

# Transgenic Tobacco Plants Expressing the *Drosophila* Polycomb (Pc) Chromodomain Show Developmental Alterations: Possible Role of Pc Chromodomain Proteins in Chromatin-Mediated Gene Regulation in Plants

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The chromodomain of the *Drosophila* Polycomb (Pc) protein has been introduced into tobacco nuclei to determine its location in the nucleus and its effect on plant development. Pc is a repressor of homeotic *Drosophila* genes that shares a well-conserved, although not identical, chromodomain with a structural heterochromatin component, Heterochromatin Protein 1. The chromodomains might therefore play a common role in chromatin repression. An analysis of transgenic plants expressing the Pc chromodomain, which was linked to the green fluorescent protein, suggested that the Pc chromodomain has distinct target regions in the plant genome. Transgenic plants expressing the Pc chromodomain had phenotypic abnormalities in their leaves and flowers, indicating a disruption in development. In axillary shoot buds of plants displaying altered leaf phenotypes, enhanced expression of a homeodomain gene, which is downregulated in wild-type leaves, was found. In *Drosophila*, Pc has been shown to possess distinct chromosome binding activity and to be involved in the regulation of development-specific genes. Our results support the assumptions that the heterologous chromodomain affects related functions in *Drosophila* and in plants, and that chromatin modification mechanisms are involved in the regulation of certain plant genes, in a manner similar to chromatin-mediated gene regulation in *Drosophila*.

## INTRODUCTION

Development of any organism requires differential gene expression comprising the induction and maintenance of expression patterns. Chromatin modification mechanisms can be involved in the control of development processes via the establishment of self-perpetuating alternative chromatin states.

In *Drosophila*, members of the Polycomb protein group (Pc-G) form repressive chromatin complexes maintaining the inactive expression states of homeotic genes and other genes (Paro, 1990; Simon, 1995). Large multiprotein complexes can consist of several members of the Pc-G (Franke et al., 1992), and many target regions are shared by individual Pc-G proteins (DeCamillis et al., 1992). The Pc protein is one of the best characterized Pc-G members. It shares a common chromodomain with Heterochromatin-Associated Protein HP1 (James and Elgin, 1986), a structural component of heterochromatin and a modifier of position-effect variegation (PEV), suggesting that Pc represses homeotic gene activity via formation of heterochromatin-like complexes (Paro and Hogness, 1991). Pc does not bind DNA di-

rectly but is recruited to its chromosomal target regions through protein-protein contacts, and targeting is abolished by mutations of the Pc chromodomain (PCcd; Messmer et al., 1992).

It is unclear whether chromatin-mediated gene regulation is also involved in the regulation of plant gene expression, but some remarkable similarities to chromatin-mediated silencing phenomena have been observed in transgenic plants when transgenes became transcriptionally silenced and hypermethylated. Reminiscent of dominant PEV, the inactive state of methylated plant transgenes could be transferred to homologous sequences that were located at allelic (Meyer et al., 1993) or ectopic (Matzke et al., 1989) positions. PEV-like variegation was observed for a marker gene that was inserted together with a genomic region that provided a hot spot of de novo methylation (Ten Lohuis et al., 1995). Because the transcriptionally silenced state of a transgene correlates with a condensed and less accessible chromatin structure (Van Blokland et al., 1997), a possible explanation for these phenomena is that transcriptional silencing in plants involves local condensation of chromatin that prevents access of *trans*-acting factors to the transgene and that homology-based transfer of silenced states is mediated by a transfer of chromatin components that form

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condensed complexes. If this assumption is correct, then it is likely that plants use chromatin modification as a tool to regulate the expression of endogenous genes as does *Drosophila*.

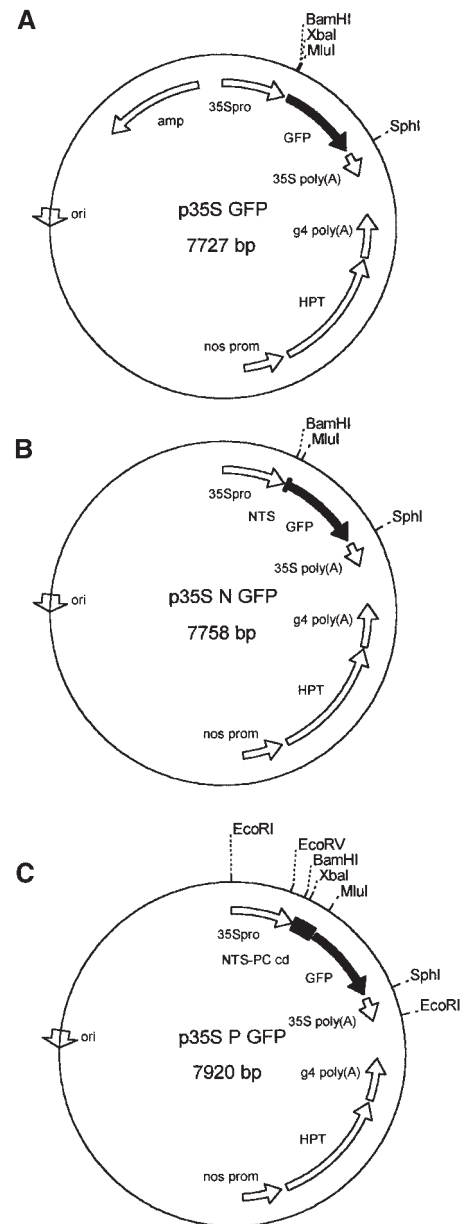
To address this possibility, we tested the effect of the PCcd in plants. In a recombinant protein expressed in tobacco, the PCcd was linked to a nuclear targeting sequence and to the green fluorescent protein (GFP) domain that served as a marker for localization of the recombinant protein (Chalfie et al., 1994). Whereas control GFP proteins were uniformly dispersed within the nucleus, the PCcd-GFP fusion protein showed a nonrandom localization to multiple nuclear regions. We could only obtain transformants with relatively low expression levels, indicating a lethal effect of strong expression of the PCcd construct. Moreover, some lines that expressed the PCcd construct showed developmental abnormalities and increased expression of a new homeodomain gene in axillary shoot buds of phenotypically altered leaves. The implications of these findings for a role of chromodomain proteins in gene regulation are discussed.

## RESULTS

### The PCcd Directs the Recombinant GFP to Defined Nuclear Regions

The PCcd sequence was fused to a GFP construct that contained a nuclear targeting sequence (Figures 1A to 1C). As a control, the GFP construct with the nuclear target sequence alone was used. The constructs were inserted into a plant transformation vector that contained a hygromycin resistance cassette, and the resulting constructs p35S P GFP and p35S N GFP (Figure 1) were transferred into protoplasts via direct gene transfer (Kartzke et al., 1990). In both constructs, the GFP coding sequence is transcribed by the 35S promoter of the cauliflower mosaic virus. In p35S N GFP, the GFP coding sequence is fused in frame to a nuclear targeting sequence (N), and in p35S P GFP, a PCcd region is inserted between N and GFP. To test the localization of the recombinant GFPs in the nucleus, we regenerated transgenic lines and used transformants expressing the GFP constructs to study GFP-specific expression in the nucleus of trichomes (Figure 2).

Figures 2A to 2D show examples of light microscopy and fluorescence microscopy of trichomes. Transformants expressing the control construct p35S N GFP displayed uniform distribution of GFP-specific fluorescence within the nucleus, indicating that the GFP does not localize at specific nuclear regions (Figures 2E to 2G and 3A to 3F). In plants transformed with the p35S P GFP construct that produced a recombinant GFP linked to the PCcd, we failed to detect any candidates that expressed the transgene at equally strong levels. We had observed strong transgene expression in some of the transformants expressing the GFP control constructs.



