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Expression of an N-Terminal Fragment of COP1 Confers a Dominant-Negative Effect on Light-Regulated Seedling Development in Arabidopsis

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***CONSTITUTIVE PHOTOMORPHOGENIC1* (*COP1*) is an essential regulatory gene that plays a role in light control of seedling development in Arabidopsis. The COP1 protein possesses three recognizable structural domains: a RING finger zinc binding domain near the N terminus, followed by a coiled-coil domain and a domain with WD-40 repeats in the C-terminal half. To determine whether COP1 acts specifically as a light-inactivable repressor of photomorphogenic development and to elucidate the functional roles of the specific structural domains, mutant cDNAs encoding the N-terminal 282 amino acids (N282) of COP1 were expressed and analyzed in transgenic plants. High-level expression of the N282 fragment caused a dominant-negative phenotype similar to that of the loss-of-function *cop1* mutants. The phenotypic characteristics include hypersensitivity of hypocotyl elongation to inhibition by white, blue, red, and far-red light stimuli. In the dark, N282 expression led to pleiotropic photomorphogenic cotyledon development, including cellular differentiation, plastid development, and gene expression, although it has no significant effect on the hypocotyl elongation. However, N282 expression had a minimal effect on the expression of stress- and pathogen-inducible genes. These observations support the hypothesis that COP1 is directly involved in the light control of seedling development and that it acts as a repressor of photomorphogenesis. Further, the results imply that the N282 COP1 fragment, which contains the zinc binding and coiled-coil domains, is capable of interacting with either downstream targets or with the endogenous wild-type COP1, thus interfering with normal regulatory processes. The fact the N282 is able to interact with N282 and full-length COP1 in yeast provided evidence for the latter possibility.**

INTRODUCTION

Plant development is a highly malleable process that is strongly influenced by environmental factors, especially light. The effects of light on plant development are especially prominent at the seedling stage (Kendrick and Kronenberg, 1994; McNellis and Deng, 1995). During skotomorphogenic or dark development, Arabidopsis seedlings develop extremely elongated hypocotyls, whereas the cotyledons remain closed and unexpanded. In contrast, during photomorphogenic or light development, hypocotyl elongation is inhibited, the cotyledons exhibit dramatic expansion and cellular differentiation patterns, and the plastids develop into photosynthetically competent chloroplasts. The expression levels of photosynthetic genes are high in the light and low in the dark. Photomorphogenic development depends on the plant being able to detect light signals. If this ability is impaired, such as when photoreceptors are disrupted by mutations, then a growing seedling assumes a somewhat etiolated developmental pattern. For example, mutations in phytochrome A gene (*PHYA*) cause reduced responsiveness to far-red light signals (Dehesh et al., 1993; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993); mutations in phytochrome B gene (*PHYB*) cause reduced responsiveness to red light stimulation (Reed et al., 1993; Wester et al., 1994); mutations in a putative blue light receptor gene, *HY4*, cause reduced responsiveness to blue light (Ahmad and Cashmore, 1993). Symptoms of reduced light responsiveness in *phyA*, *phyB*, and *hy4* mutants include long hypocotyls and reduced cotyledon expansion under certain light conditions (Koornneef et al., 1980; McNellis et al., 1994b). On the other hand, photoreceptor overexpression causes hypersensitivity to the spectral quality of light that the photoreceptor primarily absorbs. For example, overexpression of *PHYB* causes hypersensitivity to red light (Wagner et al., 1991; McCormac et al., 1993); overexpression of *PHYA* causes hypersensitivity to far-red light and red light (Boylan and Quail, 1991; McCormac et al., 1991, 1992, 1993; Whitelam et al., 1992); and overexpression of the CRYPTOCHROME1 (CRY1) blue light photoreceptor encoded by the *HY4* locus causes hypersensi-

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tivity to blue, UV-A, and green light (Lin et al., 1995). Because of the importance of light to plant survival, it makes sense that plants have developed multiple photoreceptors with partially overlapping functions. The photoreceptors work together to monitor light signals, and stimulation of any one photoreceptor class alone appears to be sufficient to induce many aspects of seedling photomorphogenesis (Kendrick and Kronenberg, 1994; McNellis and Deng, 1995).

The pleiotropic *CONSTITUTIVE PHOTOMORPHOGENIC/DEETIOLATED/FUSCA* (*COP/DET/FUS*) loci may define a group of important developmental regulators specifically involved in light control of seedling morphogenesis. Mutations at all of these loci cause seedlings to exhibit essentially all aspects of photomorphogenic development in darkness (Castle and Meinke, 1994; Miséra et al., 1994; Pepper et al., 1994; Wei et al., 1994; Kwok et al., 1996). Because all of the mutations at the pleiotropic *COP/DET/FUS* loci are recessive and cause constitutive photomorphogenic development regardless of the actual light conditions, the proteins encoded by these loci have been postulated to act as repressors of photomorphogenesis in the dark. Light signals absorbed by the various photoreceptors are thought to reverse the repressive activities of the COP/DET/FUS proteins and allow photomorphogenic development to proceed (reviewed in McNellis and Deng, 1995).

However, putative null mutations at all the *COP/DET/FUS* loci cause adult lethality and severe physiological abnormalities during seed maturation and seedling development. This has raised concern about the specificity of these genes in regulating light-mediated development (Castle and Meinke, 1994; Miséra et al., 1994). It is formally possible that the pleiotropic COP/DET/FUS proteins may be ubiquitous global cellular regulators and function mainly to set up the cellular environment necessary for light regulatory cascade. Although this alternative model is also consistent with the mutant phenotypes of those genes, it suggests that their gene products are not an integral part of the light regulatory cascade.

