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An Arabidopsis Mutant with a Reduced Level of *cab140* RNA Is a Result of Cosuppression

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We analyzed a mutant of Arabidopsis with a severely reduced level of *cab140* RNA. This mutant, named *lct* for *l*ow level of *cab140* *t*ranscript, was obtained during a selection for phytochrome signal transduction mutants. The selection was based on reduced expression of the tumor morphology shoots gene (*tms2*), an introduced counter-selectable marker under the control of the *cab140* promoter. Expression of the introduced *cab140*::*tms2* gene was also greatly reduced in *lct*, but surprisingly, expression of other phytochrome-regulated genes was not comparably affected. Furthermore, the *lct* phenotype could not be separated genetically from the T-DNA insert; thus, we suggest that this phenotype was caused by cosuppression of the introduced construct and the endogenous *cab140* gene, and that the mutation causing the cosuppression was located on the T-DNA insert. In vitro nuclear transcription experiments demonstrated that the suppression was occurring at the level of transcription. We also found that the suppressed *cab140* genes were not significantly more methylated than the nonsuppressed *cab140* genes.

INTRODUCTION

One approach to studying the function of a plant gene is to overexpress the gene in a host plant and observe the resulting phenotype. This approach has been successful in many cases (Boylan and Quail, 1989; Kay et al., 1989; Keller et al., 1989; Martineau et al., 1989; Tepperman and Dunsmuir, 1990; Wagner et al., 1991), but there have been a number of instances in which the introduction of a gene into the chromosome resulted in silencing of both the introduced and the endogenous gene. The first examples described were the result of introducing a chalcone synthase (*chs*) gene into petunia. The *chs* gene product is involved in pigment production in the petals, and the expected result of *chs* overexpression is a dark purple petal, but, instead, many plants with white petals were recovered (Napoli et al., 1990; van der Krol et al., 1990). These plants had a greatly reduced level of *chs* transcript, and the expression of the endogenous *chs* gene showed both somatic and genetic reversion, demonstrating that the gene was not permanently altered. This phenomenon was termed cosuppression. Although observations of cosuppression are becoming common in plant research (Matzke et al., 1989; Elkind et al., 1990; Smith et al., 1990; Goring et al., 1991; Gottlob-McHugh et al., 1992; Hart et al., 1992), there is only one report of a cotransfected sense gene suppressing gene expression in mammalian cells (Cameron and Jennings, 1991).

Three mechanisms that have been proposed to explain the occurrence of cosuppression are: (1) the unexpected production of antisense RNA, (2) methylation of the cosuppressed promoters, and (3) ectopic pairing of the repeated sequences of the genome (reviewed in Jorgensen, 1991). An antisense transcript could suppress gene expression post-transcriptionally by inhibiting RNA processing, transport from the nucleus, or translation. It is also possible that an antisense transcript could repress transcription by interaction with the genomic promoter region. An antisense *chs* transcript has been observed in a cosuppressed petunia line, but its function in gene suppression has not been proven (Mol et al., 1991). Promoter methylation has been correlated with silencing of an introduced gene after transformation with a second homologous gene (Matzke et al., 1989); however, methylation of a repressed native gene has not yet been reported. Evidence for ectopic pairing of nonhomologous chromosomes exists in yeast (Jinks-Robertson and Petes, 1985) and in Neurospora (Selker et al., 1987). In the case of cosuppression, repeated sequences in the chromosome have been hypothesized to interact in such a way that transcription is prevented. The many examples of cosuppression may not all result from the same phenomenon; thus, a detailed analysis of a number of cosuppressed lines is required to evaluate the potential mechanisms of gene suppression. We have undertaken such an analysis using a cosuppressed line isolated in Arabidopsis.

Our laboratory has been involved in the development of a counter-selection strategy in Arabidopsis for the isolation of signal transduction mutants defective in phytochrome-regulated chlorophyll *a*/*b* binding protein (*cab*) (recently renamed

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Lhcb1; Jansson et al., 1992) gene expression. The phytochrome-responsive Arabidopsis *cab140* gene promoter (−1326 to +14 with +1 being the start of transcription) was fused to the tumor morphology shoots gene (*tms2*) (also known as the indoleacetamide hydrolase [*iaaH*] gene; Klee et al., 1987), which converts nontoxic naphthalene acetamide (NAM) into toxic naphthalene acetic acid (NAA). This gene was introduced into Arabidopsis via T-DNA transformation, and a homozygous line with multiple copies of the fusion construct at a single site of insertion was isolated. When seedlings of this line were germinated and grown on NAM, red light treatment led to increased expression of the *tms2* gene and thus to growth inhibition (Karlin-Neumann et al., 1991). This line was mutagenized with ethylmethane sulfonate (EMS), and we sought to identify potential mutants in the phytochrome signal transduction pathway that would result in decreased activation of the *cab140* promoter after red light treatment. Such mutant seedlings would be expected to grow taller on NAM with red illumination because they would synthesize less of the *tms2* gene product and thus would produce less of the toxic product NAA.

As described in this report, an initial screening of M_2 seeds resulted in the isolation of a mutant with severely reduced levels of transgenic *cab140*::*tms2* RNA and endogenous *cab140* RNA. Genetic analysis strongly suggested that this line is not a phytochrome signal transduction mutant but most likely is a result of a mutation in the T-DNA insert that causes cosuppression. Further analysis of this line showed that the cosuppression of the *cab140* genes occurs at the level of transcription.

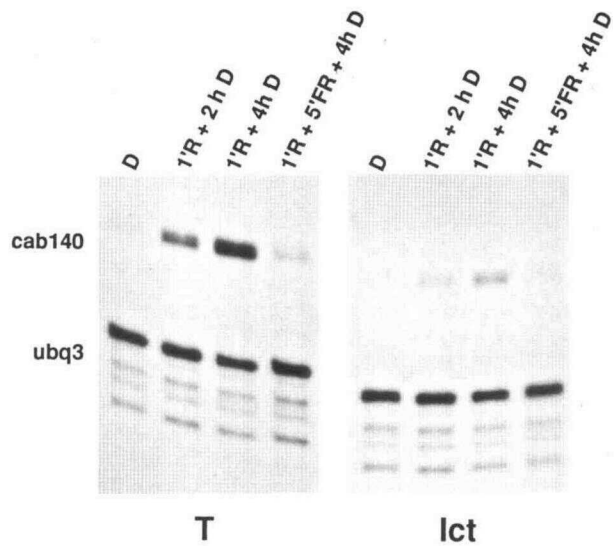

Figure 1. *cab140* RNA Levels Are Greatly Reduced in the *lct* Mutant.