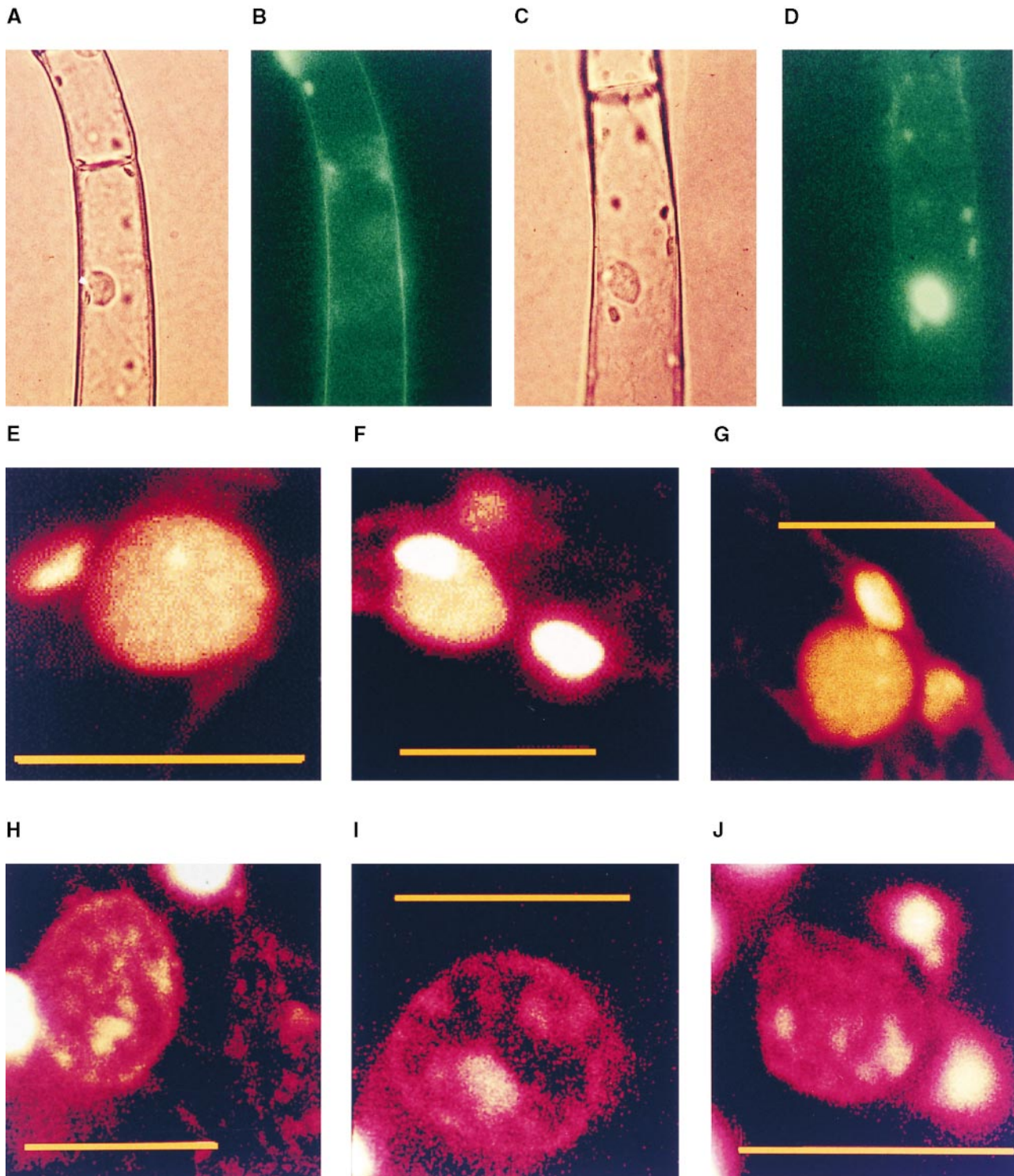
**Figure 1.** Transformation Vectors.

(A) In p35S GFP, the GFP coding sequence (GFP) was cloned between the 35S promoter (35Spro) and polyadenylation sequence (35S poly[A]) next to a hygromycin resistance gene (HPT) under the control of the nopaline synthase promoter (nos prom) and the gene 4 polyadenylation region (g4 poly[A]).

(B) Fusion of the GFP sequence to a nuclear targeting domain sequence (NTS) produced the control vector p35S N GFP, which was used to assay nuclear localization of GFP.

(C) Vector p35S P GFP contains the chromodomain of Pc (PC cd) inserted between the NTS and the GFP sequences.

Open vectors indicate genes or gene control units. Solid vectors indicate GFP and GFP fusion open reading frames. amp, ampicillin resistance gene; ori, origin of replication.



**Figure 2.** Localization of Recombinant GFP Proteins in the Nucleus of Transgenic Tobacco Cells.

(A) to (D) Light microscopy ([A] and [C]) and fluorescent light microscopy ([B] and [D]) of trichome cells of an untransformed control plant ([A] and [B]) and a plant expressing p35S N GFP ([C] and [D]). (D) shows that the GFP is targeted to the nucleus.

(E) to (G) Confocal microscopy of nuclei from p35S N GFP transformants. GFP-specific fluorescence is shown in yellow. Autofluorescence of the chloroplasts is also displayed in yellow and serves as a reference for the intensity of the fluorescent signals. The GFP is uniformly distributed within the nucleus.

(H) to (J) Confocal microscopy of nuclei from p35S P GFP transformants. The GFP linked to the PCcd localizes to distinct nuclear regions.

Bars in (E) to (J) = 10 μm.

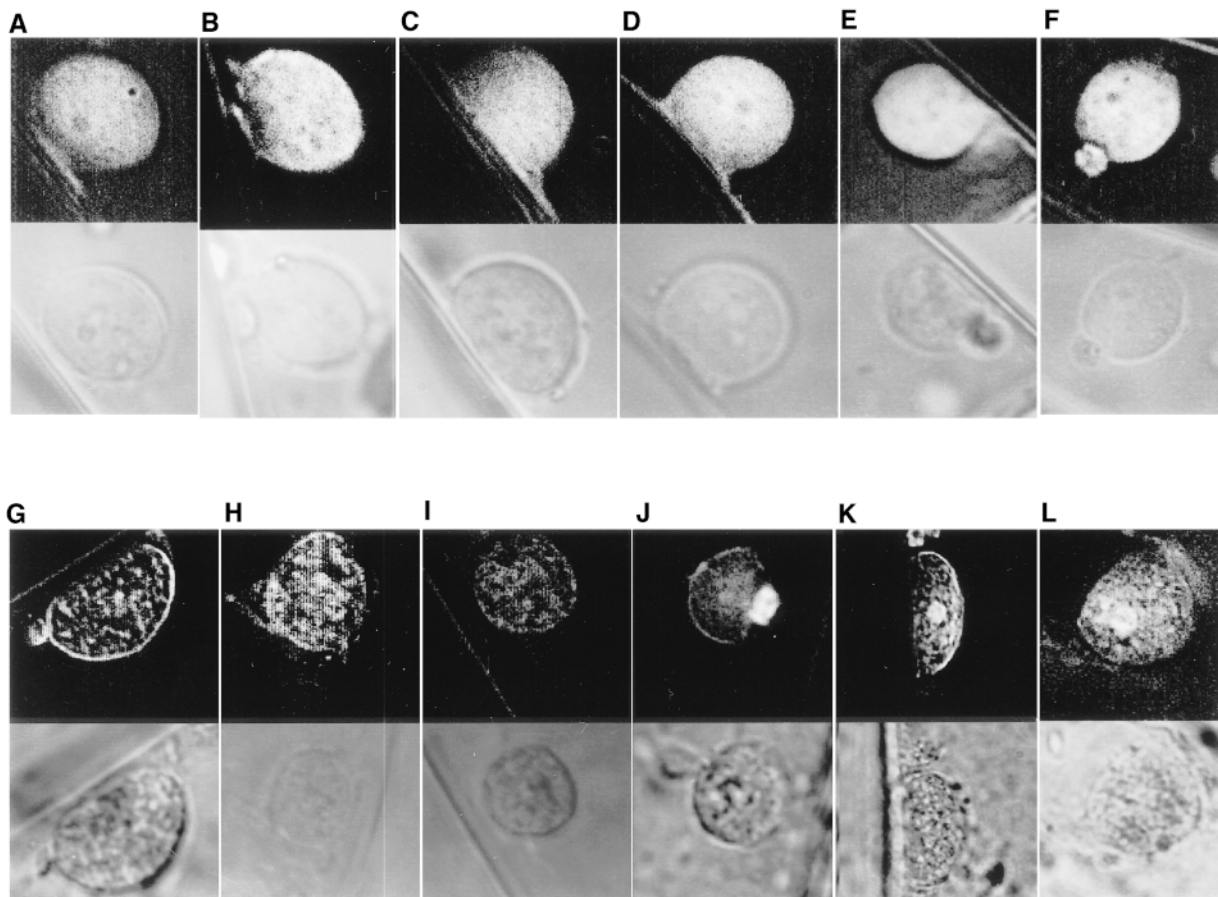
Figures 2H to 2J show examples of p35S P GFP transformants with weak GFP fluorescence in the nucleus. GFP-specific fluorescence is not universally distributed within the nucleus but is localized to specific regions, many of which are located at the periphery of the nucleus (Figures 2H to 2J and 3G to 3L). From the nonuniform distribution of GFP within the tobacco nuclei, we conclude that the PCcd has multiple target regions.

