved among plant proteins (e.g., 61.7% identity between *Arabidopsis* and carrot) and between plants and animals (e.g., 56% identity/70% similarity between *Arabidopsis* and mouse). The 3D structures of mouse HP1 (MoMOD1) chromo and chromo shadow domains have a similar global organisation, with a three-stranded anti-parallel β-sheet structure, folded against one or two carboxy-terminal α-helices (Ball et al., 1997; Brasher et al., 2000; Cowieson et al., 2000). Both CD and CSD structures are organised around a hydrophobic core of well conserved residues in animals. The CD hydrophobic core (MmMOD1 residues 23, 26, 38, 40, 42, 58, 60, 63, 64, 67) is well conserved in LHP1, except for residue 60. The two glycine residues (34 and 44, in MmMOD1) involved in loop formation between β strands and proline 54 (in MoMOD1), which moves the main chain out of the β-sheet plan, are also conserved between plants and animals. In *Drosophila*, the mutation Y24F disrupts the function of HP1, affects gene silencing (Platero et al., 1995) and is thought to play a crucial role in the 3D structure (Ball et al., 1997). This amino acid substitution is also observed in the carrot homologue and may suggest a non-functional plant counterpart or differences reflecting plant specificity.

The chromo shadow domain is highly conserved among the three plant proteins (from 67.8 to 74.6% identity), but is less conserved between animals and plants compared to the chromo domain (36% identity between *Arabidopsis* and *Drosophila*), especially in the N-terminal part of the domain. The hydrophobic core is still present, but only the central residues of the β strand and α helix are similar. Differences were found in β2, β3, α1 and β1, the latter being apparently much longer in plants. Major differences occur in the junction regions between the β and α structures. However, the plant and animal α2 helix are very well conserved. This is consistent with the motif being involved in HP1 dimerisation (Brasher et al., 2000). This region contains the residues involved in the main contacts between two molecules (I161, Y164, L168). Other residues involved in self-association (A125, L132, N153, P157 W170) are also generally conserved between plants and animals.

Dimerisation of the *Arabidopsis* LHP1 protein requires the chromo shadow domain

In vivo studies have suggested that HP1 proteins can dimerise to participate in heterochromatin assembly complexes (Platero et al., 1995). The ability of HP1-like proteins to form homodimers was shown to be conserved in yeast (Wang et al., 2000), worms (Epstein et al., 1992) and mammals (Le Douarin et al., 1996; Ye et al., 1997).

To test whether self-association of the HP1-like protein also occurs in plants, the yeast two hybrid system was used with the full-length LHP1 protein fused to the GAL4 DNA-binding domain (BD) or to the GAL4 transcriptional activation domain (AD) (Fig. 5A). When the BD-LHP1 and AD-LHP1 fusion proteins were expressed together in yeast, the reporter genes *HIS3*, *ADE2* and *lacZ* were activated (Fig. 5B). This suggests