Molecular cloning of four pleiotropic *COP/DET/FUS* genes (Deng et al., 1992; Castle and Meinke, 1994; Pepper et al., 1994; Wei et al., 1994) has provided tools for testing those competing models. Recently, moderate overexpression of COP1 in Arabidopsis has been shown to partially suppress blue and far-red-light–mediated inhibition of hypocotyl elongation and blue light–mediated cotyledon expansion (McNellis et al., 1994b). Those effects are the only phenotype that can be detected in the transgenic plants; therefore, they were interpreted as evidence supporting the direct involvement of COP1 in the light signaling cascade. However, overexpression of full-length COP1 failed to have any detectable effect on the phytochrome B–mediated red light inhibition of hypocotyl elongation (McNellis et al., 1994b), possibly due to the low levels of overexpression. In addition, if COP1 functions directly within the light regulatory cascade and acts as a light-inactivable repressor of photomorphogenic development, ideally COP1 overexpression should also affect light control of plastid development and light-regulated gene expression. Limited overexpression of the COP1 protein (fourfold or less) is clearly unable to cause detectable effect on the light-regulated gene expression and plastid development (McNellis et al., 1994b). Therefore, the previous overexpression studies could not critically rule out the possibility that COP1 overexpression coincidentally influenced cell elongation in the hypocotyl and cell expansion in the cotyledon under our experimental conditions through a mechanism unrelated to photomorphogenesis.

To overcome the limitations of the full-length COP1 overexpression studies, we initiated an effort to overexpress specific mutated forms of COP1 to look for possible dominant-negative effects. COP1 is a 76.2-kD protein with an N-terminal RING finger zinc binding domain, followed by a putative α-helical domain and multiple WD-40 repeats in the C-terminal half that are similar to the β subunit of trimeric G-proteins (Deng et al., 1992; von Arnim and Deng, 1993; McNellis et al., 1994a). We reasoned that the distinct structural motifs of COP1 may represent modular domains that interact specifically with upstream and downstream partners in the light regulatory cascade, if COP1 is indeed an integral part of such a signaling cascade. Overexpression of mutated COP1 proteins containing different domains would potentially compete with endogenous COP1 for interacting partners and cause dominant-negative interference with the normal COP1 function. Here, we report COP1 overexpression studies using stably transformed Arabidopsis plants containing transgenes encoding the N-terminal half of COP1, which contains both the Zn binding motif and the putative coiled-coil domain, but depleted of the C-terminal half of COP1, which contains the entire WD-40 repeat domain. Overexpression of the N-terminal 282–amino acid fragment (N282) of COP1 caused a dominant-negative interference with the ability of the endogenous wild-type COP1 to suppress multiple photomorphogenic development processes, including cellular differentiation, plastid development, and gene expression. This effect of N282 overexpression seems to be specific for light-regulated development, because it has no effect on stress- and pathogen-inducible gene expression.

RESULTS

Production of Stably Transformed Transgenic Lines

A cDNA fragment encoding the N-terminal 282 amino acids (N282) of COP1 was placed under the control of the strong cauliflower mosaic virus 35S RNA promoter for overexpression. The construct included proper start and stop codons as well as a viral translational leader sequence to improve the efficiency of translation (see Methods). Figure 1A shows a schematic diagram of the predicted N282 compared with wild-type COP1. N282 is a truncated COP1 protein with a predicted molecular mass of 33 kD. The construct was stably introduced into Arabidopsis by Agrobacterium-mediated transformation. A total of 14 independent transgenic lines with the transgenes encoding N282 were generated and are summarized in Table 1.

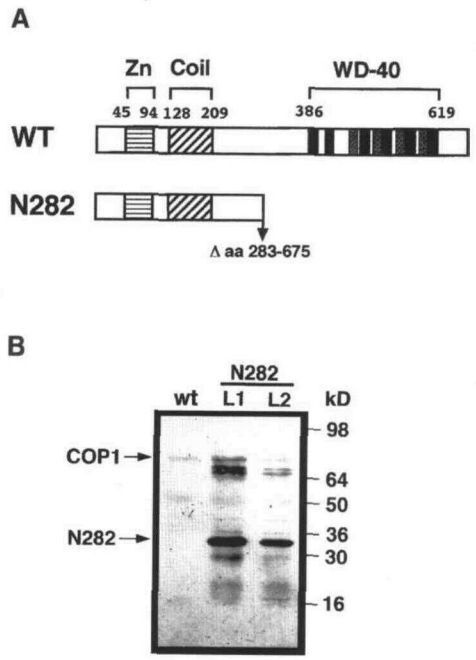

Figure 1. Summary of N282 COP1 Protein Fragment and Its Expression in Transgenic Plants.

(A) N282 is shown in comparison with the wild-type COP1 protein (675 amino acids), with the locations of the RING finger zinc binding (Zn), coiled-coil (Coil), and G_β domains. The G_β domain is made up of multiple repeats of the WD-40 motif, which consists of A (gray rectangles) and B (black rectangles) subrepeats. aa, amino acids; WT, wild type.
(B) Protein gel blots of two representative N282 transgenic lines are compared with the wild type (wt). Equal amounts of total protein extracts (~10 μg) were analyzed. The N282 protein (33 kD, marked by arrows) in the transgenic lines accumulated to a level about eight- to 10-fold higher than the level of the wild-type full-length COP1 protein (76 kD). The N282 transgenic lines also accumulated two other bands at ~60 and 70 kD, the identities of which are not clear. Molecular mass markers (in kilodaltons) are indicated at right.

Among the 14 transgenic lines, 10 of the lines (L1 to L10) contained a single T-DNA insertion locus, three of the lines (L11 to L13) contained two insertion loci, and one line (L14) contained at least three insertion loci. Homozygous plants were isolated from all 10 lines with single T-DNA insertions. Immunoblot analysis of seedlings from these homozygous lines indicated that they all accumulated copious quantities of the 33-kD N282 protein. Figure 1B shows a representative immunoblot for the two representative lines L1 and L2. The amount of N282 protein accumulating in the different lines varied from 8 to 10 times as strong as the intensity of the wild-type COP1 protein signal. The nature of the doublet at ~64 to 70 kD in the transgenic lines is not clear, although the bands appear to be specific to the transgenic lines. It is important that the level of the endogenous, full-length COP1 protein in all transgenic lines examined appeared to be unaltered. Therefore, the phenotypes are due to the overexpression of N282 rather than suppression of the endogenous wild-type *COP1* gene expression.