Levels of *cab140* and *ubq3* RNA were determined by RNase protection analysis using 5 μg of total RNA from each sample. Seedlings were grown for 5 days in darkness, then left untreated (D), or given the light treatments described above each lane (R, red; F, far-red), and then returned to darkness for the times indicated. T, parental line transformed with a T-DNA containing the *cab140*::*tms2* gene fusion; *lct*, mutant line selected from M_2 seeds of the T line.

RESULTS

Isolation of the *lct* Mutant

We performed the counter-selection for phytochrome signal transduction mutants on the M_2 seedlings of the mutagenized transgenic line described in the Introduction. We screened 75,000 seedlings for resistance to NAM when grown for 5 days with intermittent red light (Karlin-Neumann et al., 1991) and selected 159 seedlings that had noticeably longer hypocotyls. The M_3 progeny recovered after selfing of these potential mutants were rescreened for NAM resistance to eliminate false positives. They were also screened for resistance to the auxin product NAA, because auxin-resistant mutants were an expected byproduct of the screen. Of the 159 M_3 lines tested, 86 (54%) were false positives, 36 (23%) were auxin resistant, and 37 (23%) were resistant to NAM but sensitive to NAA.

These 37 NAM^r, NAA^s lines were then tested for phytochrome control of endogenous *cab140* gene expression. Figure 1 shows that one of these lines, *lct* (for *l*ow level of *cab140* *t*ranscript), had a significantly reduced *cab140* RNA level compared to the original transformed line (T), but that the level of a control transcript encoding polyubiquitin (*ubq3*; Norris et al., 1993) was not altered. The *cab140* RNA was still somewhat increased by red light, but the relative increase was 3.5 times less than in the parental line in this experiment. *lct* was backcrossed three times to the parental line, and the reduced levels of *cab140* and *cab140*::*tms2* RNAs were consistently observed in the *lct* F_2 segregants. The mean hypocotyl length of *lct* seedlings was significantly greater (5.6 ± 1.2 mm) than that of the T line (2.5 ± 1.2 mm) when seedlings were grown for 5 days under intermittent red light on the NAM substrate, indicating that the low level of the *cab140*::*tms2* transcript resulted in resistance to NAM.

lct Phenotype

We next tested whether the reduction in the *cab140* RNA level observed in the *lct* mutant was a general phenomenon affecting other phytochrome-regulated genes. Figures 2A to 2C show the levels of *cab140*::*tms2*, *cab140*, *cab165*, *cab180*, small subunit of ribulose bisphosphate carboxylase [*rbcS-1A*], and *ubq3* RNAs after various light treatments. The level of the *ubq3* RNA was equivalent in T and *lct*. In Figure 2A, both *cab140*::*tms2* and *cab140* RNA levels were decreased to a similar extent in *lct*. In the dark, the *cab140* RNA level in *lct* was one-third of that seen in the T line, but after 1 min of red light

(R) or in continuous white light (WL), the level of *cab140* message was reduced to one-seventh of the T line.

Expression of two other closely linked members of the *Lhcb1* gene family is shown in Figure 2B. As previously observed (Karlin-Neumann et al., 1988), the transcript levels for *cab180* and *cab165* are low compared to *cab140* in R-treated seedlings, but all three genes are expressed at similar levels in seedlings grown in WL. The *cab180* RNA levels were identical in *lct* and T, but the *cab165* RNA levels were reduced 2.5-fold in the *lct* mutant after R and in WL. The expression of another phytochrome-regulated gene, *rbcS-1A*, is shown in Figure 2C.

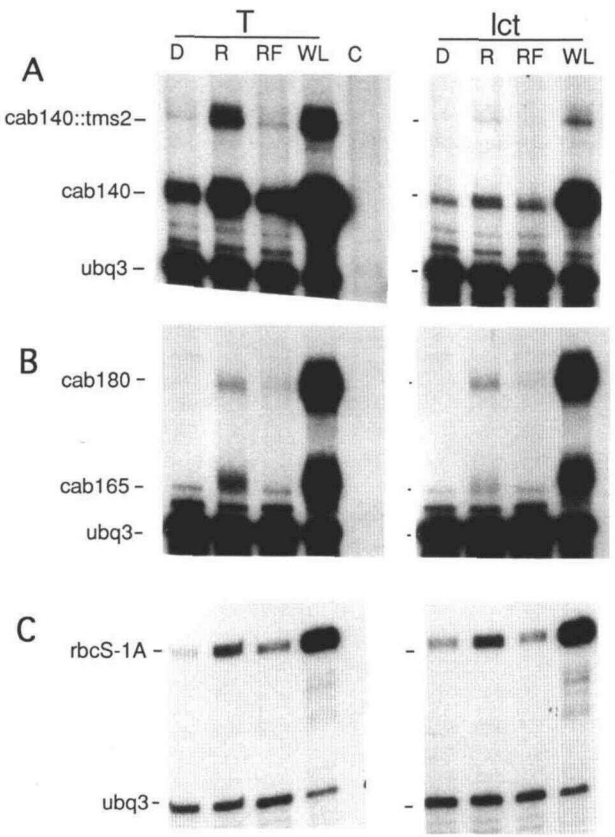

Figure 2. RNase Protection Analysis of Phytochrome-Regulated Transcripts in T and *lct*.

Seedlings were grown for 5 days in darkness, then left untreated (D) or given 1 min of red light plus 4 hr darkness (R), or 1 min of red light immediately followed by 10 min of far-red light plus 4 hr of darkness (RF). Another set of seedlings was grown for 5 days in white light (WL). Five micrograms of total Arabidopsis RNA from each sample plus 10 μg of yeast tRNA or 15 μg of yeast tRNA for control (C) was treated with RNase after hybridization with the indicated probes. The antisense *ubq3* riboprobe was used as a nonphytochrome-regulated control in all lanes.