### Plants Expressing the PCcd Construct Show a Variety of Developmental Abnormalities in Leaves and Flowers

P35S P GFP transformants showed either stable or variegated expression of the transgene. Among the transformants,

we found plants with a single transgene copy integrated into the genome or with several integrated transgenes that were rearranged or truncated (Figure 4). In particular, the latter ones do not always show uniform expression of the transgene but display variegated GFP patterns. This is in accordance with the observation made in other transgenic plants that multicopy transgenes have an increased tendency to become silenced (Meyer and Saedler, 1996).

The lack of strongly expressing p35S P GFP transformants indicates a possible lethal effect of higher doses of the PCcd-GFP protein. This assumption is strengthened by phenotypic analysis of p35S P GFP transformants. In line with variable expression of the p35S P GFP construct, silencing of the construct correlated with the development of branches similar to those of plants with a wild-type pheno-

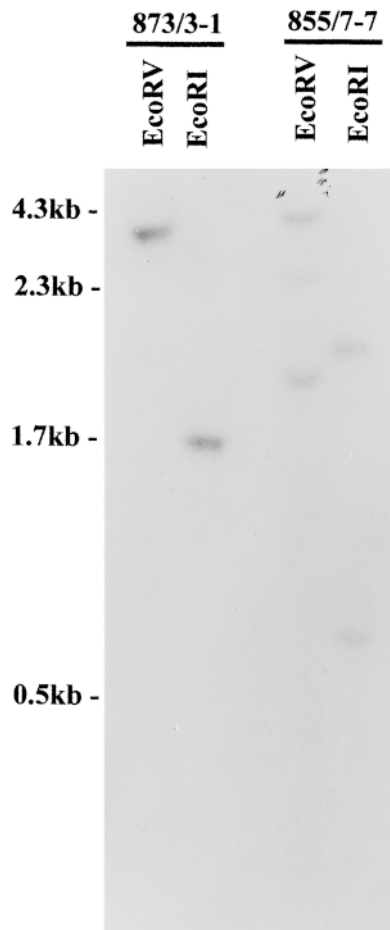


**Figure 3.** Deconvoluted Fluorescence and Light Microscopy of Nuclei of p35S N GFP Transformants and p35S P GFP Transformants.

(A) to (F) Nuclei from control plants show uniform distribution of GFP within the nuclei of trichome cells of fully expanded leaves ((A) to (C)) and trichome cells of young leaves ((D) to (F)).

(G) to (L) Nuclei from plants expressing GFP linked to the PCcd show GFP localization to defined nuclear regions, especially in the nuclear periphery. Staining of nucleoli is preferentially observed in young leaves ((J) to (L)), indicating developmental stage-specific changes of localization patterns.

In (A) to (L) at top, deconvoluted fluorescence is shown, with light microscopy images shown below. In (A) to (F), p35S N GFP transformants are shown. In (G) to (L), p35S P GFP transformants are shown.



**Figure 4.** DNA Gel Blot Analysis of Transgenic Plants.

DNA from transformant 873 (line 3-1) and transformant 855 (line 7-7) was analyzed. To determine the integrity of the transgene, we digested genomic DNA with EcoRI that cuts left and right of the GFP construct. To determine the number of integrated copies, we digested the DNA with EcoRV that cuts once in the transferred vector. The location of these restriction sites is shown in Figure 1C. Hybridization with a transgene-specific probe shows that plant 873 from line 3-1 contains a single transgene copy with a complete 1.7-kb EcoRI GFP fragment, whereas plant 855 from line 7-7 contains three partly truncated transgene copies. Numbers at the left indicate marker fragment sizes in kilobases.

type. However, in branches that expressed the construct, leaves developed abnormally, producing thin, corkscrew leaves and flowers that failed to produce normal petals, which indicates that PCcd-GFP accumulation can interfere with developmental processes.

A more detailed analysis of p35S P GFP lines revealed a variety of phenotypical abnormalities in leaves and flowers (Figure 5 and Table 1). These pleiotropic phenotypes occurred randomly in a background of normally developed tis-