Transgenic Lines Accumulating the N282 Protein Display a Short Hypocotyl Phenotype in White Light

When seeds from the N282 primary transformants were germinated under normal long-day white light conditions, it became immediately obvious that 13 of the 14 lines were segregating a short hypocotyl phenotype (Table 1 and Figures 2A and 3A). The strength of the short hypocotyl phenotype varied among the lines. The one line that showed normal, wild-type hypocotyl elongation (~2.5 mm) in white light was L14, a line with three or more T-DNA insertion loci. The reasons for this exception are not currently known. The other lines fell into two basic classes of hypocotyl length (Table 1). Those with the more extreme phenotype were categorized as very short, having an average hypocotyl length of no more than 1 mm in cycling white light. Nine of the N282 lines displayed a very

Table 1. N282 Transgenic Line Summary

Line	T-DNA Insertion Loci[a]	Hypocotyl Length[b]
UR-1[c]	0	Wild type[d]
L1	1	Very short[e]
L2	1	Very short
L3	1	Short[f]
L4	1	Very short
L5	1	Very short
L6	1	Very short
L7	1	Short
L8	1	Short
L9	1	Short
L10	1	Very short
L11	2	Very short
L12	2	Very short
L13	2	Very short
L14	3+[g]	Wild type

[a] The number of insertion loci was determined by kanamycin resistance segregation in the T_1 generation, as described in Methods.
[b] Hypocotyl length after 5 days of growth in cycling white light conditions, as described in Methods.
[c] UR-1, an untransformed line regenerated during the transformation process.
[d] The average wild-type hypocotyl length was 2.5 mm ± 0.5 mm.
[e] "Very short" indicates that the average hypocotyl length was <1 mm.
[f] "Short" indicates that the average hypocotyl length was >1 mm but <2.0 mm.
[g] Fifty-eight T_1 seeds were screened from this line, and all of them were found to be kanamycin resistant, indicating the presence of at least three insertion loci.

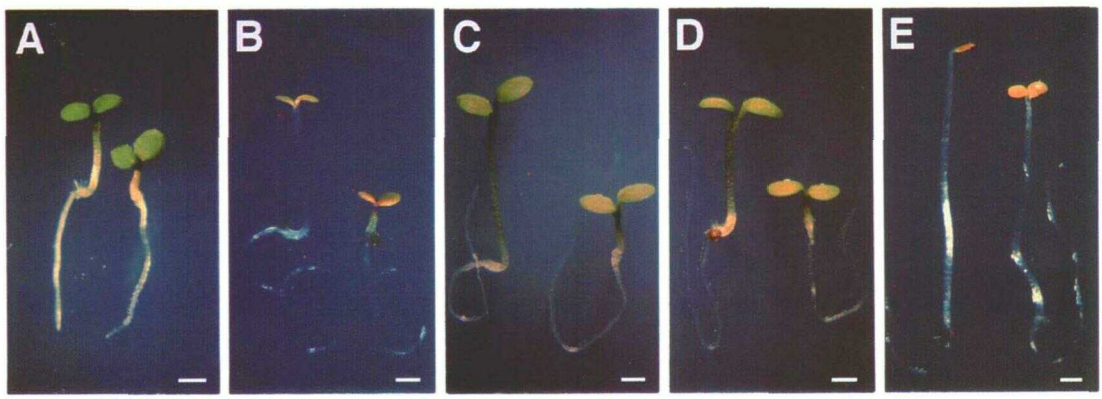

Figure 2. Morphogenetic Comparison of Wild-Type and N282 Seedlings (L1) Grown under Various Light Conditions.

(A) A wild-type and an N282 seedling grown for 5 days in cycling (16 hr of light/8 hr of dark) white light.
(B) A wild-type and an N282 seedling grown for 5 days in continuous far-red light.
(C) A wild-type and an N282 seedling grown for 5 days in continuous red light.
(D) A wild-type and an N282 seedling grown for 5 days in continuous blue light.
(E) A wild-type and an N282 seedling grown for 5 days in complete darkness.
In **(A)** to **(E)**, the wild-type seedling is on the left and the transgenic seedling is on the right. Different magnifications were used for each panel. Bars = 1 mm.

short hypocotyl phenotype, and they were all very similar in the degree of hypocotyl length inhibition. The lengths of the hypocotyl in those light-grown seedlings are essentially identical to that of the light-grown *cop1-1* and *cop1-4* mutants (Figure 3A). Four of the N282 lines were classified in the short hypocotyl group. These lines had average hypocotyl lengths of >1.0 mm but <2.0 mm. There appeared to be a general correlation between the degree of hypocotyl shortening and the level of N282 accumulation (data not shown). The short hypocotyl phenotype in the N282 lines always segregated with the T-DNA (see the following sections). This finding, together with the fact that 13 of 14 N282 transgenic lines displayed a short hypocotyl phenotype, suggests that the short hypocotyl phenotype was caused by the accumulation of N282 protein.

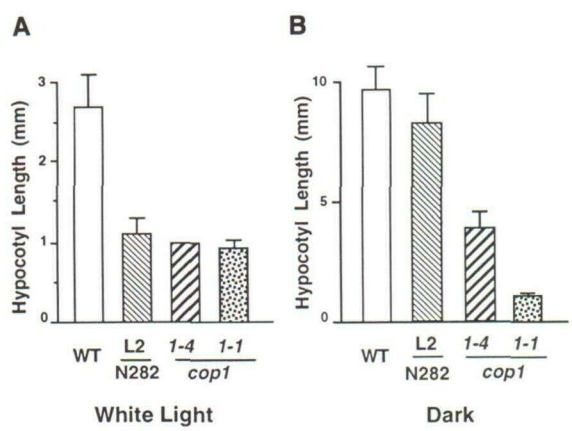

Figure 3. Effect of the N282 Protein on Hypocotyl Elongation in White Light and in Darkness.

(A) The average hypocotyl lengths of wild-type (WT), N282 (L2), *cop1-4*, and *cop1-1* seedlings after 5 days of growth in a 16-hr white light/8-hr dark photoperiod.
(B) The average hypocotyl lengths of wild-type (WT), N282 (L2), *cop1-4*, and *cop1-1* seedlings after 5 days of growth in darkness.
The error bars indicate standard deviations from the mean. A minimum of 30 seedlings were measured for each line in each growth condition.