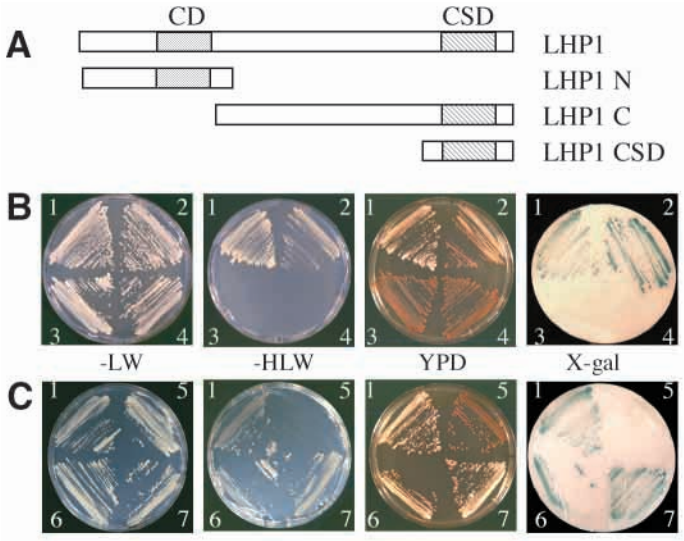

Fig. 5. LHP1 homodimerisation in the yeast two-hybrid system. (A) Schematic representations of LHP1 and truncated LHP1 proteins. LHP1N: N-terminal region (aa 1-194). LHP1C: long C-terminal region (aa 162-445). LHP1CSD: short C-terminal region (aa 378-445). (B,C) Growth of different yeast strains containing combinations of fusion proteins. Selective media lacking particular amino acids (L: leu, W: trp, H: his) or rich YPD medium lacking adenine are indicated under each plate. On medium lacking adenine, interacting proteins result in white colonies, non-interacting proteins results in red colonies. X-Gal plates show staining for β-galactosidase activity. 1, positive control. The plasmids pTD1-1 and pVA3-1 encoding proteins p53 and SV40 are known to interact in vivo. 2, pBD-LHP1 and pAD-LHP1. 3, pBD-LHP1 and pTD1-1. 4, pAD-LHP1 and pVA3-1. 5, pBD-LHP1N and pAD-LHP1N. 6, pBD-LHP1C and pAD-LHP1C. 7, pBD-LHP1CSD and pAD-LHP1CSD.

that dimerisation of LHP1 occurred. As a negative control, the BD-LHP1 and AD-LHP1 fusion proteins do not activate the reporter genes when expressed separately with a random protein fused to the appropriate AD or BD domain (Fig. 5B).

To delineate the domains involved in dimer formation in *Arabidopsis*, different regions of the LHP1 protein were tested; an N terminal region (aa 1-194), a long C-terminal region (aa 162-445), and a short C-terminal region (aa 378-445). Through co-transformation of these truncated LHP1 proteins fused to the BD or AD domains, it was shown that the C-terminal region, which contains the chromo shadow domain and part of the hinge region, could form homodimers but that the N-terminal, which only has the chromo domain, could not (Fig. 5C). Furthermore, the chromo shadow domain region (aa 378-445) was sufficient for dimer formation (Fig. 5C).

In complementation experiments (Fig. 2B), the binary pCaES plasmid encoded for a truncated LHP1 protein (1-434 aa) with key C-terminal residues Y435 and L439-K440-Y441 missing. This truncated protein was unable to rescue the mutant phenotype, supporting the importance of the conserved C-terminal end of the chromo shadow domain.

LHP1 is ubiquitously expressed

To determine at which developmental stages *LHP1* may act, we studied the expression of *LHP1* in the wild type and *lhp1-1* mutant by semi-quantitative RT-PCR. The level of transcription was too low for northern blot analysis. In wild-type plants, *LHP1* transcripts were detected before and after the reproductive transition and in all wild-type tissues examined: roots, rosette leaves, stems, young floral buds, flowers and siliques with a slightly lower level at the two cotyledon stage (Fig. 6). The gene is ubiquitously expressed, and therefore, any regulation may be at the cellular or post-transcriptional level.

Major differences could be detected between the wild type and the mutant. In *lhp1-1* mutants, transcripts were detected but expression was strongly reduced at all developmental stages and in all types of tissues tested (Fig. 6A). This suggested that *lhp1-1* was not a null allele. This observation can be explained by the fact that the insertion of the T-DNA in the *lhp1-1* genome is located in the promoter region of *LHP1*, only 22 bp upstream of the 5′ end of the isolated cDNA (Fig. 2B). The decrease in transcript levels was particularly noticeable in flowers and siliques. Therefore, the insertion does