(A) *tms2* and *cab140*, 14-hr exposure.

(B) *cab180,cab165*, 14-hr exposure.

(C) *rbcS-1A*, 2-hr exposure.

The *rbcS-1A* transcript levels were identical in *lct* and T in all conditions tested. These data show that all members of the *Lhcb1* gene family are not affected equally in the *lct* mutant and that the *lct* mutant is not a result of a lesion in the early stages of phytochrome signal transduction, which would affect all phytochrome-regulated genes.

The *lct* line had no visible phenotype compared to the T line when grown without NAM in WL, long-day (16:8 hr), or short-day (8:16 hr) conditions. Because *cab140* is a member of a gene family encoding the major antenna proteins of photosystem II, we reasoned that the *lct* line might be delayed in chlorophyll accumulation. However, when *lct* seedlings were grown for 3 days in darkness, then transferred to WL, they accumulated chlorophyll at the same rate as the T line (data not shown). This observation demonstrated that the low level of *cab140* RNA (Figures 1 and 2) coupled with normal levels of RNAs from other members of the *Lhcb1* gene family are sufficient for normal rates of greening and normal growth and development.

Mapping the *lct* Mutant

To determine whether *lct* was recessive or dominant, the mutant was crossed to WT (wild type containing a glabrous [*gl1*] marker) and to the parental T line, and the F_1 plants were analyzed for *cab140*::*tms2* and *cab140* RNA levels. If the mutation were recessive, then normal high transcript levels would be expected in the F_1 plants; if dominant, then low transcript levels would be expected. Figure 3 shows the *cab140*::*tms2*, *cab140*, and *ubq3* RNA levels of the T and *lct* lines used in the crosses and of four individual F_1 plants from each cross. *ubq3* was used as a control RNA because its level was not affected in *lct* (Figure 1). The average *cab140*::*tms2* and *cab140* transcript levels (normalized to *ubq3)* are shown at the bottom of Figure 3. The F_1 plants from WT × *lct* had *cab140* RNA levels that were intermediate between the parental lines, indicating that *lct* was semidominant. F_1 plants from T × *lct* had *cab140* RNA levels that were lower than in the individuals from WT × *lct*, suggesting that the T-DNA in the T line had some influence on *cab140*::*tms2* and *cab140* RNA levels when in the presence of *lct*.

lct was mapped with respect to visible markers by crossing to five marker lines (W3, W6, W7, W8, W9), each containing three recessive visible markers on a single chromosome. F_1 plants were allowed to self, F_2 plants displaying visible mutations were grown to maturity, and F_3 seed was collected from these individuals. *cab140* RNA levels were measured in each set of F_3 seedlings and used to score *lct*. If a visible marker is not linked to *lct*, then one-quarter of the F_2 lines (or their F_3 progeny) with this visible marker should have the low levels of the *cab140* transcript characteristic of *lct*. On the other hand, if a marker is linked to *lct*, then less than one-quarter of the F_2 lines (or their F_3 progeny) homozygous for the marker will also display low *cab140* RNA only as a result of

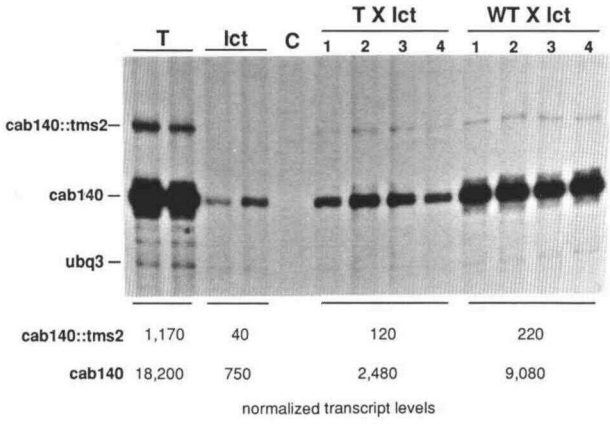

Figure 3. F_1 Progeny from T × *lct* or WT × *lct* Crosses Show Intermediate Levels of *cab140* and *cab140*::*tms2* RNAs.

Five micrograms of total RNA from 3-week-old rosette leaves isolated from individual plants was analyzed by RNase protection for *cab140*::*tms2*, *cab140*, and *ubq3* transcripts. Two individuals from two of the parental lines (T, *lct*) are shown in the first four lanes, and the C lane contains only yeast tRNA. Four F_1 individuals (1 to 4) are shown for each of the crosses. The WT line is a trichomeless mutant (*gl1*) from the Columbia background. The average values of the *cab140*::*tms2* and *cab140* transcript levels (cpm) that have been normalized to *ubq3* (cpm) are shown below the figure.

a crossover between the marker and *lct*. Analysis of F_3 lines showed close linkage of *lct* to the brevipedicellus (*bp*) marker on chromosome 4. The F_3 progeny from 55 individual F_2 *bp* lines from W8 (*bp*, eceriferum-2 [*cer2*], apetala-2 [*ap2*]) × *lct* demonstrated that *lct* was located 1.7 map units from *bp*. Fifty-three of 55 *bp* lines had a normal level of the *cab140* transcript, whereas the other two lines had an intermediate level of *cab140* RNA.

Genetic Linkage between *lct* and the T-DNA

To test whether the *lct* mutation affected *cab140* gene expression independently of the T-DNA insert used to set up the counter-selection, we attempted to separate these two loci genetically. F_3 progeny from 46 F_2 individuals from the WT × *lct* cross were scored both for the presence of the T-DNA insert and for the reduced *cab140* RNA levels characteristic of *lct*. We used resistance to hygromycin, a gene included in the T-DNA insert, to score the presence of the T-DNA. The T-DNA insert was found to segregate in a ratio of 1:2:1 for 2 copies of the T-DNA (n = 9):1 copy of the T-DNA (n = 25):0 copies of the T-DNA (n = 12). The *cab140* RNA levels showed identical segregation, with lines containing 2 copies of the T-DNA having low levels of the *cab140* transcript, lines with 1 copy of the T-DNA having intermediate levels of *cab140* RNA, and lines with no copies of the T-DNA having normal, high levels of *cab140* RNA. Six lines of each type are shown in Figure 4. These data show that *lct* cosegregated with the T-DNA insert.