High-Level Accumulation of the N282 Protein Causes Hypersensitivity to Far-Red, Red, and Blue Light

To investigate the nature of the photoreceptors involved in the hypersensitivity of the N282 seedlings to light signals, N282 seedlings from different N282 lines were examined in detail under far-red, red, and blue light conditions designed primarily to stimulate the photoreceptors phyA, phyB, and CRY1, respectively (Figures 2B to 2D). Figure 4A shows the average hypocotyl lengths for wild-type seedlings and seedlings from three representative N282 lines grown in continuous far-red, red, and blue light. Under all three light conditions, lines L1 and L2 have an extremely short average hypocotyl (~1 mm), whereas line L3 has an intermediate hypocotyl length. These results correlated well with the severity of the phenotype observed for these lines under white light growth conditions (see Table 1). Again, the hypocotyl length of the severe transgenic lines is very similar to that of the *cop1* mutants under the same conditions (Figure 4A), with the only minor difference in red light. Whereas the *cop1-4* seedlings under red light have very uniformly short hypocotyls of ~1 mm, the hypocotyl lengths of some transgenic lines (such as L1) are slightly more vari-

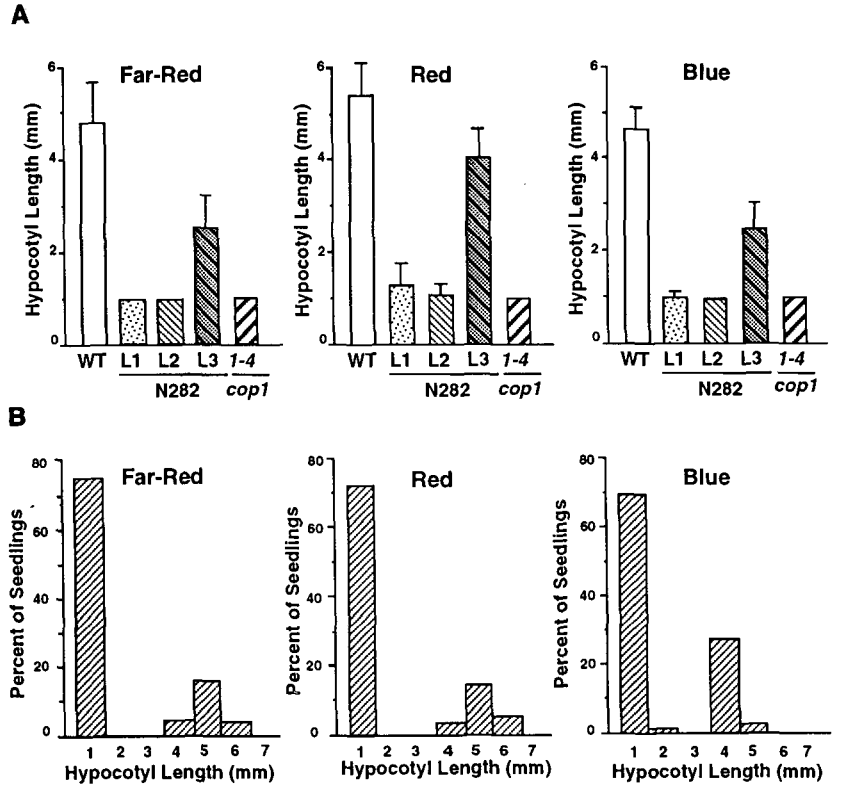

Figure 4. Effects of the N282 Protein on Hypocotyl Elongation in Far-Red, Red, and Blue Light.

(A) N282 expression resulted in hypersensitivity of hypocotyl elongation to inhibition by multiple light wavelength regions. The average hypocotyl lengths of wild type (WT) seedlings, seedlings from three representative homozygous N282 lines, and *cop1-4* mutant seedlings after 5 days of growth in continuous far-red, red, or blue light are shown. The error bars indicate standard deviations from the mean. A minimum of 30 seedlings were measured from each line for each experiment. For details on light experiments, see McNellis et al. (1994b).

(B) Phenotypic segregation of an N282 line (L1) heterozygous for the transgene at a single genomic locus suggested that the transgene acts in a genetically dominant manner. The seedlings were grown under continuous far-red, red, or blue light for 5 days, and the distributions of seedlings according to hypocotyl length were plotted. In each case, a short and a long hypocotyl phenotypic group was evident, and the approximate ratio of short to long was 3:1, as predicted for a dominant trait.

able, as reflected by the large error bar in Figure 4A. The hypersensitivity of the N282 transgenic lines to far-red, red, and blue light mimics that caused by overexpression of Arabidopsis *PHYA* (Boylan and Quail, 1991), *PHYB* (Wagner et al., 1991), and *CRY1* (Lin et al., 1995) photoreceptors, respectively. This implies that overexpression of N282 resulted in hypersensitivity to light stimulation mediated by at least three independent photoreceptors.

Hypersensitivity to blue light is complicated by the fact that phytochromes also absorb blue light, and it cannot be ruled out that an increase in responsiveness to PhyB stimulation by blue light contributes to the short hypocotyl phenotype of the N282 lines in blue light. However, although the N282 plants had a phenotype similar to that of PhyB overexpressers in red light, they had a stronger phenotype in blue light than the PhyB overexpressers (Wagner et al., 1991). This suggests that hypersensitivity to blue light photoreceptor stimulation must play a role in causing the phenotype of the N282 lines in blue light.

Although COP1 was placed downstream of phyB based on double mutant analysis, our previous full-length COP1 overexpression analysis failed to show any measurable effect on PhyB-mediated red light inhibition of seedling hypocotyl elongation (McNellis et al., 1994b). Therefore, the hypersensitivity of N282 lines to continuous red light provides clear evidence supporting the conclusion that COP1 also functions downstream of PhyB in addition to phyA and CRY1.