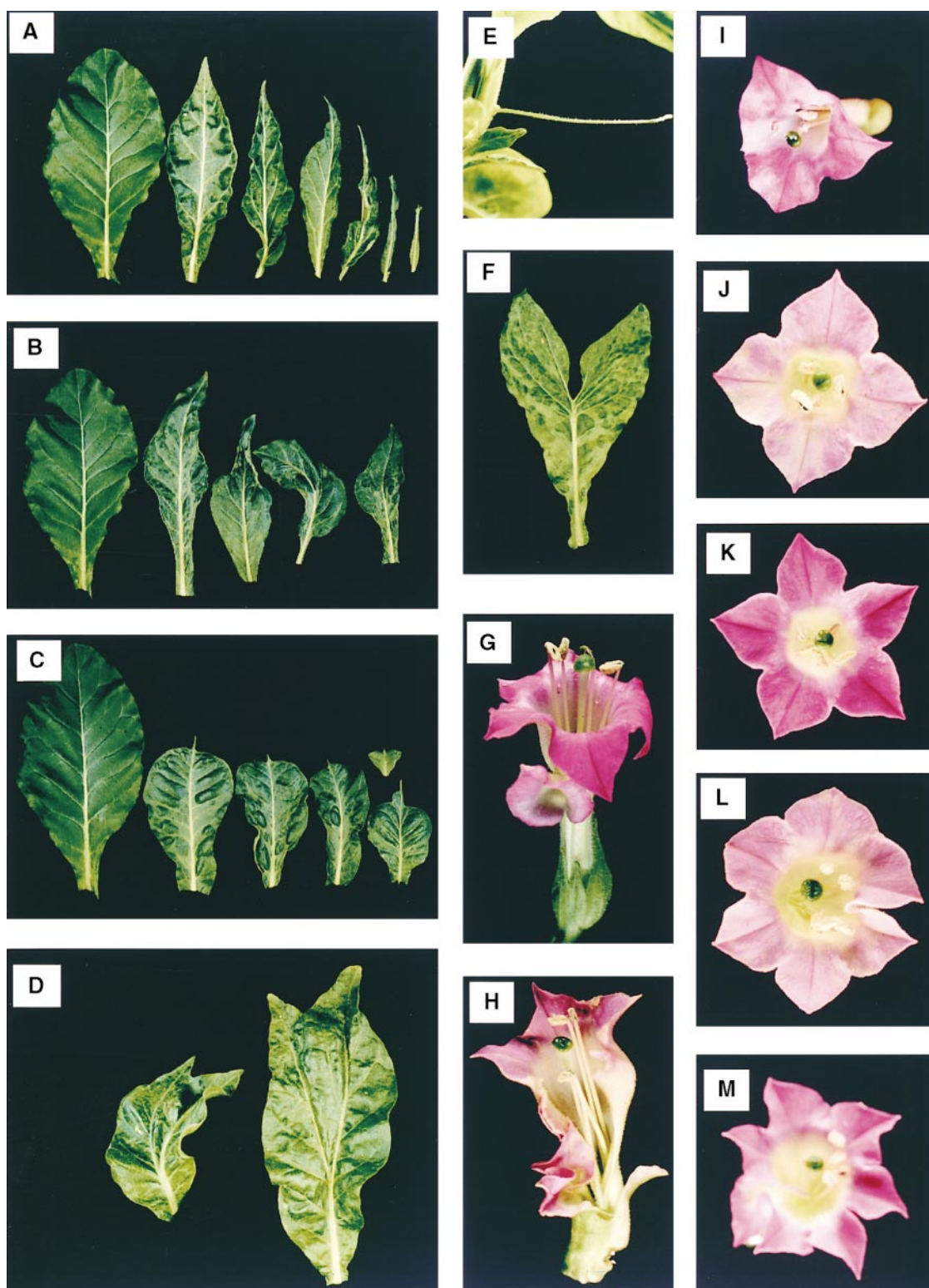
sue at frequencies of 21 to 36% for leaf phenotypes and 1 to 2% for flower phenotypes, but they were not observed in SR1 control plants or in  $F_1$  progeny plants that did not contain the transgene or did not express it (Table 1). Transformants expressing the PCcd-GFP construct also showed an increase in the percentage of floral buds that dropped off before maturity from an average of ~5% in control lines to 22 to 26% in expressing transformants (Table 1). Among the leaf modifications, we observed thin leaf or needlelike phenotypes (Figures 5A and 5E), with a reduced or absent lamina, bent- or curled-leaf phenotypes (Figure 5B), broad-leaf phenotypes (Figures 5C and 5F) displaying shortened leaves with a broad lamina in the upper region of the leaf, and double-vein phenotypes (Figure 5D) producing leaves with two vein systems. Abnormal flower phenotypes included homeotic mutations (Figures 5G and 5H) and changes in organ number, which ranged from three to seven (Figures 5I, 5J, 5L, and 5M), in contrast to five organs in wild-type plants (Figure 5K).

Scanning electron microscopy showed that leaves with needlelike phenotypes failed to produce a leaf blade, although midrib-specific cells were unaltered (Figure 6B), and that those with broad-leaf phenotypes showed some characteristic alterations in the blade. In comparison to wild-type tissue (Figures 6A and 6C), the numbers of cells per area was reduced in broad leaves. For the epidermis, an enlargement of cells compensated for this, whereas in the palisade parenchyma, cells had lost their close spacing and their characteristic columnar shape, and spongy mesophyll cells were separated by large air space regions (Figure 6D). We conclude from these data that a major cause of the developmental abnormalities appears to be a partial or complete deficiency in the initiation of leaf blade development or in the proper differentiation of blade-specific tissue.

To assess the correlation of phenotypic abnormalities with the expression of the transgene, we analyzed  $F_1$  progeny plants derived from a cross of one line with SR1 (Figure 7 and Table 2). Five progeny plants expressed the transgene, four of which showed the characteristic abnormal leaf phenotypes, and three of these four plants also showed a small percentage of abnormal flowers. None of the plants that did not express the transgene produced an abnormal phenotype, but we also could not detect any significant abnormalities in one of the expressing plants, line 14 (Table 2). In agreement with our previous observations (Table 1), we conclude that expression of the PCcd construct is necessary but not always sufficient for the development of abnormal phenotypes. Leaf phenotypes occur at a higher frequency than do floral phenotypes and can appear in some plants without being accompanied by floral phenotypes.

Because it has been suggested that leaf mutants might respond to temperature because of chromatin effects (Pickett et al., 1996), we grew  $F_1$  plants at different temperatures, but we did not observe any significant differences in the frequencies at which leaf mutant phenotypes occurred (data not shown).





**Figure 5.** Pleiotropic Effects of PCcd Expression.

Transformants expressing the PCcd-GFP construct displayed a variety of leaf- and flower-specific phenotypes that varied in intensity.

**Table 1.** Frequencies of Leaf and Flower Phenotypes in Transgenic Lines and SR1 Control Plants

Line <sup>a</sup>	No. of Plants	Average No. of Leaves	Average No. of Abnormal Leaves	Abnormal Leaves (%)	Average No. of Flowers	Average No. of Abnormal Flowers	Abnormal Flowers (%)	Average No. of Floral Buds	Average No. of Buds Dropped	Dropped Buds (%)
SR1	3	16.3	0	0	82.7	0	0	87.6	5	5.7
3-1 hom. ex	6	12.5	3.33	26.6	57.0	0	0	76.6	20	26.1
8-1 hom. ex.	12	14.1	5.09	36.8	64.7	0.92	1.41	88.5	19.6	22.1
8-1 het. ex.	5	14.8	3.20	21.6	58.4	1.02	2.05	68.7	17	24.7
8-1 het. not ex.	23	14.3	0	0	60.2	0	0	76.2	3.7	4.9

<sup>a</sup>F<sub>1</sub> plants derived from a cross of SR1 with transformants that are either homozygous (hom.) or heterozygous (het.) for the transgene were grouped according to the expression (ex) or lack of expression (not ex.) of the transgene.

### PCcd Transformants Show Altered Expression of a Homeodomain Gene

The phenotypic alteration suggested that the PCcd fusion protein could interfere with relatively early steps in the regulation of leaf development. Therefore, we tried to identify endogenous genes that were affected in their expression pattern by the expression of the PCcd construct. Due to the unpredictable appearance of abnormal leaf phenotypes, it was difficult to select young, undifferentiated tissue that would give rise to phenotypic alterations. To increase the likelihood of isolating the appropriate tissue, we selected a transformant that had developed several extremely thin leaves, and we isolated RNA from the axillary shoot buds of the thin leaves (Figures 8A and 8B). As a control, we isolated RNA from axillary shoot buds from SR1 that were of similar size and were located in a comparable stem region.

Considering the role of Pc in its regulation of homeotic genes in *Drosophila*, homeodomain genes could be possible candidates that might be affected by expression of the PCcd. Because no leaf-related homeodomain genes have been described for SR1, we designed primers for a homeobox consensus sequence based on the homeobox se-

quences from five other species (see Methods). After isolating and cloning the polymerase chain reaction (PCR) products that had been amplified by the consensus primers, we found that the consensus primers had amplified 346 and 352 bp of two different homeodomain sequences, which we termed Nt-HD1 and Nt-HD2. The two genes are closely related, differing by only 11 base substitutions and a six-base insertion. However, these differences were sufficient to design specific primers for both genes to compare their transcript levels in quantitative PCR experiments (Figure 9A). Despite their high degree of similarity, the two genes were differentially affected by the expression of the PCcd construct. Whereas Nt-HD1 expression was moderately reduced, Nt-HD2 transcription was significantly increased (Figure 9A).