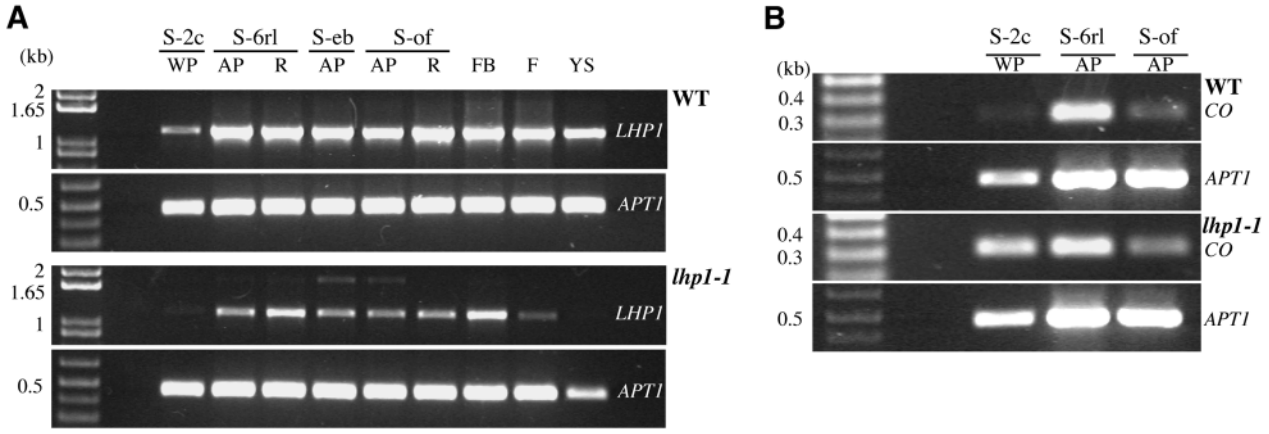

Fig. 6. RT-PCR transcriptional analyses of *LHP1* (A) and *CONSTANS* (B) in the wild type and *lhp1-1* mutant. (A) *LHP1* expression is ubiquitous during development in wild-type plants whereas it is downregulated in the mutant. (B) *CO* expression is upregulated in *lhp1* at an early vegetative stage. Expression of the constitutive *APT1* gene was used as a positive control and to normalise the amounts of cDNA. Different organs or tissues were collected from plants grown in LD, at the following developmental stages: S-2c (two cotyledons), S-6rl (6 rosette leaves), S-eb (early bolting), S-of (first open flower). The tissues were WP (whole plants), AP (aerial parts), R (roots), FB (floral buds), F (mature flowers) and YS (young siliques).

not prevent transcription, but interferes with normal *LHP1* regulation and is sufficient to cause the mutant phenotype.

CONSTANS transcription is upregulated at an early stage in *lhp1-1*

CONSTANS (*CO*) is a key transcription factor that promotes flowering in response to day length (Redei, 1962). Transcriptional regulation of *CO* is involved in regulating flowering time and by increasing levels of *CO*, early flowering occurs (Putterill et al., 1995). Therefore, to reveal a possible mechanism for the early flowering of *lhp1* mutants, *CO* transcription was analysed by RT-PCR experiments. The expression of *CO* was specifically upregulated in the *lhp1-1* mutant compared to wild type, at an early developmental stage (Fig. 6B). Indeed, a high level of *CO* transcripts was detected at the 2-cotyledon stage in the *lhp1-1* mutant whereas the level of *CO* expression was very low in the wild type at the same stage. Later during development, the levels of *CO* expression were not significantly different in the mutant compared to wild type. Therefore, ectopic or increased *CO* expression could be partly involved in the early floral transition of the mutant.