The presence of the T-DNA was also tested in the progeny of the 55 *bp* F_2 lines discussed above. The T-DNA insert was lacking in the same 53 lines that had the normal *cab140* RNA level, and 1 copy of the T-DNA insert was found in the two lines that had an intermediate level of the *cab140* transcript. Thus, we observed cosegregation of *lct* and the T-DNA insert in 101 of 101 cases, demonstrating that these two loci are closely linked (less than 0.5 map units).

The T-DNA insert in *lct* behaved differently from the T-DNA insert in the unmutagenized parental T line. The original T line had normal levels of *cab140* RNA, and even after mutagenesis other potential mutants did not show the *lct* phenotype. In homozygous *lct* plants, there were 2 copies of the T-DNA from *lct*, and, as seen in Figure 3, *cab140* RNA levels were barely detectable. In the F_1 siblings generated from T × *lct*, again there were 2 copies of the T-DNA, 1 from *lct* and 1 from T; yet the *cab140* and *cab140*::*tms2* RNA levels were threefold higher than in the *lct* homozygotes and threefold lower than in *lct* heterozygotes that had only 1 T-DNA copy. The fact that the T-DNA insert of *lct* acted differently from the T-DNA insert in the T line suggests that the *lct* mutation resides in the T-DNA. The result of this mutation is the cosuppression of both *cab140*::*tms2* and *cab140* gene expression.

DNA Gel Blot Analysis of the *lct* Mutant

We performed DNA gel blot analysis to determine whether any insertions, deletions, or changes in copy number had occurred in the T-DNA insert in the *lct* line. Figure 5A shows a map of the T-DNA insert containing the *cab140*::*tms2* gene fusion as well as a nopaline synthase (*nos*) gene. Probes used for hybridization (Figures 5B to 5D) are shown as thick lines below the map. We determined previously that the T-DNA integrated into the chromosome in a complex pattern which resulted in the presence of multiple copies of the *cab140*::*tms2* gene at a single site of insertion in the T line (Karlin-Neumann et al., 1991). Figure 5B shows that a probe derived from the *nos* gene hybridized to the identical pattern of bands for both T and *lct*, demonstrating that there were no gross rearrangements in the *lct* T-DNA insert. This pattern of bands segregated as a single locus, and the nature of the complexity is not known. Bands corresponding to tail-tail (tt), head-tail (ht), and an internal fragment (i) that hybridized to the probe are indicated.

A probe from the *cab140* promoter region was hybridized to BamHI-digested DNA, and the results are shown in Figure 5C. This probe recognized an 11-kb chromosomal fragment and a 1.5-kb fragment from the introduced gene. The presence of the 11-kb fragment demonstrated that the *cab140* locus was not interrupted in T or *lct*. The intensity of the 1.5-kb band was ~10 times the intensity of the endogenous gene, indicating a copy number of 10 per haploid genome for the introduced gene in both the T and *lct* lines. We do not know the nature of the additional BamHI fragments from WT DNA that hybridize to the *cab140* promoter probe.

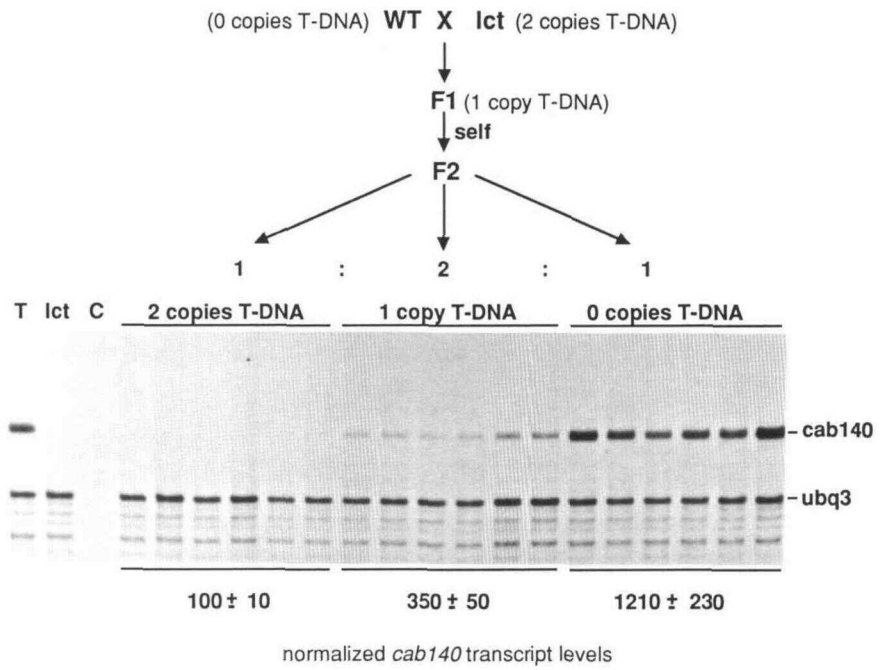

Figure 4. Reduced *cab140* RNA Level Characteristic of *lct* Cosegregates with the T-DNA Insert.