Quantitative reverse transcription-PCR analysis of different tissues showed almost identical transcription profiles for Nt-HD1 and Nt-HD2 (Figures 9B, 10B, and 10C). Highest transcription levels were found for both genes in flowers, internodes, and young shoot buds, whereas barely any expression occurred in leaves (Figure 10). It is possible that PCcd complexes may be involved in the differential gene expression, at least of Nt-HD2, and that the expression of the PCcd

**Figure 5.** (continued).

(A) to (C) Modified leaf phenotypes, with wild-type leaves shown at far left. In the thin-leaf phenotype (A), the leaf lamina was reduced compared with the midrib. In extreme forms of the thin-leaf phenotype, leaves were reduced to a midrib only, generating a needle phenotype shown in (E). In some leaves, the reduction of leaf lamina development was more pronounced on one side or in the top region of the leaves, which caused bent or curled phenotypes (B). In the broad-leaf phenotypes (C), leaves were shortened with a broad lamina in the upper region of the leaf. Midrib size was reduced due to a shortened distance between branching veins.

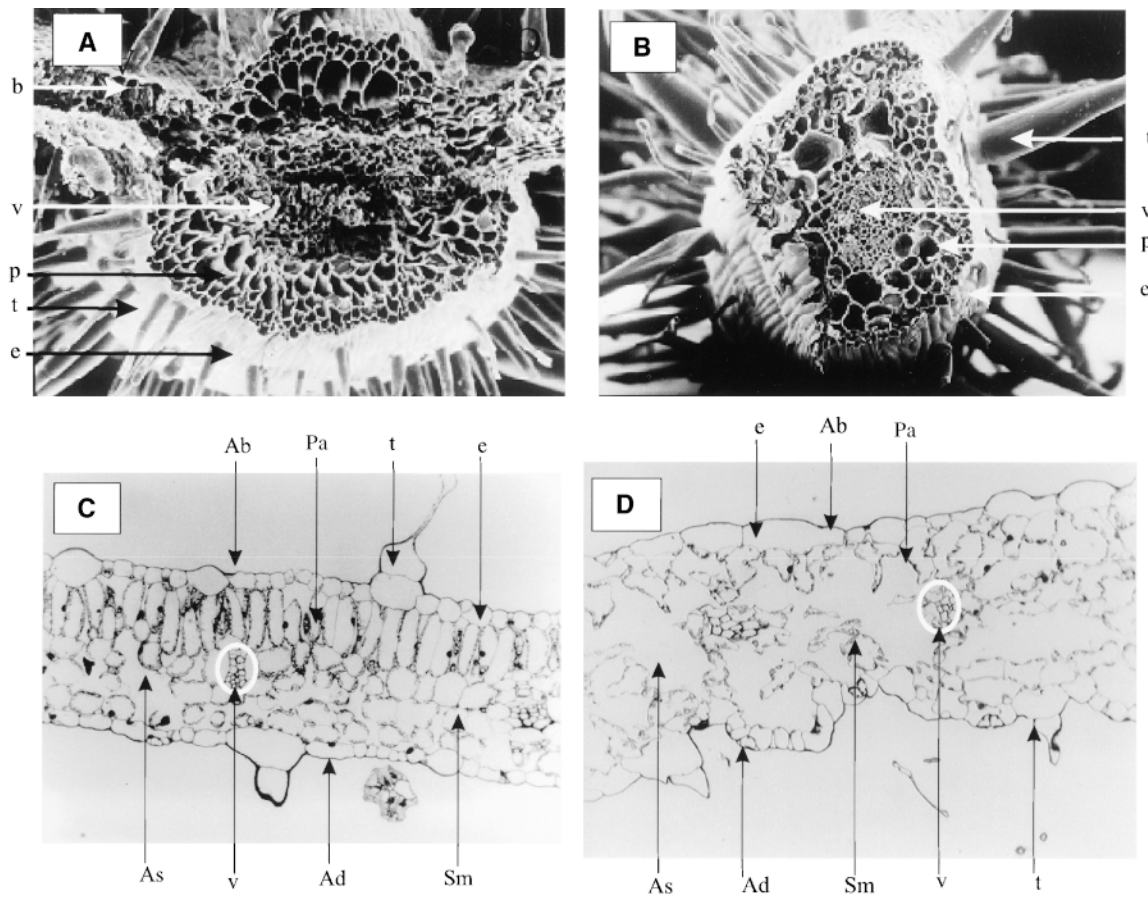
(D) In the double-vein phenotype, two vein systems emerged from the base and separated into two leaf tips.

(E) In the needle phenotype, leaves contain only the midrib but no blade.

(F) An extreme form of the broad-leaf phenotype with a strong reduction of the midrib that is "overgrown" by the leaf lamina.

(G) and (H) Among the abnormal flower phenotypes, we observed homeotic mutations with a stamen converted into a petal (G) or a sepal converted into a petal (H).

(I) to (M) Other phenotypes affected organ numbers. In contrast to the tobacco wild-type flower that contains five petals (K), we found flowers with three (I), four (J), six (L), or seven (M) petals.



**Figure 6.** Histological Analysis of the Effects of PCcd Expression on Leaf Anatomy.

(A) and (B) Scanning electron microscopy of a transverse section of a wild-type leaf and a needle leaf, respectively, reveal no significant differences in the anatomy of cells in the midrib, whereas the leaf blade is not formed in the needle phenotype.

(C) and (D) Transverse sections through the blade of a wild-type leaf and a broad leaf show disruptions of the tissue context in the broad leaf. Epidermal cells are enlarged, palisade cells are distorted, and palisade and spongy mesophyll cells are unorganized and separated by large air space regions.

Ab, abaxial surface; Ad, adaxial surface; As, air spaces; b, blade; e, epidermis; p, parenchyma; Pa, palisade; Sm, spongy mesophyll; t, trichome; v, vascular tissue.

interferes with this regulation, thus inducing changes in the expression levels.

## DISCUSSION

The *Drosophila* proteins HP1 and Pc share significant similarity in their chromodomains, which suggests that there is a common mechanistic basis for the two proteins in establishing repressive chromatin complexes. Pc regulates silencing of homeotic genes as part of a large multiprotein complex (Paro, 1990). The PCcd has no DNA binding activity, but it mediates the recruitment of Pc to distinct chromosomal

sites through protein-protein interactions (Mueller, 1995). The repressive Pc complexes that mediate homeotic silencing involve cooperative interactions of multiple Pc-G proteins, because antibodies against the Pc protein precipitate a multimeric protein complex also containing other members of the Pc-G family (Franke et al., 1992). The high degree of cooperative interactions makes the complex highly sensitive to initial concentrations of components and leads to an all-or-nothing character of complex formation (Pirota and Rastelli, 1994).

We were interested in examining the potential role of PCcd proteins in the establishment of chromatin complexes in plants. Transcriptionally silenced transgenes can acquire highly methylated (Matzke et al., 1989; Meyer et al., 1994)