A characteristic subnuclear localisation of LHP1 in foci

To understand the cellular action of LHP1, we analysed its localisation within the cell. The presence of five nuclear localisation signals (NLS) and two chromo domains strongly suggested a nuclear localisation for LHP1. To test for nuclear targeting, translational fusions of *LHP1* to the GFP marker were made and tested in transient expression assays using tobacco mesophyll protoplasts. To avoid possible conformational artefacts or inactivation of the fluorescent activity due to protein fusions, LHP1 was fused to the N- or C-terminal region of GFP. The orientation of the fusion had no effect on the localisation: both types of protein fusion presented the same pattern. Controls included GFP alone or GFP fused to a plant functional NLS (VirD2 of *Agrobacterium*) (Tinland et al., 1992; Howard et al., 1992; Citovsky et al., 1994) (Fig. 7). As expected, GFP alone was detected both in the cytoplasm and in the nucleus: its small size (26 kDa) allows passive diffusion throughout nuclear pores (Fig. 7A,B). To observe a nuclear confined localisation of GFP, at least one functional nuclear localisation signal was required (Fig. 7C,D). GFP-NLS/VirD2 localisation was uniform throughout the nucleus, including the nucleolus, which likely corresponds to a basic and classical localisation of nuclear proteins (Fig. 7C,D). In contrast, both N- and C-terminal LHP1-GFP protein fusions showed a novel pattern (Fig. 7E-H). LHP1-GFP was targeted to the nucleus suggesting that at least one NLS is functional among the five NLS. Furthermore, a specific subnuclear localisation of LHP1 was observed; a diffuse fluorescence was detected throughout the nucleoplasm but was excluded from the nucleolus. In addition, numerous local accumulations in discrete rounded foci (speckles) were detected. These speckles (approximately 1 μm diameter) were distributed throughout the nucleus with a tendency to accumulate around the nucleolus as shown in serial sections (Fig. 8). Although not precisely determined, their number varied from one protoplast to another.

Additional experiments suggest that the observed localisation of the full-length fusion reflected the localisation of a functional LHP1 protein. Indeed GFP fusions with truncated LHP1 proteins showed different localisation patterns (unpublished data). Furthermore preliminary results of