A cross between WT (*gl1*) and the *lct* mutant gave rise to F_1 progeny that were allowed to self-fertilize, producing F_2. Individual F_2 plants were allowed to self, producing F_3 seeds that were tested for resistance to hygromycin (30 μg/mL), a gene located on the T-DNA insert contained in the *lct* line. Segregation of the F_3 with respect to hygromycin resistance was used to determine the genotype of the F_2. Six F_3 populations from F_2 lines containing either 2 copies of the T-DNA insert, 1 copy of the T-DNA insert, or 0 copies of the T-DNA insert were then analyzed by RNase protection for *cab140* and *ubq3* RNA levels. F_3 seedlings and the original T and *lct* lines were grown for 5 days in the dark, treated with 1 min of R, and then returned to D for 4 hr before harvest. RNA levels were determined by RNase protection analysis using 5 μg of total RNA and 10 μg of yeast tRNA. Lane C contains 15 μg of tRNA only. After electrophoresis and autoradiography, protected fragments from *cab140* and *ubq3* were cut out and quantified using a scintillation counter. *cab140* was normalized to *ubq3*, and the average values ± SD are indicated below each group.

cab140 Promoter Regions Are Not Methylated in the *lct* Mutant

Because methylation has been correlated with gene silencing (Matzke et al., 1989), the methylation status of the *cab140* promoters was tested with the methylation-sensitive enzyme MspI, which recognizes the site CCGG but will not digest ^{m}CCGG sites. In plants, cytosines in either the CNG or CG context can be methylated (Grueunbaum et al., 1981). An MspI site is located 740 bp upstream from the start of transcription, and Figure 5D shows that this site was not extensively methylated in the T or *lct* lines for either the introduced (*) or the endogenous *cab140* gene (o). The restriction enzyme HpaII, which recognizes the same site but will not cleave ^{m}CCGG or C^{m}CGG, produced a similar pattern of digestion (data not shown). Thus, although the introduced *cab140* promoter in *lct* was partially methylated, it was not significantly more methylated than in the T line, demonstrating that methylation is not correlated with cosuppression of *cab140*::*tms2* and *cab140* gene expression in this case.

Transcription of *cab140* and *cab140*::*tms2* Is Repressed in the *lct* Mutant

It has been proposed that cosuppression could operate via a post-transcriptional mechanism (Jorgensen, 1991); however, this possibility seemed unlikely in our case because the introduced *cab140*::*tms2* gene contained only 14 nucleotides of the *cab140* transcript. To test whether silencing occurred by a post-transcriptional mechanism, nuclei were isolated from T and *lct* leaves grown for 3 weeks in WL. As shown in Figure 3, *cab140* RNA levels were reduced 25-fold in *lct* compared to T leaves at this time. Nuclei were used in in vitro transcript elongation reactions, labeled transcripts were hybridized to plasmid DNA encoding *tms2*, *rbcS*, or the upstream untranslated copy-specific 69-bp region of *cab140* (Leutwiler et al., 1986), and the results are shown in Figure 6. The *rbcS* gene was used as a control, and its transcription was equivalent in both T and *lct*. However, both the *cab140*::*tms2* and the endogenous *cab140* gene were transcribed at fivefold lower rates in *lct* than in the parental T line, demonstrating that a

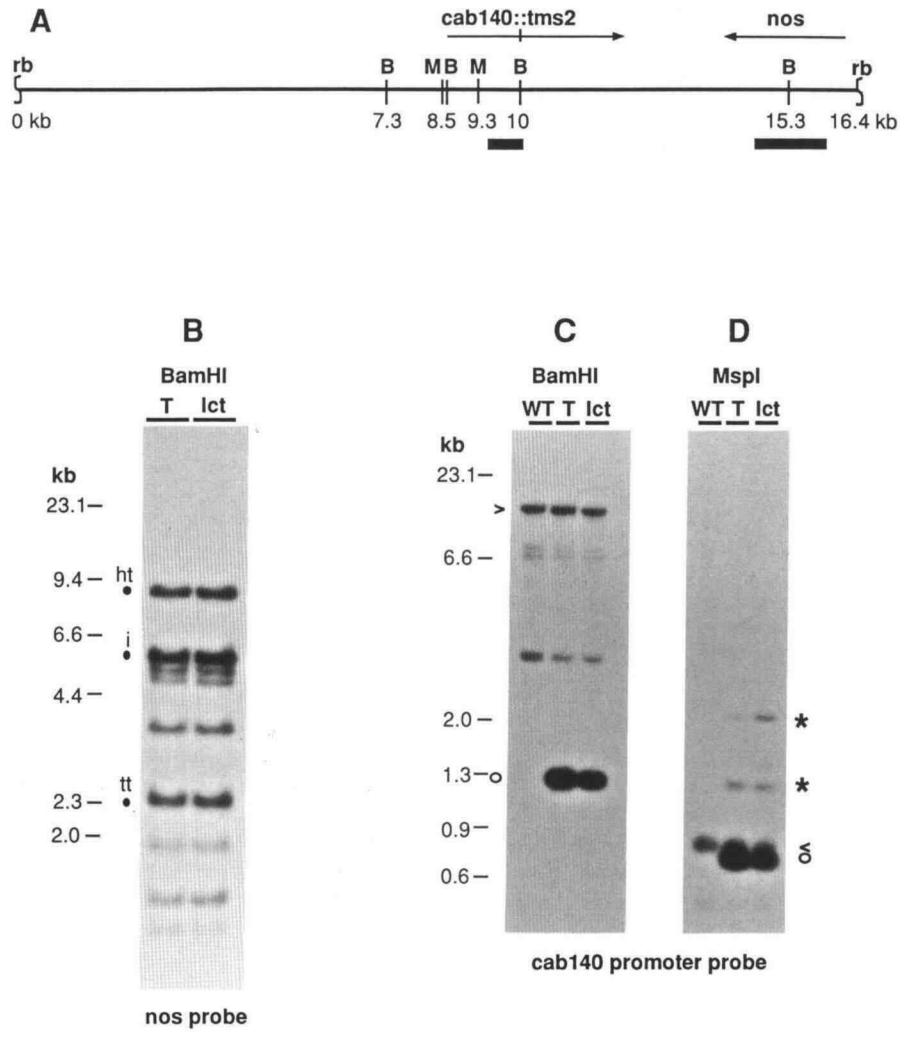

Figure 5. DNA Gel Blot Analysis of WT, T, and *lct* Lines.

(A) Map of a single unit of the T-DNA insert showing BamHI sites (B), relevant MspI sites (M), the right border (rb), and the location of the *cab140*::*tms2* fusion and the *nos* genes. The regions where the probes hybridize are shown as bars below the map. Scale is in kilobases (kb).

(B) Chromosomal DNA digested with BamHI and hybridized to the *nos* probe after electrophoresis and blotting. Dots correspond to the following bands: ht, head-to-tail; i, internal 5.3-kb BamHI fragment; tt, tail-to-tail.