The Short Hypocotyl Phenotype Observed in the N282 Transgenic Lines Is Genetically Dominant

To establish the causal relation of N282 expression and the hypocotyl phenotype, the progeny of a representative plant heterozygous for a single T-DNA insertion locus of the N282

transgene (line L2) were analyzed for phenotypic segregation in far-red, red, and blue light conditions. The results are summarized in Figure 4B. In each case, the seedlings segregated into two clearly distinct phenotypic classes: very short (~1 mm) and wild type. The short hypocotyl phenotype always cosegregated with the T-DNA: all seedlings with short hypocotyls were positive for the T-DNA resistance marker, and all wild-type–like siblings lacked the T-DNA locus (data not shown). In far-red light, the ratio of the number of short to the number of long seedlings was ~3:1, indicating that the N282 transgene had a completely dominant effect in far-red light ($P > 0.1$). Similar results were obtained in red light ($P > 0.9$) and blue ($P > 0.99$) light, indicating that the N282 transgene had a dominant effect under those conditions as well.

Accumulation of the N282 Protein Causes Partial Photomorphogenic Development and Cell Differentiation in Darkness

The dramatic short hypocotyl phenotype of the N282 transgenic lines under different light qualities is reminiscent of the phenotype of weak *cop1* mutants (Deng and Quail, 1992). This prompted us to investigate whether production of the N282 protein could cause constitutive photomorphogenic development in the dark. As shown in Figure 2E, dark-grown seedlings expressing N282 do indeed have open and expanded cotyledons, a characteristic of deetiolation or photomorphogenic development. Scanning electron microscopy examination of the cotyledon surface cells (Figures 5A to 5D) indicated that dark-grown N282 seedlings have epidermal cell expansion and differentiation patterns similar to the cotyledons of light-grown seedlings, including mature stomatal structures. However, the deetiolation effects of N282 expression seem to be restricted to cotyledon development, because dark-grown N282 seedlings did not display significant inhibition of hypocotyl elongation relative to the wild type, contrasting to the dark-grown *cop1* mutants (Figure 3B). Only seedlings from the N282 lines that were classified as being very short (Table 1) exhibited photomorphogenic development in the dark, whereas those N282 lines with weaker light-grown phenotypes resembled wild-type seedlings when grown in the dark (data not shown). This finding implies that a high threshold level of available N282 was required to cause constitutive photomorphogenic

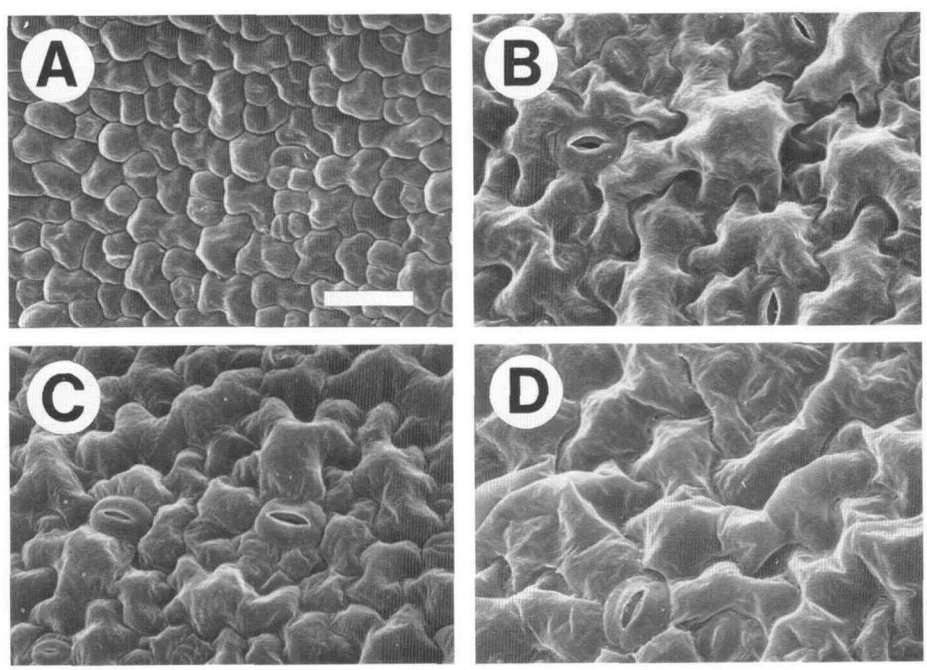

Figure 5. Comparison of Cotyledon Epidermal Cell Differentiation between Wild-Type and N282 Transgenic Seedlings as Examined by Scanning Electron Microscopy.

(A) Cotyledon from a 6-day-old dark-grown wild-type seedling.
(B) Cotyledon from a 6-day-old light-grown wild-type seedling.
(C) Cotyledon from a 6-day-old dark-grown N282 transgenic (L2) seedling.
(D) Cotyledon from a 6-day-old dark-grown *cop1-4* seedling.
In the cotyledons of dark-grown wild-type seedlings, only guard cell progenitors or immature guard cells are visible; the dark-grown N282 seedling cotyledons exhibited mature and open stomatal structures similar to those of light-grown wild-type and dark-grown *cop1-4* seedlings. The same magnification was used in **(A)** to **(D)**. Bar in **(A)** = 0.02 mm for **(A)** to **(D)**.