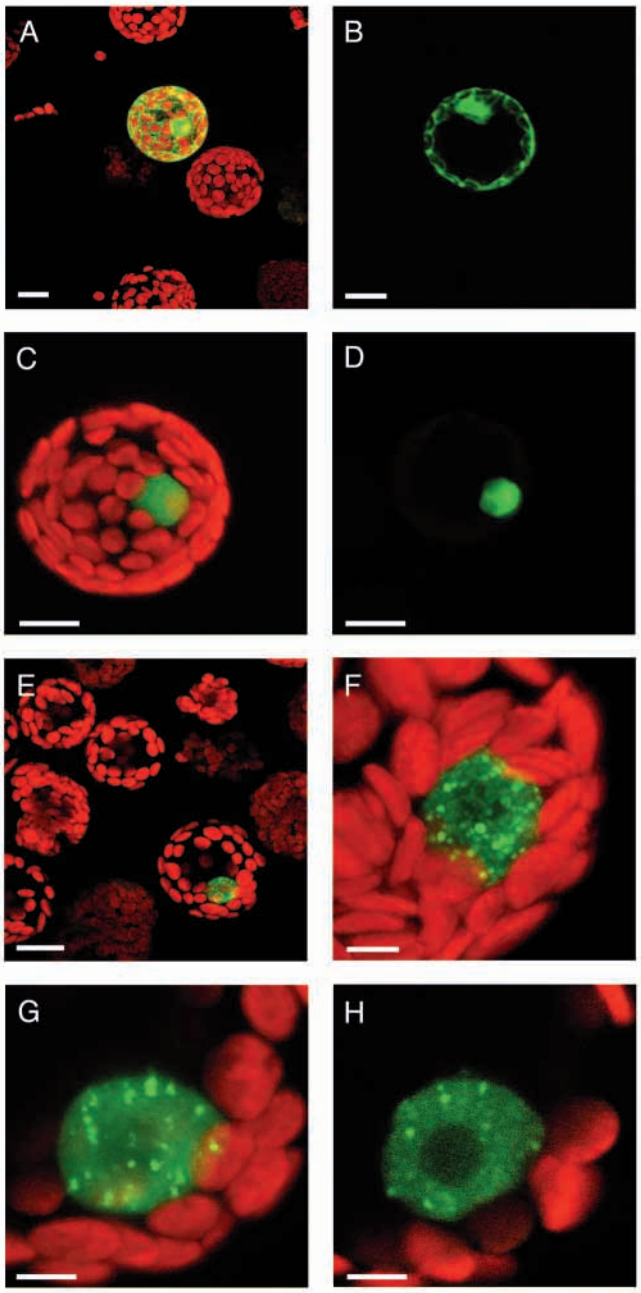

Fig. 7. LHP1 has a specific subnuclear localisation in tobacco mesophyll protoplasts, in transient assays. Chloroplasts appear red and GFP fluorescence is green; when the two fluorescences overlap, the yellow colour appears. (A,B) Protoplasts expressing GFP alone (pAVA121 plasmid). (A) Projection. (B) Section. (C,D) Protoplast expressing GFP-VirD2NLS. (C) Projection. (D) Section. The GFP fluorescence is uniformly distributed throughout the nucleus. (E-H) Protoplasts electroporated with the LHP1-GFP construct. A diffuse nucleoplasmic distribution and discrete particles are observed. (E) Projection of several protoplasts. One protoplast expresses the LHP1-GFP fusion in the nucleus, the others are not transformed. (F) Close-up view of the nucleus (projection). (G-H) Sections. Scale bar, (A-E) 10 μm; (F-H) 2 μm.

complementation experiments of the *lhp1-1* mutant indicate that 35S::LHP1-GFP is able to partly complement the mutant phenotype (unpublished data).

DISCUSSION

In plants, a major developmental change occurs upon transition from the vegetative to the flowering phase. The change occurs when developmental and environmental signals are appropriate (Simpson et al., 1999). By analysing *lhp1* mutants, we showed that chromatin components are likely to be important regulators of this timing and repression mechanism. The *lhp1* mutants have a pleiotropic phenotype with an overall reduction in plant size, organ size, modification of cell expansion and defects in leaf morphology, suggesting changes in other developmental pathways as well. *LHP1* encodes a heterochromatin-like protein, the *Arabidopsis* homologue of the *Drosophila* HP1, with characteristic chromo and chromo shadow motifs.

LHP1 and the chromo domain protein superfamily

LHP1 belongs to the large family of chromo domain proteins, which has emerged during the last ten years. Originally identified as a common motif in heterochromatin protein 1 (HP1) and Polycomb (Pc) from *Drosophila* (Paro and Hogness, 1991), this conserved domain was found in a large variety of proteins from different species such as yeast, nematodes, insects, mammals and plants (Eissenberg and Elgin, 2000). This signature for chromatin association is present in proteins with diverse functions, with specificity being generated through combinations with other motifs. Globally, the chromo domain proteins appear to be either structural components of large chromatin complexes or proteins involved in remodelling chromatin structure (Jones et al., 2000).

Despite a well characterised structure, the function of the chromo domain is still a matter of debate. The chromo domain shows some similarity to two small DNA-binding histone-like proteins found in archeabacteria, but the overall negative surface charge of the MmMOD1 chromo domain does not seem to be compatible with DNA/RNA binding activity (Ball et al., 1997; Zhao et al., 2000). However, a recent study has shown that two chromo domains are protein-RNA interaction modules (Akhtar et al., 2000). It has also been suggested that it is involved in protein-protein interactions, although only few partners have been identified (Cavalli and Paro, 1998; Jones et al., 2000). Recently, it was demonstrated that the HP1 chromo domain interacts with histone H3, a basal and conserved component of the nucleosome particle, through a methylated lysine (Bannister et al., 2001; Lachner et al., 2001). The question is open whether these properties are general features of all chromo domain proteins or restricted to particular subfamilies of the chromo domain superfamily. The conservation of the chromo domains and their residues critical to the 3D structure throughout the plant and animal kingdoms suggests a similar folding of the plant chromo domain as observed in animals and evolutionary conserved interactions and partners, such as methylated histones. The properties and partners of LHP1 require future investigation in *Arabidopsis.*