(C) Chromosomal DNA digested with BamHI and hybridized to a probe from the region upstream of the *cab140* open reading frame that recognizes the native *cab140* promoter region (> or <) and the *cab140*::*tms2* promoter region (o).

(D) Chromosomal DNA digested with MspI and hybridized to the same probe as in **(C).** Restriction fragments resulting from partial digestion due to methylation of the MspI site located 730 bp upstream of the *tms2* gene are indicated (*).

transcriptional mechanism is involved in the cosuppression of both genes.

DISCUSSION

We performed a counter-selection for phytochrome signal transduction mutants using a line of Arabidopsis in which a *cab140* promoter, fused to the *tms2* gene from Agrobacterium, was introduced into the genome via T-DNA transformation. An EMS-induced mutant with strikingly reduced RNA levels of both the introduced *cab140*::*tms2* fusion gene and the endogenous *cab140* gene was isolated. This mutant, named *lct*, had no visible phenotype, and the RNA levels for *ubq3*, *rbcS-1A*, and *cab180* were normal (Figures 1 and 2). Genetic analysis showed that the T-DNA insert in *lct*, which contains the *cab140*::*tms2* gene, cosegregated with the reduced level of

cab140 RNA in 101 of 101 cases. This tight linkage indicated that the mutant mapped at or near the T-DNA insert, which was located close to the *bp* marker on chromosome 4.

Severely reduced expression of an introduced gene and its homologous endogenous gene was observed previously and was termed cosuppression (Napoli et al., 1990; van der Krol et al., 1990). In *lct*, as in other examples of cosuppression, the silencing is reversible upon genetic separation of the T-DNA from the endogenous gene (Matzke et al., 1989; Napoli et al., 1990; Goring et al., 1991), and the dosage of the introduced T-DNA affected the level of native *cab140* gene expression (Elkind et al., 1990; de Carvalho et al., 1992).

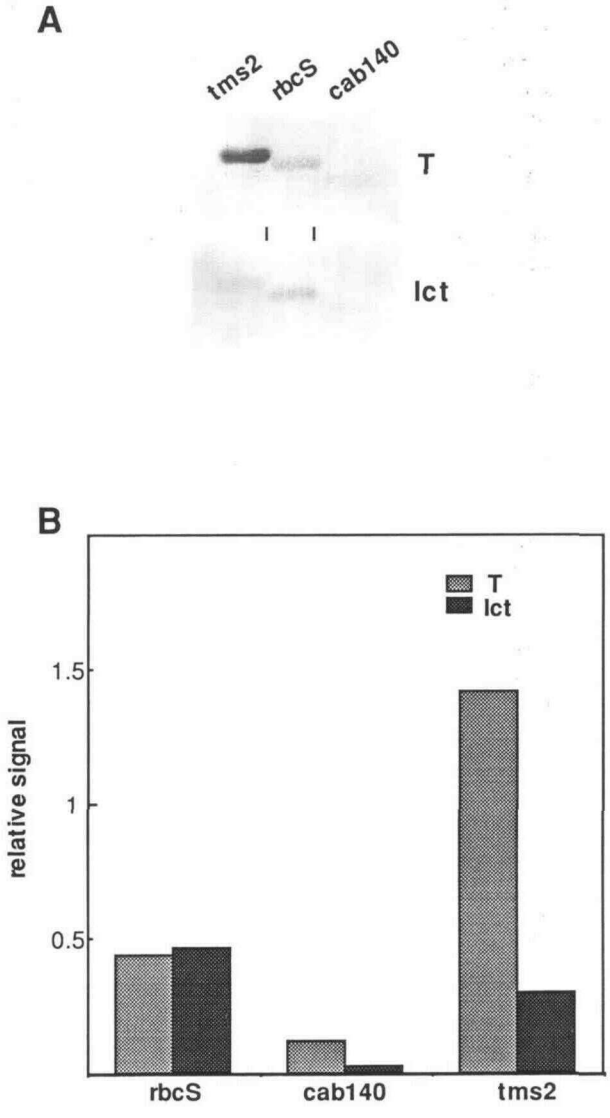

Figure 6. In Vitro Transcription Analysis of T and *lct* Lines.

In vitro transcription reactions were performed using nuclei that were isolated from plants grown for 3 weeks in WL.

(A) Hybridization of labeled transcripts to electrophoretically separated cloned DNA fragments from *tms2*, *rbcS*, and *cab140* detected by a PhosphorImager.

(B) Quantitation of hybridized transcripts. The average values of two separate blots are shown for each probe.

In all cases of cosuppression reported so far, the silencing has occurred after transformation and was not a result of mutagenesis. However, the *lct* line was isolated after mutagenesis. We therefore considered the possibility that a mutation was located on the T-DNA insert itself. It is clear that the T-DNA in *lct* is in some way different from the T-DNA in the parental T line; F_1 plants containing 1 copy of each T-DNA had higher *cab140* and *cab140*::*tms2* RNA levels than were observed in lines with 2 copies of the T-DNA from *lct* (see Figure 3; T × *lct* F_1 versus *lct*). If such a mutation were located in a *cab140* promoter region, it might bind with higher affinity and thus sequester a factor important for *cab140* transcription. Alternatively, a mutation that somehow altered the structure of the T-DNA and thus promoted cosuppression is another plausibility: the mutation did not result in gross rearrangement of the T-DNA (Figure 5B). It is probable that *lct* is a point mutation because it was induced by EMS, which has been shown to produce mostly GC-to-AT transitions (Coulondre and Miller, 1977).

It is formally possible that the reduced levels of the *cab140* RNAs are a result of a separate semidominant mutation located less than 0.5 map units from the T-DNA insert. The affected gene could encode a positive transcription regulator that specifically activates the *cab140* promoter throughout plant development. We think that this is unlikely and favor cosuppression as an explanation because the *lct* phenotype is so similar to the other reported examples of this phenomenon.

The T-DNA insert might have disrupted the *cab140* gene; however, the 11-kb BamHI fragment containing the endogenous *cab140* gene was not interrupted (Figure 3C). Another possibility is that the T-DNA inserted near the *cab140* locus and this somehow led to allelic interactions that inhibited transcription. Silencing of allelic genes has been reported previously in Drosophila (Dreesen et al., 1991) and in the plant Antirrhinum (Bollmann et al., 1991). If this were the case, one would expect to detect cosuppression in all of the progeny of the T line, but this was not observed.