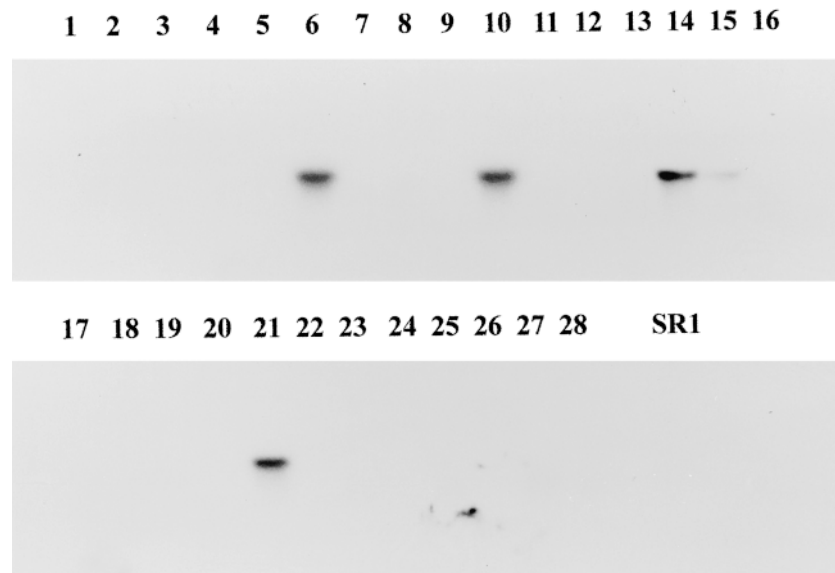
and condensed chromatin states (Van Blokland et al., 1997) that can be transferred to ectopic or allelic homologous sequences (Matzke and Matzke, 1995), which is reminiscent of chromatin-mediated inactivation of homologous sequences in *Drosophila* (Dreesen et al., 1991; Dorer and Henikoff, 1994). These observations suggested that condensed chromatin complexes could exert a similar function in the regulation of gene expression in plants as in *Drosophila*, and chromodomain-containing proteins might also be involved in complex formation in plants. If this assumption is correct, we expect that recombinant proteins containing a PCcd should bind to the chromodomains of nuclear chromatin complexes that are associated with defined nuclear regions. Moreover, if Pc-specific chromodomain complexes are involved in the regulation of homeotic or other development-specific plant genes, then binding of a recombinant PCcd protein might interfere with the sensitive cooperative nature of such complexes, which could lead to abnormal expression of plant genes that are regulated by chromatin complexes.

Our results appear to support this assumption. When linked to the PCcd, a GFP reporter protein is targeted to distinct nuclear regions, suggesting that the PCcd finds interacting partner molecules in the plant nucleus. PCcd proteins appear to localize preferentially to the nuclear rim, and the localization pattern appears to change for different developmental stages because nucleoli are especially labeled in younger leaves.

Random insertion of transgenes into the plant genome

usually generates transformants with a wide spectrum of expression intensities of the transgene. GFP transformants showed this expected range of expression, and even plants with the highest expression levels did not display any developmental abnormalities. In contrast, only PCcd transformants could be isolated that expressed relatively low levels of the transgene, which indicates selection against strongly expressed transgenes due to a lethal effect of the PCcd protein, if we assume that the PCcd transcript is not specifically degraded post-transcriptionally. Among the PCcd transformants, we found a variety of developmental abnormalities that strictly correlate with expression of the transgene, because branches of transformants in which the transgene had been silenced developed normally. Our data suggest that the PCcd protein interferes with complex formation at multiple loci and that the different phenotypes resemble a stochastic interference with chromatin-mediated control of multiple development-specific genes.

The phenotypes of the PCcd-expressing plants suggest that chromodomain proteins are especially involved in the control of genes involved in leaf development and, to a lesser extent, flower development. The homeotic phenotypes observed in the flower of PCcd transformants indicate that one group of target genes might be the homeotic flower genes or genes upstream of the pathway responsible for the expression of homeotic flower genes. The predominant effect of PCcd expression, however, is the alteration of leaf development.



**Figure 7.** Segregation Analysis of Abnormal Phenotypes in an  $F_1$  Population Derived from a Cross of a Transformant with an SR1 Wild-Type Plant.

Expression analysis of the transgene in the 28 progeny plants is shown. Phenotype analysis for the 28 plants is provided in Table 2. Among the five plants expressing the transgene, four display phenotypic abnormalities, indicating that transgene expression is necessary but not always sufficient for phenotype generation.

**Table 2.** Segregation Analysis of Abnormal Phenotypes<sup>a</sup>

Plant	Abnormal Flowers (%)	Abnormal Leaves (%)
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	28	4.6
7	0	0
8	0	0
9	0	0
10	14	0
11	0	0
12	0	0
13	0	0
14	0	0
15	35	3.2
16	0	0
17	0	0
18	0	0
19	0	0
20	0	0
21	35	1.5
22	0	0
23	0	0
24	0	0
25	0	0
26	0	0
27	0	0
28	0	0

<sup>a</sup>Plants derived from a cross of a transformant with an SR1 control plant. An expression analysis for the transgene is shown in Figure 7.

The first Pc group gene described for plants was *CURLY LEAF (CLF)* (Goodrich et al., 1997). CLF, which does not contain a chromodomain, is required for repression of transcription of the floral homeotic gene *AGAMOUS* in Arabidopsis leaves, inflorescence stems, and flowers. Like the PCcd transformants, *clf* mutants display pleiotropic effects on flowers and leaves, but development of roots, hypocotyl, or cotyledons is not affected, which suggests a common participation of different Pc-G proteins in the formation of certain complexes, similar to Drosophila (Kennison, 1995). Recently, a chromodomain has been found in a "chromomethylase" (CMT1) of Arabidopsis, where it is embedded within the catalytic region of a predicted DNA methyltransferase (Henikoff and Comai, 1998). Considering a common role for chromodomain proteins in Drosophila and plants, the DNA methyltransferase activity might be used in plants to support the maintenance of repressive expression states of homeotic genes.

A possible target gene for repressive chromodomain complexes might be the Nt-HD2 gene. Nt-HD2 is specifi-

cally repressed in leaf tissue of control plants, and enhanced expression has been observed in young axillary buds of PCcd transformants when compared with those of wild-type plants. It is tempting to speculate that the controlled repression of Nt-HD2 is a prerequisite for differentiation of leaf-specific cell types and that the abnormal phenotypes observed for PCcd transformants may therefore be the result of an inefficient or delayed downregulation of Nt-HD2. In accordance with this model, several homeodomain genes have been shown to follow a similar expression pattern as Nt-HD2, being downregulated as leaf primordia are initiated (Jackson et al., 1994; Lincoln et al., 1994).

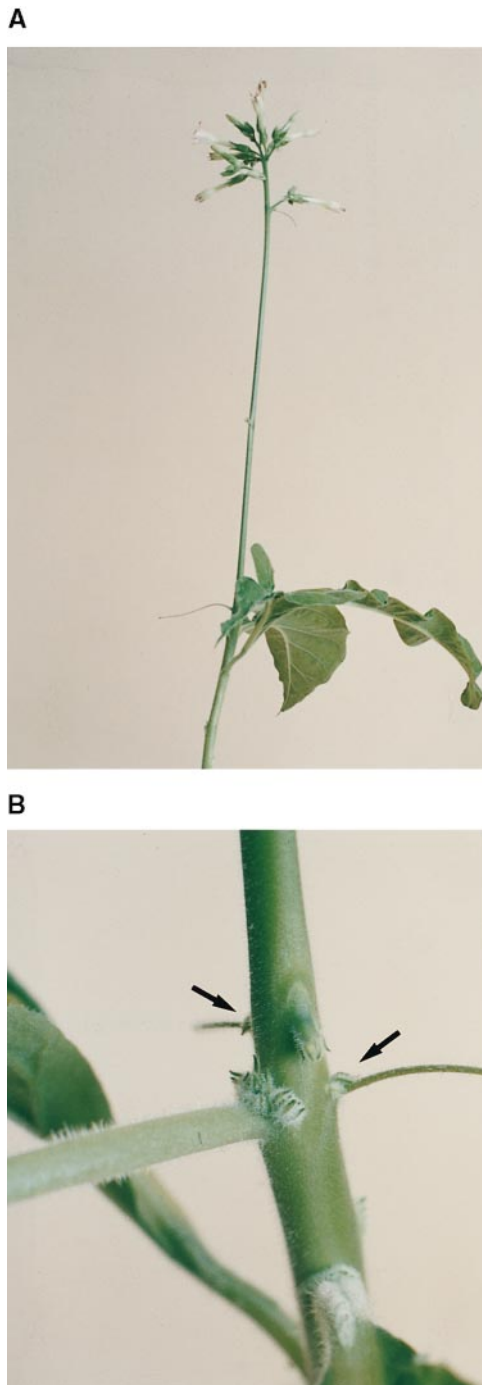
Moreover, expression of homeodomain genes, such as *OSH1* of rice (Matsuoka et al., 1993), *KNOTTED1* and *KNAT1* in Arabidopsis (Lincoln et al., 1994), or *Rs1* of maize (Schneeberger et al., 1995), causes abnormal leaf morphology and altered cell fates, which is reminiscent of the effects created by PCcd expression. For *Rs1* and *KNOTTED1*, which change the fate of the blade cells to sheath when ectopically expressed, it has been proposed that these homeodomain proteins repress genes that specify blade identity or retard the acquisition of blade identity by competing with other homeodomain proteins required for blade fate (Freeling, 1992), or that they promote an indeterminate state of leaf cells, preventing them from acquiring a blade fate (Hake, 1992). In accordance with this model, a delay in Nt-HD2 suppression could repress or delay blade development or differentiation of blade-specific cell types, causing the observed bladeless phenotypes or the reduction of blade-specific cell types.