LHP1 is a typical member of the HP1 subfamily

The chromo domain superfamily can be divided into subfamilies according to the presence of other functional domains. The HP1-like protein subfamily is characterised by the presence of a second related chromo domain, the chromo shadow domain (Aasland and Stewart, 1995). The original member, the *Drosophila* HP1, was identified as a non-histone chromosomal protein associated with centromeres and telomeres but also with discrete regions of euchromatin (James and Elgin, 1986; James et al., 1989; Fanti et al., 1998). *Drosophila* HP1 is also known as the dominant suppressor of

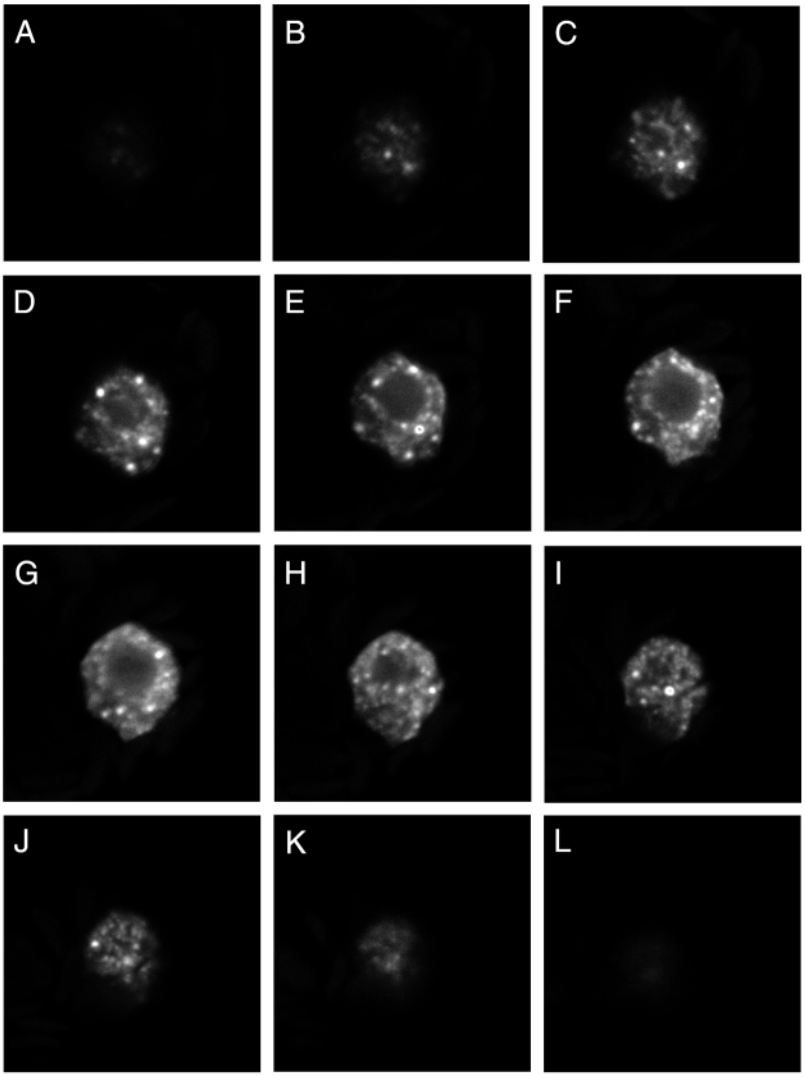

Fig. 8. Sequential confocal optical sections through a nucleus expressing the LHP1-GFP fusion. A diffuse fluorescence was detected in the nucleoplasm but was excluded from the nucleolus. Discrete nuclear bodies were observed which tended to accumulate around the nucleolus.

position-effect variegation (PEV) encoded by the *Su(var)2-5* locus which exerts dosage-dependent effects on PEV (Eissenberg et al., 1990; Eissenberg et al., 1992).