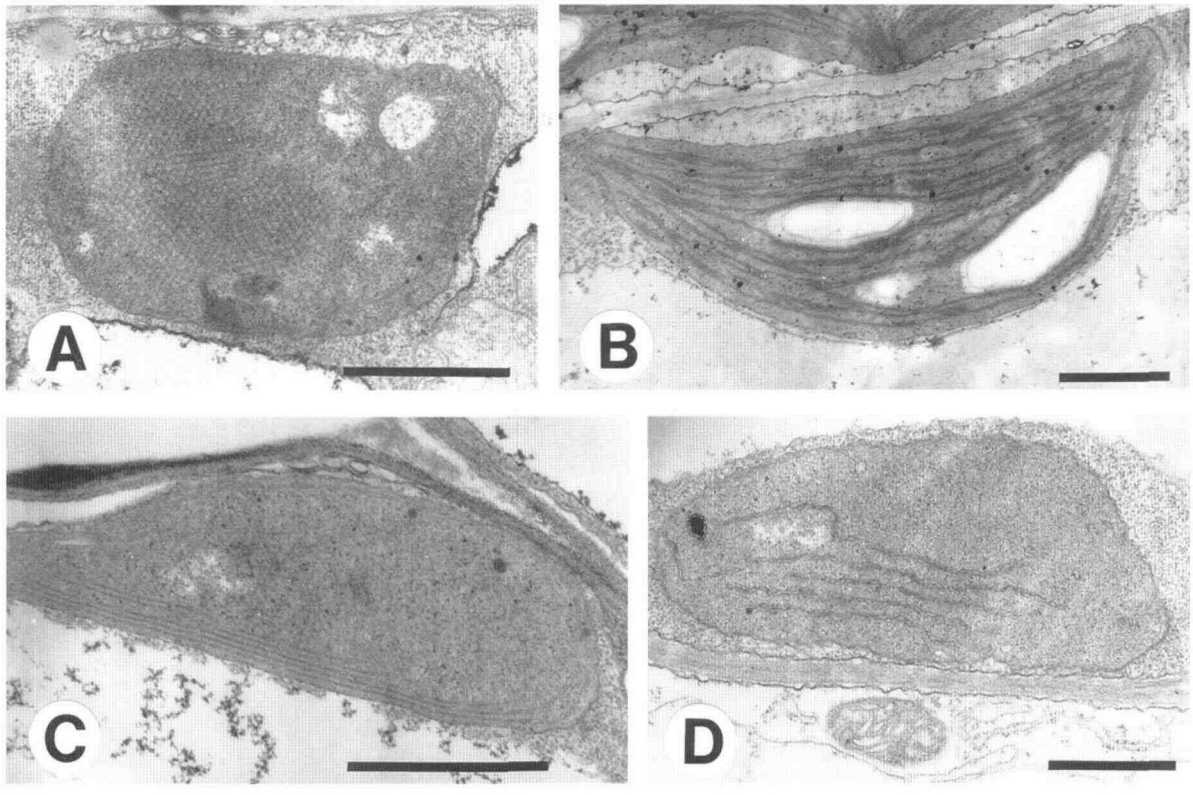

Figure 6. Comparison of Cotyledon Plastid Differentiation between Wild-Type and N282 Transgenic Seedlings as Examined by Transmission Electron Microscopy.

(A) A plastid from a 6-day-old dark-grown wild-type seedling.
(B) A plastid from a 6-day-old light-grown wild-type seedling.
(C) A plastid from a 6-day-old dark-grown N282 transgenic seedling (L2).
(D) A plastid from a 6-day-old dark-grown *cop1-4* seedling.
The plastid from dark-grown N282 seedlings lacks the prolamellar bodies of its wild-type counterpart, and it is similar to the plastid from the dark-grown *cop1-4* mutant. Bars = 1 μm.

cotyledon development in the dark. It may also imply that photomorphogenic hypocotyl development in the dark requires an even higher threshold level of N282.

Expression of N282 Results in Partial Chloroplast Development and Activation of Normally Light-Inducible Genes in the Absence of Light

To determine whether the deetiolated phenotype of the N282 lines included any alterations in plastid development and gene expression, plastids of dark-grown N282 seedlings were examined by transmission electron microscopy, and the dark expression levels of light-regulated genes in the N282 lines were determined by RNA gel blot analysis. As shown in Figure 6, the plastids of dark-grown N282 seedlings have a morphology very similar to that of dark-grown *cop1-1* and *cop1-4* mutants (Deng et al., 1991; Deng and Quail, 1992). The plastids of dark-grown N282 seedlings in general do not have the prolamellar bodies characteristic of etioplasts but instead have slightly more extensive thylakoid membrane structures that are similar to those found in immature chloroplasts. Therefore, N282 expression prevented etioplast development and promoted partial chloroplast development.

To examine the effects of N282 expression on light-regulated gene expression, mRNA levels of three representative genes (*cab*, the nuclear gene encoding the chlorophyll *a*/*b* binding protein; *rbcS*, the nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase; and *psbA*, the plastid gene encoding the 32-kD protein of the photosystem II reaction center) were examined in 6-day-old dark- and light-grown seedlings. As shown in Figure 7, RNA gel blot analysis indicated that all of the genes examined exhibited elevated levels of mRNA in dark-grown N282 seedlings in comparison to that

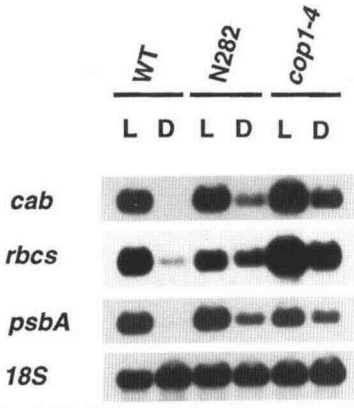

Figure 7. Effects of the N282 Protein on Expression of Light-Regulated Genes.

RNA levels of selected genes in 6-day-old light- (L) and dark- (D) grown seedlings were examined by RNA gel blot analysis. Equal amounts of total RNA (2.5 μg) were used in each lane. The membrane used for *psbA* was reprobed with the 18S rRNA probe to confirm the equal loading. For details, see Methods. WT, wild type.

of wild type, although the dark mRNA level was not as high as the level observed in light-grown seedlings. This elevated dark expression of genes that normally are only highly expressed in the light is very similar to that caused by the weak *cop1-4* mutation. Therefore, expression of N282 not only induced photomorphogenic seedling development but also led to abnormal plastid development and failure to repress light-inducible genes in complete darkness.