Our data suggest that the PCcd interferes with the expression of homeobox genes, like Nt-HD2, either by targeting the recombinant PCcd protein to genomic regions that harbor such genes or by binding to cellular proteins involved in the regulation of homeodomain genes. Expression of the PCcd interferes with developmental processes; this resembles the function of the PCcd in Drosophila and suggests that plant proteins containing a PCcd have a similar role in chromatin-mediated gene regulation as the members of the Pc family in Drosophila. This strengthens the assumption that chromatin complexes in plants are used in the repression of certain homeodomain genes and that the specificity of the chromodomain is conserved in plants and Drosophila. The identification of target regions of chromodomain complexes should therefore be a major goal for future experiments to elucidate the details of chromatin-mediated gene regulation in plants.

## METHODS

### Construction of Expression Vectors

The basic expression vector p35S GFP was constructed by inserting the 714-bp open reading frame (ORF) of the green fluorescent



**Figure 8.** Axillary Buds from Which RNA Was Isolated for Reverse Transcription-PCR Experiments.

(A) A transformant that produced a number of extreme thin-leaf phenotypes in the upper part of the stem.

(B) A close-up of two thin leaves with arrows labeling the axillary buds that were used for RNA isolation.

protein between the 35S promoter and polyadenylation region of a hygromycin resistance plant transformation vector. The GFP ORF was isolated by polymerase chain reaction (PCR) from plasmid pGFP10.1 (Chalfie et al., 1994), which was kindly provided by M. Chalfie (Columbia University, New York). The PCR was performed with a 5' GFP primer, 5'-GGTCTAGACGCGTCGTCATCTGTTATGAGTAAAGGAGAAGAAGAACTTTTC-3', and a 3' GFP primer, 5'-GGGCATGCTTATTTGTATAGTTCATCC-3'. In the PCR product that was generated with these primers from pGFP10.1, the GFP ORF was preceded by 15 bp encoding the RRASV recognition sequence for the catalytic subunit of cAMP-dependent protein kinase from heart muscle (Kaelin et al., 1992). This sequence serves as a spacer to separate GFP from the Polycomb (Pc) chromodomain (PCcd) in the translational fusion and as a potential tag for kinase labeling of translational fusion proteins. The PCR product was restricted with XbaI and SphI and inserted into the XbaI-SphI-restricted hygromycin resistance transformation vector (Figure 1A).

The PCcd was isolated by PCR from plasmids containing the cDNAs of Pc (Paro and Hogness, 1991) kindly provided by R. Paro (University of Heidelberg, Germany). The primers for isolation of the PCcd were 5' PCCR, 5'-GGGGATCCAAGCTTACAATGGCTCCCA-TGTACGCGG-3', and 3' PCCR, 5'-GGACGCGTGATGTCGATG-GAGCGGCG-3'. PCR of the cDNA plasmid with the two primer sets produced a 0.2-kb fragment with BamHI and MluI sites at the ends, encoding the PCcd, preceded by the MAPKKRNV nuclear localization signal of simian virus 40 (Van der Krol and Chua, 1991). The PCR product was restricted with BamHI and MluI and inserted into BamHI-MluI-restricted p35S GFP, resulting in plasmid p35S P GFP (Figure 1C).

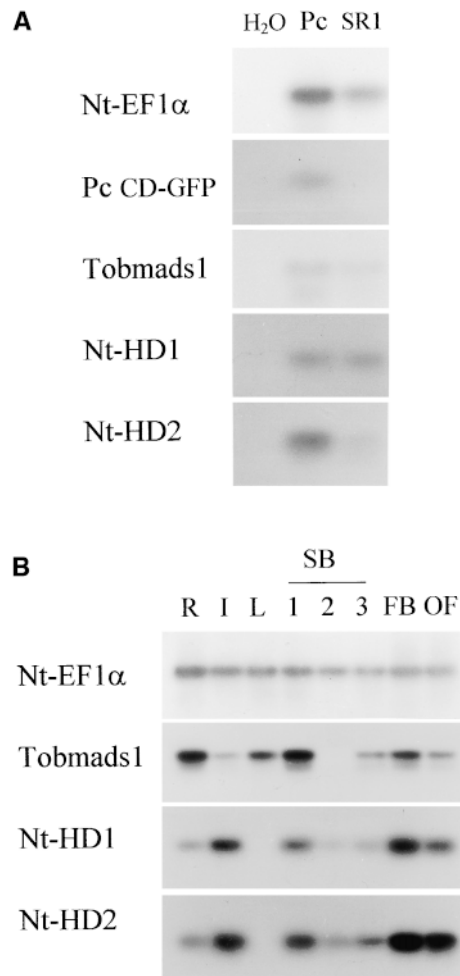
To construct a control vector, we changed p35S N GFP, which contained the nuclear localization signal fused to the GFP ORF, the XbaI site in p35S P GFP, into an MluI site via PCR. The resulting MluI fragment encoding the chromodomain was deleted by MluI restriction and religation of the remaining vector, which gave the control p35S N GFP (Figure 1B). Sequencing confirmed that in p35S N GFP, the nuclear localization sequence was linked in frame to the GFP sequence.

### Electron Microscopy

Tissue samples from *Nicotiana tabacum* cv SR1 were fixed overnight in 5% glutaraldehyde, washed twice for 10 min in distilled H<sub>2</sub>O, and dehydrated in an ethanol series for 30 min each in 20, 40, and 60% ethanol, with dehydration overnight in 80% ethanol and an additional 30 min of dehydration in 100% ethanol. Tissues for sections were immersed in propylene oxide for 2 hr and overnight in 50% propylene oxide plus 50% Araldite (Sigma-Aldrich, Poole, UK), followed by 6 hr in 25% propylene oxide plus 75% Araldite, and finally embedded in 100% Araldite, which was cured at 60°C overnight. Sections of 0.5 mm were cut with a glass knife on a microtome and stained with toluidine blue. Tissue for scanning electron microscopy had the ethanol removed by critical point drying; samples were mounted and sputter coated in gold.

### Analysis of GFP Expression

Thin slivers of live tobacco material were mounted in water and examined using a  $\times 100$  lens. GFP was excited at 395 nm by using a monochromator (Photonics, Planegg, Germany) and visualized



**Figure 9.** Reverse Transcription-PCR Was Used to Determine Transcript Levels of Four Classes of Endogenous Genes.

Total RNAs from different tissues were isolated and tested for the presence of transcripts of Nt-EF1 $\alpha$ , which served as an internal control, the Tobmads1 transcript, and the Nt-HD1 and Nt-HD2 transcripts. PCR products were blotted and probed with the corresponding sequenced PCR products. A control containing H<sub>2</sub>O instead of RNA shows the absence of contamination during reverse transcription-PCR.