HP1-like proteins have been observed in yeast, insects, fish, amphibians and mammals (Eissenberg and Elgin, 2000). LHP1 is the first example of a functional HP1-like protein reported in plants. The plant HP1-like proteins seem to be larger than their animal counterparts (e.g., *Arabidopsis*, 445 aa; carrot, 392 aa; *Drosophila*, 206 aa), with a longer hinge sequence between the two chromo domains. As in *Drosophila*, the gene is unique in the *Arabidopsis* genome whereas three isoforms have been reported in mouse and man (Saunders et al., 1993; Ye and Worman, 1996; Le Douarin et al., 1996; Minc et al., 1999).

Interaction studies in yeast showed that LHP1 behaves similarly to HP1 and could homodimerise. This is dependent on the presence of a chromo shadow domain, as has been shown for MmMOD1 self-association. The importance of this motif was further highlighted in planta as transformation with a truncated form of LHP1, lacking the C-terminal part of the chromo shadow domain, was not able to rescue the mutant phenotype. In vitro experiments have shown that the MmMOD1 homodimer structure is required for further protein interactions with TIF1β, a transcriptional intermediary factor, and CAF1p150, the large subunit of chromatin assembly factor 1 (Brasher et al., 2000). These results suggest that dimerisation through the chromo shadow domain may be a first step essential for some of the functions of HP1-like proteins, in both the plant and animal kingdoms. Despite a similar mechanism of dimerisation, sequence divergence of plant chromo shadow domains suggests interactions with evolutionary divergent partners.

A subnuclear localisation in foci which suggests multiple targets

LHP1 showed a nuclear localisation, consistent with the presence of the five nuclear localisation signals and the two chromo domains. The localisation of LHP1 fused to GFP revealed both a diffuse nucleoplasmic distribution and discrete particles in interphasic nuclei. In plants, micro-punctuate localisation patterns reminiscent of those observed with LHP1 were only described for the *Arabidopsis* COP1 protein, a key repressor of plant photomorphogenesis and light responses (von Arnim et al., 1998; Stacey and von Arnim, 1999) and for the phytochrome B photoreceptor (Yamaguchi et al., 1999). However, we do not yet know if any of these patterns overlap or are distinct, since their biochemical nature and functions are predicted to differ.

Only a few in vivo localisation studies of HP1 have been reported in interphasic nuclei (Minc et al., 1999; Yamada et al., 1999). The three mammal HP1 isoforms were compared and did not localise exactly to the same positions in the nucleus (Minc et al., 1999). HsHP1α was located in a few masses in condensed chromatin areas, HsHP1β was dispersed in multiple smaller foci, while HsHP1γ localisation was more complex and fluctuated. Some similarities with the in vivo plant pattern can be drawn; for instance, both *Arabidopsis* and human proteins are excluded from the nucleolus. *Arabidopsis* LHP1 localisation pattern seems to be more closely related to the HsHP1β pattern. However, it is difficult to stretch the comparison too far since the organisation of the genomes are not similar, nor are their content in heterochromatin and its dispersion throughout the genome.

In *Drosophila*, similar punctuate patterns of localisation were also described for the Polycomb protein (Dietzel et al., 1999), which is involved in the repression of euchromatic genes by compacting the corresponding chromatin regions. The localisation of the GFP protein fused to the *Drosophila* Pc chromo domain was studied in transgenic tobacco. It was found in distinct nuclear regions, many of which were localised at the nuclear periphery (Ingram et al., 1999). This localisation differs from the LHP1 pattern, probably reflecting differences between the HP1 and Pc chromo domain functions.