Both N282 Accumulation and *cop1-4* Mutation Have Minimal Defect in Pathogenesis-Related Gene Expression or Induction

To test the specificity of the effects of N282 expression on light-regulated development and gene expression, we examined whether the N282 transgenic lines exhibited any altered expression of the Arabidopsis pathogenesis-related (PR) genes (Uknes et al., 1992). The PR genes are repressed in normal wild-type plants but can be induced to high levels by a variety of signals, including pathogen infection, elicitors, salicylic acid, UV-B stress, and synthetic compounds such as 2,6-dichloroisonicotinic acid (INA) (Uknes et al., 1992; Chen et al., 1993; Cao et al., 1994; Green and Fluhr, 1995). We anticipated that if COP1 were a global regulator affecting overall gene regulation in plants, then the N282 lines and *cop1* mutants might exhibit an altered pattern of PR gene expression. As Figure 8 shows, the expression patterns of two PR genes, *PR-1* and *PR-2* (Uknes et al., 1992), are very similar in N282 transgenic, *cop1-4*, and wild-type plants. In the absence of INA, an agent that induces systemic acquired resistance and PR gene expression, the two PR genes were completely repressed and no measurable mRNA accumulation was detected. This undetectable PR gene expression is observed in both light-grown (Figure 8) and dark-grown (data not shown) wild-type and mutant seedlings. While in the presence of 0.1 mM INA, the mRNAs of the two PR genes accumulated to high levels in wild-type, N282-expressing, and *cop1-4* seedlings. The expression of PR genes in the N282 or *cop1-4* mutant lines is slightly higher than that of the wild type in the presence of INA. However, this difference is minor; therefore, we concluded that neither N282 expression nor the *cop1-4* mutation had a significant effect on the expression and inducibility of the two Arabidopsis PR genes examined.

N282 Interacts with Both Full-Length COP1 and N282 Itself in Yeast

All of the phenotypic characteristics of the N282 expressing lines indicated that the presence of N282 somehow interferes with or blocks the normal function of the endogenous wild-type COP1. At least two general mechanisms could account for this effect. As shown in Figure 9A, one possibility is that N282 interacts with wild-type COP1 to form an unproductive association, which leads to a dominant phenotype. It is also equally possible that N282 may interact with and titrate out the normal downstream targets of COP1 action, thus preventing the wild-type COP1 protein from performing its function normally (Figure 9B).

As a first step to gain insight into the possible mechanism involved, and by using the yeast two-hybrid assay (Gyuris et al., 1993), we tested whether N282 indeed has the capacity

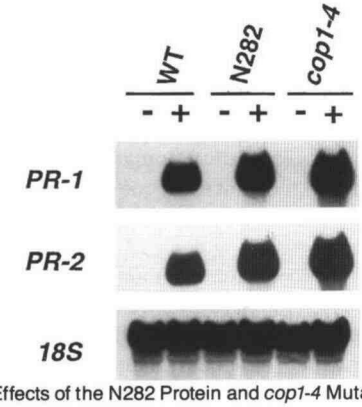

Figure 8. Effects of the N282 Protein and *cop1-4* Mutation on Expression of Pathogen-Inducible Genes.

RNA levels of selected genes in 10-day-old light-grown seedlings grown in GM medium with (+) or without (−) 0.1 mM INA were examined by RNA gel blot analysis. Equal amounts of total RNA (20 μg) were used in each lane for the PR genes. To control for equal loading, one membrane was reprobed with the 18S rRNA probe. For details, see Methods. WT, wild type.

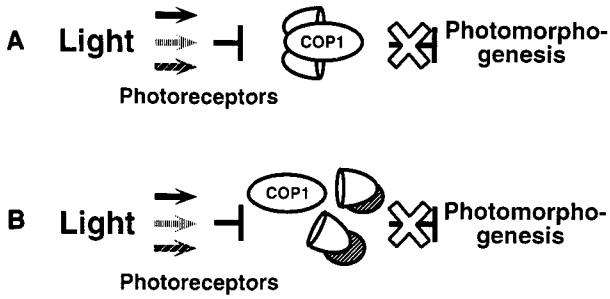

Figure 9. Two Possible Mechanisms by Which the N282 Protein May Cause the Observed Dominant-Negative Phenotype in Transgenic Arabidopsis.

(A) N282 may interact with endogenous wild-type COP1 and form a nonproductive association and thus interfere with normal COP1 function.
(B) N282 may compete with wild-type COP1 for interaction with the normal COP1 downstream target(s) and thus inhibit normal COP1 function.

to interact with COP1. As shown in Figures 10A to 10C, the two proteins of interest were fused to either the LexA DNA binding domain (bait) or the yeast transcription activation domain (prey). If the bait and prey interact, the reporter *lacZ* gene, which contains the LexA binding site in its promoter, will be activated. The results of this assay clearly suggested that N282 is indeed able to interact with full-length COP1 in yeast. Most strikingly, the interaction between two N282 molecules was even stronger than that between N282 and full-length COP1. Because N282 does not interact with either the LexA or the activation domain alone, our results clearly indicate that N282 protein contains the domains responsible for intermolecular interaction of COP1. The reason for a stronger interaction between the N282 molecules than that between N282 and COP1 is not clear. However, one possible explanation is that the presence of the C-terminal half of the protein may somehow weaken the protein–protein interaction through steric hindrance or regulatory inhibition of the COP1 intermolecular interaction. It is also possible that the full-length COP1 in yeast was not properly expressed or subcellularly localized.

DISCUSSION

This study revealed that expression of N282 caused a dominant-negative phenotype in both light- and dark-grown seedlings. The expression of N282 interfered with the ability of the endogenous COP1 protein to repress photomorphogenesis and resulted in increased seedling sensitivity to a variety of light signals and partial deetiolation in total darkness. The fact that the level of wild-type COP1 protein accumulation in the N282 transgenic seedlings did not appear to be reduced as a proportion of total protein (see Figure 1B) clearly ruled out the possibility that cosuppression of the endogenous wild-type *COP1* gene may be responsible for the observed phenotype. These phenotypic characteristics not only confirmed a specific involvement of COP1 in the light regulatory cascade but also allowed us insights into the functional roles of the structural domains residing in the N-terminal portion of the COP1 molecule.

The Dominant-Negative Effects of N282 Suggest a Specific Role for COP1 in the Signaling Cascade Mediating Light Control of Seedling Development

If COP1 is an integral part of the light signaling cascade mediating the light control of seedling development, it would be expected that a dominant-negative interference with COP1

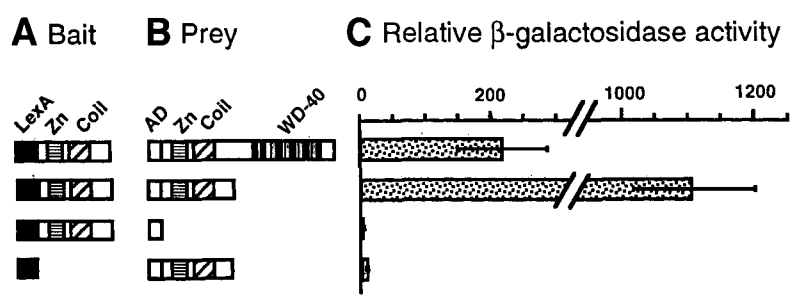

Figure 10. N282 Is Capable of Interacting with Both Full-Length COP1 and N282 Itself in the Yeast Two-Hybrid Assay.