**(A)** Total RNA was extracted from axillary shoot buds of SR1 and Pc-transformed plants (Figure 8). The presence of the transcripts corresponding to the transgene (Pc CD-GFP) was tested by using reverse transcription-PCR as well. Whereas Nt-HD1 transcript levels are moderately reduced in Pc plants, Nt-HD2 transcript levels are significantly increased.

**(B)** Total RNA was prepared from roots (R), internode parts of the stem (I), leaves (L), 1- to 2-mm-long shoot buds (SB1), 2- to 5-mm-long shoot buds (SB2), 5- to 10-mm-long shoot buds (SB3), flower buds (FB), and open flowers (OF).

through a 515- to 565-nm band pass filter. Pictures were obtained with a CCD camera (model C4880; Hamamatsu, Hamamatsu-city, Japan) and deconvoluted using an Improvion software package (Improvion, Coventry, UK).

#### RNA Isolation and Reverse Transcription-PCR

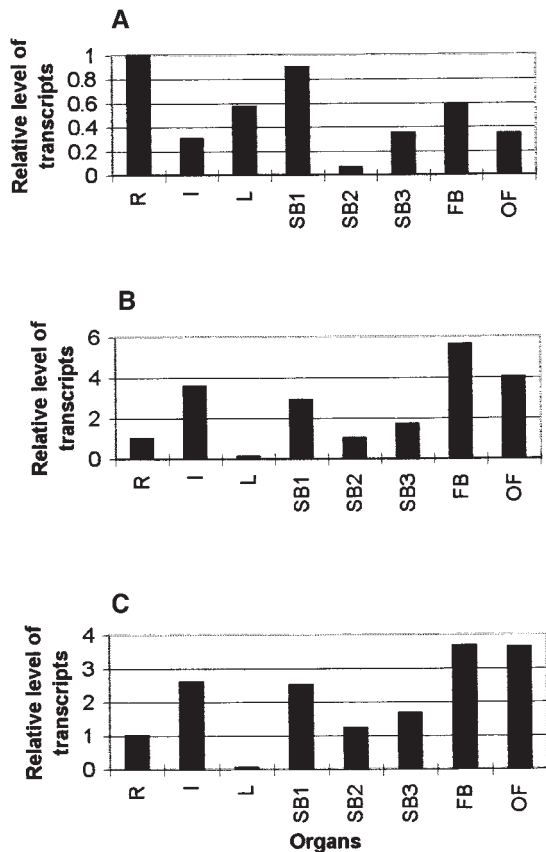
The different tissues analyzed were collected from tobacco plants grown in the greenhouse. Fully developed leaves, internodes of stem, flower buds, and open flowers were collected and frozen at once in liquid nitrogen. Shoot buds at different developmental stages were collected the same way: stage 1, 1 to 2 mm long; stage 2, 2 to 5 mm long; and stage 3, 5 to 10 mm long. Roots were collected after washing them under tap water and drying them quickly with paper tissues, and they were immediately frozen in liquid nitrogen. Axillary buds from phenotypically modified leaves and SR1 control leaves were collected from adult plants grown in the greenhouse. RNA was prepared using the RNeasy plant mini kit from Qiagen (Chatsworth, CA).

Multiple transcript analysis by reverse transcription-PCR was performed according to Sambrook et al. (1989), with some modifications. Ten micrograms of total RNA was subjected to DNase I treatment (40 mM Tris-HCl, pH 7.5, 6 mM MgCl<sub>2</sub>, 20 units of RNA-guard RNase inhibitor, 7.5 units RNase-free DNase I [Pharmacia]) at 37°C for 30 min. The RNAs were purified using the RNeasy kit. For reverse transcription, 2  $\mu$ g of DNA-free RNA was incubated for 1 hr at 37°C with 160 units of Moloney murine leukemia virus H superscript reverse transcriptase (Gibco BRL) in a 30- $\mu$ L reaction mixture containing 100 pmol of oligo(dT)<sub>12-18</sub>, the corresponding buffer (Gibco BRL), 10 mM DTT, 0.8 mM deoxynucleotidetriphosphate, and 14.4 units of RNAGuard.

Equal amounts of cDNAs corresponding to one-tenth of the reaction mixture were used for amplification in 100  $\mu$ L of a PCR mixture containing 1  $\times$  PCR buffer (Appligene, Durham, UK), 0.12 mM deoxynucleotidetriphosphate, 50 pmol of the required 5' and 3' primers, and 0.5 units of Taq polymerase (Appligene).

The amplification of the cDNA coding for the elongation factor (EF1 $\alpha$ ; GenBank accession number X16430) (Kumagai et al., 1995) served as a control for cDNA synthesis and PCR efficiency in the different samples. For EF1 $\alpha$  cDNA amplification, we used oligonucleotide EF3(5'), 5'-AGACCACCAAGTACTACTGC-3', which is localized on exon 1, and oligonucleotide EF2(3'), 5'-GTCAAGAGCCTC-AAGAAGAG-3', which is localized on exon 2. For the amplification of the Tobmads1 cDNA (GenBank accession number X76188) (Mandel et al., 1994), we used the oligonucleotides Tob1, 5'-TAAAGACAA-AGTTCAACCTG-3', and Tob2, 5'-AGCTAAACAGGTGCTTAAC-3'.

The PCR amplification of the transgene cDNA was performed using the oligonucleotide P1, 5'-TAACTAGTACAATGGCTCCAAG-3', which is specific for the nuclear target sequence region, and oligonucleotide P4, 5'-TAGGATCCCGATGAGGCGGCGATCCAGG-3', which is specific for the PCcd. To amplify the Nt-HD cDNAs, we designed the degenerated oligonucleotides HD1 and HD2 from a consensus sequence obtained by comparison of homeobox sequences of rice (GenBank accession number D16507), barley (GenBank accession number Af022390), maize (GenBank accession number X61308), apple tree (GenBank accession number Z71979), tomato (GenBank accession number U32247), and *Arabidopsis thaliana* (GenBank accession number U32344). The sequence of HD1 is 5'-GA(C,T)-CAGTTCATGGA(G,T)GC-3', and the sequence of HD2 is 5'-TA(A,T)-GGCCA(C,T)TTGTA(G,T)TG-3'. The two clones identified after cloning the Nt-HD PCR products were named Nt-HD1 and Nt-HD2. Spe-



**Figure 10.** Histograms Showing the Relative Levels of Transcripts in SR1 Plants.

For each experiment, after subtracting the data obtained for the background signal, all the data were standardized on the corresponding Nt-EF1 $\alpha$  value. The values obtained for each organ are shown in relation to the root values that were arbitrarily set as 1. Abbreviations are as given in the legend to Figure 9.

(A) Tobmads1.

(B) Nt-HD1.

(C) Nt-HD2.

cific oligonucleotides were designed for each of them: HD3, 5'-CTCTGAATCTGCTCTAG-3', was used with oligonucleotide HD2 to amplify specifically Nt-HD1, and oligonucleotide HD4, 5'-TGTGAACTGTGTCAGC-3', was used with oligonucleotide HD2 to amplify specifically Nt-HD2.

Amplifications were performed using the following conditions: Nt-EF1 $\alpha$ , 20 and 25 cycles at 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min; Tobmads1, 25 and 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; PCcd-GFP cDNA, 35 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; Nt-HD, 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; Nt-HD1, 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min; Nt-HD2, 30 cycles at 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min.

The products of the PCR experiments were subjected to electrophoresis through a 1.5% agarose gel and transferred to a nylon membrane (Amersham). Filters were hybridized with Nt-EF1 $\alpha$ , Tobmads1, and Nt-HD probes obtained after cloning and sequencing of the PCR products.

The sequences of the cloned PCR products for Nt-HD1 and Nt-HD2 have EMBL accession numbers AJ006179 and AJ006180.

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