We suggest that these discrete particles are enriched in LHP1 proteins, probably associated with various distinct nucleoproteins and represent heterochromatin or heterochromatin-like structures at multiple targets in the genome. The targeting of LHP1 and the functional regions of the protein involved in this process, or the mechanisms involved in foci formation and maintenance require further investigation. The nucleus seems to be a very dynamic but stable organelle, exhibiting plasticity in terms of size, shape, position and maintenance of its compartments (Shaw, 1996; Lamond and Earnshaw, 1998; Misteli, 2001). The diffuse localisation observed for LHP1 could be explained by there being a pool of free nucleoplasmic LHP1 in equilibrium with the assembly of the foci structure. It will be necessary to follow how LHP1 localisation changes in such a dynamic environment and how this is linked to regulation of developmental processes.

Relation between localisation and function

How can the phenotype of the *Arabidopsis lhp1* mutants be interpreted? On the one hand, these mutants show a pleiotropic phenotype with a modification of flowering time and severe defects in plant architecture. On the other hand, the LHP1 protein has structural and functional similarities to animal subunits of heterochromatin involved in higher order chromatin structure, mediating gene silencing.

In the *lhp1-1* mutant, a reduced level of transcription was observed suggesting that the LHP1 protein content is lower than in the wild type. We propose that the absence or a lower content of this plant heterochromatin-like protein might release silencing of a subset of critical genes controlling flowering time, leaf development and general plant architecture, by putting them in a more favourable transcriptional context. To test this hypothesis, we analysed the transcription of *CONSTANS*, a transcriptional activator of flowering time (Putterill et al., 1995). *CO* up-regulation was observed in the *lhp1* mutant at an early developmental stage and could participate in the early flowering phenotype of the mutant, with *CO* being one possible target under LHP1 control although we can not exclude an indirect effect of the mutation on *CO* expression. However, this would be consistent with LHP1 controlling developmental pathways such as flowering transition by mediating gene silencing. This needs to be further tested. Investigating any mis-regulation of other flowering time genes or genes controlling affected pathways may identify other targets or interacting components. Furthermore, LHP1 protein content may be particularly critical at some developmental stages, such as an early vegetative stage, to establish or maintain particular chromatin environments for

gene regulation. Is this lower LHP1 content sufficient to promote flowering? This could involve an interesting dosage effect of LHP1, reminiscent of the *Drosophila* and mouse dosage effect on PEV. Studying the effects of variations in *LHP1* content may provide interesting information. However, *Drosophila* and human *HP1* regulation occurs both at transcriptional and post-transcriptional levels and mechanisms of homeostasis may compensate for induced deregulation in plants.

Because of some common phenotypic characteristics between the *lhp1* and *clf* mutants and their similar modes of action in chromatin architecture, investigation of the interactions between LHP1 and the Polycomb-group gene *CLF* may shed light on how heterochromatin-like complexes regulate gene expression. Does *CLF* recruit *LHP1* to mediate its repression during the vegetative phase? Is this repression differentially mediated later in development? In animals, HP1 interacts with a diversity of partners involved in forming multiprotein complexes associated with higher orders of chromatin organisation (Jones et al., 2000). Therefore, a full understanding of LHP1 awaits many potential partners in the control of *Arabidopsis* development.

We are grateful to the EEC for financial support. T. J. was supported by an INRA fellowship and G. Z. was an assistant professor supported by the Chinese government. We thank Y. Chupeau for his precious support, M. Tepfer, J. Traas, H. Vaucheret for critical reading of the manuscript and D. Bradley and J. Goodrich for interesting discussions. We thank V. Cudo and S. Peyramaure for their contributions to this work, and our colleagues M.-C. Chupeau for advice with protoplast culture, A.-M. Jaunet for SEM support, A. Baudry for useful discussions on the yeast system, J.-D. Faure for the yeast vectors and H. Mireau for the gift of the VirD2NLS. We thank Dr Chae Oh Lim for sending the *B. rapa* clone (PMBBRC, Gyeongsang University, Korea).

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