Mechanism of Cosuppression in *lct*

There are no well-documented mechanisms by which genes introduced into the genome can suppress the expression of nonallelic homologous genes. In vitro transcript elongation reactions using nuclei isolated from the parental T line and *lct* demonstrated that there was a fivefold reduction in the rate of transcription for both the introduced *cab140*::*tms2* gene and the native *cab140* gene (Figure 6), suggesting a similar mechanism is operating at the level of transcription for both promoters. Ectopic pairing of chromosomes has been suggested as a mechanism of cosuppression (Jorgensen, 1991), and evidence for such interactions has been observed in yeast

(Jinks-Robertson and Petes, 1985) and in Neurospora (Selker et al., 1987). In our example, lines with only 1 copy of *lct* T-DNA (WT × *lct* F_1) were less cosuppressed than lines with 1 copy of the *lct* T-DNA plus 1 copy of the T-DNA from the parental T line (T × *lct* F_1; see Figure 3). This observation suggests that the *lct* mutation might promote interactions with homologous sequences on the parentally derived T-DNA that result in a further reduction in the expression of the introduced and the endogenous *cab140* gene promoters.

Further evidence supporting the ectopic pairing hypothesis is the partial repression of the *cab165* gene while the *cab180* gene remains unaffected (Figure 2). The *cab140* and *cab165* genes are located 2 kb apart and share divergent promoter regions. If 1.4 kb of the *cab140* promoter region were altered by ectopic pairing, the *cab165* promoter region might be affected to some extent as well. The *cab180* promoter, located 4 kb from *cab140*, might not be expected to show such an effect.

Methylation has been correlated with the silencing of genes during plant development (Spena et al., 1983; Watson et al., 1987; Bianchi and Viotti, 1988; Burn et al., 1993) and with the silencing of genes that have been introduced into the genome (Hepburn et al., 1983; Amasino et al., 1984; van Slogteren et al., 1984; Matzke et al., 1989; Kilby et al., 1992). However, in the *lct* mutant, neither the introduced *cab140* promoter region nor the promoter region of the native *cab140* gene was heavily methylated. A lack of correlation between cosuppression and methylation has also been observed in other cases (Goring et al., 1991; Hart et al., 1992).

The formation of antisense RNA has also been proposed as a mechanism of cosuppression (Mol et al., 1991). We considered the possibility that an antisense RNA was being made in *lct* and this RNA was silencing all of the introduced genes as well as the endogenous *cab140* gene. It is, in fact, possible that the 14 nucleotides of the transcribed region could be an effective antisense RNA. Previously, a 15-mer antisense oligonucleotide directed to the cap site of the hepatitis B virus surface antigen was shown to decrease protein levels by 89%, but unlike our situation, this was achieved post-transcriptionally (Goodzari et al., 1990). Antisense RNA operating at the level of transcription has been reported for the c-*myc* gene, but such inhibition was observed when the full-length antisense RNA product was expressed at extremely high levels (Yokoyama and Imamoto, 1987). We have tried to detect an antisense RNA in *lct*, but such a product has not been apparent on RNA gel blots using double-stranded probes (data not shown).

We cannot rule out the possibility that a post-transcriptional component is also involved in the gene suppression observed in *lct* because the half-lives of the *cab140* transcripts are not known. Suppression was demonstrated to occur post-transcriptionally for the introduced β-1,3-glucanase gene in transgenic tobacco, and, in this example, the suppression was developmentally regulated, only occurring after 4 weeks of growth (de Carvalho et al., 1992). Similar developmental control of cosuppression has been observed for the polygalacturonase gene in transgenic tomato (Smith et al., 1990). In our case of cosuppression, *cab140* was silenced from early development through maturity. Also, non-Mendelian segregation of silenced lines has been reported (Hart et al., 1992), but selfed *lct* lines always produced cosuppressed progeny. These disparate observations suggest that mechanisms of silencing may differ for the many examples of cosuppression.

In conclusion, we have isolated a mutant of Arabidopsis that displays cosuppression of an introduced *cab140*::*tms2* construct and the native *cab140* gene. The identity between these genes is the *cab140* promoter region (−1326 to +14). Cosuppression is occurring at the level of transcription, and methylation of the *cab140* promoters is not correlated with the gene silencing. The mutation that causes cosuppression maps at or near the T-DNA insert. Two models consistent with our observations are a mutation that promotes ectopic pairing of the repeated sequences of the genome, thereby mediating suppression of transcription, and a mutation in one of the *cab140* promoter regions in the T-DNA that binds and sequesters a factor specifically required for *cab140* gene transcription.

METHODS

Screening Conditions

Screening was performed using an ethylmethane sulfonate (EMS)-mutagenized *cab*(+14)B line (20 mM EMS; Karlin-Neumann, 1991) on medium containing 1 × Murashige and Skoog salts (GIBCO BRL), 0.6 g/L 2-(*N*-morpholino)ethane-sulfonic acid, 3% sucrose, B_5 vitamins, 0.7% phytagar (GIBCO BRL), 4.5 μM α-napthalene acetamide (NAM) (Sigma), 112.5 μM β-NAM (Michigan State University Synthesis Laboratory, East Lansing), pH 5.7. Seeds were surface sterilized in 10% commercial bleach, 0.02% Ivory dishwashing liquid (Proctor & Gamble, Cincinnati, OH) for 10 min, and then sown onto 7.5-cm filter papers (Whatman) using an apparatus described previously (Karlin-Neumann, 1991). The filter papers with seeds were transferred to plates containing 25 mL of solid medium, and the plates were placed in the dark at 4°C for 2 days, warmed to 25°C, and given 15 min of white light (WL) to stimulate germination. The plates were then placed in a light-tight box in which they received 1 min of red light (R) (1.25 $\mu Em^{-2}\ sec^{-1}$) every 2 hr or kept in complete darkness for 5 days. The plates were then brought into the light, and seedlings that were as tall as dark-grown controls were selected and transferred to the above medium without the NAM compounds and grown for 1 week under WL. Seedlings were then transferred to soil and grown in the greenhouse for M_3 seed production and subsequent rescreening. Rescreening was done without filter paper on 3 μM α-NAM and 75 μM β-NAM or on 10 μM α-napthalene acetic acid (NAA) (Sigma) for auxin resistance in the same medium described above.

Mapping Lines

A glabrous (*gl1*) marker in the Columbia background was a gift from Ry Meeks-Wagner (Washington State University, Pullman), and the W3, W6, W7, W8, and W9 lines in the Landsberg *erecta* background

were gifts from Chris Somerville (Michigan State University, East Lansing). For mapping, *low* level of *cab140* transcript (*lct*) was crossed to each of the marker lines, and F_1 plants were allowed to self. F_3 seeds were pooled from each F_2 individual and analyzed for *cab140* RNA levels as described below.

RNase Protection Analysis

M_3 lines that were resistant to the NAM compounds and sensitive to α-NAA were sown in sets of three plates (300 seeds/plate) as described previously (Brusslan and Tobin, 1992). After 5 days of growth in the dark, one set of seeds was given 1 min of R (3 μEm^{-2} sec^{-1}) and then returned to the dark for either 2 or 4 hr, another set was given 1 min of R immediately followed by 10 min of far-red (F) light (0.01 μEm^{-2} sec^{-1}) and then returned to the dark for 4 hr, while the last set was treated by the green safelight (0.05 μEm^{-2} sec^{-1}) only. For mapping experiments, a single set of plates that received 1 min of R and then 4 hr of the dark was used. For other experiments, seedlings were grown in WL (60 μEm^{-2} sec^{-1}) for 5 days. For the analysis of individual F_1 adult plants, five to seven rosette leaves were removed for RNA isolation from 3-week-old plants grown in WL. Total RNA was isolated as described previously, and RNase protections were performed using riboprobes also described previously (Brusslan and Tobin, 1992).

DNA Gel Blot Analysis

DNA was extracted from ~8000 etiolated seedlings grown for 5 days on the MS medium, described above, using a procedure modified from Leutwiler et al. (1986). The grinding was performed at 25°C in a proteinase K buffer containing 0.2 M Tris, pH 8.0, 0.1 M EDTA, pH 8.0, 1% sarkosyl, and 100 μg/mL proteinase K. Ground tissue was incubated at 48°C for 1 hr and the DNA banded on a CsCl gradient. Chromosomal DNA (3 μg) was digested with BamHI (Bethesda Research Laboratories) or MspI (New England BioLabs, Beverly, MA), separated by electrophoresis, and blotted to Zeta-Probe (Bio-Rad, Hercules, CA) according to manufacturer's instructions. The probe spanning the nopaline synthase (*nos*) region is a 1.8-kb SstII-HpaI fragment isolated from pMON410 (Rogers et al., 1987), and the probe for the region upstream of *cab140* is a combination of two fragments (−546 to −249 and −249 to +12). These fragments were cloned into pBluescript KS−, released by XbaI and HindIII double digests, separated by gel electrophoresis, and purified using a nucleic acid chromatography system (NACS™-52 PREPAC™) column (Bethesda Research Laboratories). Probes were labeled using the random prime reaction (Feinberg and Vogelstein, 1983), and hybridizations were done in formamide according to the Zeta-probe (Bio-Rad) instruction manual. Final washes were performed at 65°C in 0.5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS.

Isolation of Nuclei and In Vitro Transcription Reactions

Nuclei were isolated from 6 g of 3-week-old leaves grown on soil in WL using a procedure modified from Oscar Vorst and Sjef Smeekens (University of Utrecht, The Netherlands). Leaves were ground for 7 min using a mortar and pestle in 50 mL of ice-cold modified Honda buffer (0.44 M sucrose, 25 mM Tris, pH 8.5, 10 mM $MgCl_2$, 2 mM spermine, 2.5% [w/v] Ficoll 400, 5% [w/v] dextran 40, 0.5% [v/v] Triton X-100, 10 mM β-mercaptoethanol), and the homogenate was filtered through one layer of Miracloth (Calbiochem, La Jolla, CA). The filtrate was centrifuged at 4°C in an HB-4 rotor at 4000 rpm for 7 min, and the pellet was washed twice with 10 mL of the same buffer without spermine and once in a buffer containing 50 mM Tris, pH 8.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 20% [v/v] glycerol. Nuclei were finally resuspended in 100 μL 50 mM Tris, pH 8.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 50% [v/v] glycerol, and stored under $N_{2(l)}$. Nuclei were resuspended using a camel hair brush during washes, but gentle shaking was sufficient for the final resuspension.

In vitro transcription reactions were performed according to the method of Silverthorne and Tobin (1984) except that 1 mCi of 3000 Ci/mmol α-^{32}P-UTP (Amersham Corp.) was used in each 600-μL reaction. Labeled transcripts were hybridized to 100 ng of cloned DNA fragments blotted to Zeta-probe (Bio-Rad) after electrophoresis. The tumor morphology shoots gene (*tms2*) DNA was a 650-bp BamHI-HindIII fragment from pBluescript SK− αtms2RV, which contained homology to the *tms2* open reading frame (Karlin-Neumann et al., 1991). The ribulose bisphosphate carboxylase (*rbcS*) DNA was a 490-bp HindIII-EcoRI fragment containing the first two exons as well as the first intron of *rbcS-1A* (Krebbers et al., 1988), which had been cloned into pGEM-11Zf− to create pGEMrb-NS. The *cab140* DNA was a 350-bp BglII-BamHI fragment isolated from pBluescript SK−/cab140Δ(+69) (Karlin-Neumann, 1991), which contained 69 bp of transcribed region specific to the *cab140* gene as well as the upstream promoter region. Hybridizations were done at 40°C in the formamide buffer recommended by the manufacturer plus 100 μg/mL tRNA, and blots were washed in 2 × SSC, 1% SDS at 50°C. Low-stringency washes were necessary to prevent the *cab140* transcripts from being washed off of their 69-bp AT-rich target. Hybridizations were performed in duplicate, and filters were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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