(A) The bait constructs. The LexA DNA binding domain was used either alone or as a fusion with N282.
(B) The prey constructs. The synthetic yeast transcription activation domain (AD) was used alone and as a fusion with N282 or full-length COP1.
(C) The relative LacZ reporter activity in yeast cells with four different combinations of bait and prey constructs as shown in **(A)** and **(B)**. LacZ activity in combinations 3 and 4 represent the background levels in yeast cells. For each pairwise combination, 10 individual transformants were used to measure relative LacZ activity. Error bars represent standard deviation.
In **(A)** and **(B)**, abbreviations are as given in the legend to Figure 1.

function would lead to pleiotropic phenotypic defects that are specific for light-regulated processes. Our experimental data are entirely consistent with this prediction. In the dark, the N282 lines displayed a partial but pleiotropic photomorphogenic developmental pattern, including cotyledon cell differentiation, plastid development, and gene expression. This implies that in the transgenic plants, the endogenous wild-type COP1 protein is unable to repress photomorphogenesis completely due to the presence of the N282 protein. Also, the N282 transgenic seedlings showed hypersensitivity of hypocotyl-length inhibition under all light conditions tested, similar to that of the *cop1* loss-of-function mutations. It is hypothesized that as a negative regulator of photomorphogenesis, COP1 may be inactivated to varying degrees by light signals, depending on their intensity or quantity (McNellis et al., 1994b; McNellis and Deng, 1995).

The hypersensitivity of the N282 transgenic lines to light suggests that the endogenous wild-type COP1 is less efficient at inhibiting photomorphogenic responses in these lines and more easily inactivated by light. Further, the hypersensitivity of the N282 lines to far-red, red, and blue light indicates that the N282 protein renders the seedlings more sensitive to signals from multiple photoreceptors, including PHYA, PHYB, and a blue light photoreceptor. This observation agrees with genetic evidence from *cop1* mutants and evidence from transgenic plants overexpressing full-length COP1. This result suggests that signals from multiple photoreceptors converge to modulate COP1 activity (Deng et al., 1991; McNellis et al., 1994b; McNellis and Deng, 1995). It is especially worth mentioning the effects of N282 expression on responses to red light, because the overexpression of full-length COP1 failed to cause any observable effect on red light–mediated hypocotyl elongation (McNellis et al., 1994b). The experiments described here therefore provide critical evidence, in addition to double mutant analysis (Ang and Deng, 1994), that COP1 acts downstream of phyB.

The specificity of the effects of N282 expression on light-regulated development is supported by two lines of evidence. First, the phenotype of all of the transgenic N282 lines is limited to the light control of seedling development, and little effect was detected on other developmental process and normal adult development. Second, both the expression of N282 and the *cop1-4* mutation had almost no effect on the expression levels or induction of stress- or pathogen-responsive PR genes examined (Figure 8). These results strongly suggest that photomorphogenic development is the process that is most sensitive to the modulation of COP1 activity. Together with the fact that COP1 activity is modulated by multiple photoreceptors (see above) and that its nuclear localization is light regulated (von Arnim and Deng, 1994), our results support the conclusion that COP1 is involved specifically in a signaling cascade mediating light control of seedling development. However, our result cannot rule out the possibility that COP1 plays a role in the developmental processes that were unaffected by N282 expression. Thus, it is formally possible that COP1 may also play roles in other developmental processes or that the activity of COP1 may be regulated by other cellular or external signals.

Implications for the Function of the COP1 N-Terminal Domains

In our opinion, at least three plausible mechanisms could be responsible for the dominant-negative phenotype caused by the expression of N282 (Figure 9). These models assume that the N282 COP1 protein fragment interferes with the signaling processes of the wild-type COP1 protein directly. First, the N282 fragment may be able to interact with some factor or factors that are necessary for COP1 to exert its repressive influence on photomorphogenesis (Figure 9B). Potential candidates include light signal transduction proteins or promoter targets of downstream genes, because the RING finger zinc binding domain of COP1 may be able to bind DNA. In the N282 transgenic plants, the high levels of N282 protein may titrate out such factors, reducing their availability to the endogenous full-length COP1 protein. This would result in a decrease in the ability of the wild-type COP1 protein to inhibit photomorphogenesis, and it would explain the partially deetiolated phenotype of the N282 transgenic seedlings in darkness as well as their increased sensitivity to a variety of light signals. In this case, it is implied that N282 contains the protein motifs necessary for mediating those proposed interactions. Second, N282 has a domain mediating COP1–COP1 interaction, and the presence of N282 in the cells leads to a nonproductive N282–COP1 association (Figure 9A). Although we have yet to analyze these interactions in Arabidopsis, the ability of N282 to interact with COP1 in yeast is consistent with this possibility.

The third mechanism is a combination of the two mentioned above. Our preliminary results seem to be consistent with this combined option, because it appears that N282 contains distinct motifs that mediate interactions with COP1 and other novel cellular proteins (K.U. Torii and X.-W. Deng, unpublished data). The proposal that N282 interferes with possible in vivo protein–protein interactions is consistent with the observation that the level of N282 is important in achieving the dominant-negative phenotype. For example, the degree of inhibition of hypocotyl elongation in different N282 lines correlates with the abundance of N282 protein. It is also consistent with the observation that the *cop1-4* mutation results in the production of a very low level of N282 protein, and it behaves as a completely recessive mutation (McNellis et al., 1994a).

In summary, it seems clear that N282 of COP1 are involved in protein–protein or protein–nucleic acid interactions that are essential for the light control of seedling development by COP1. It will be necessary to assess the function of the zinc binding and coiled-coil domains of COP1 separately, because both of these domains were included in the N282 fragment. This may be done in a similar manner by using deletions of specific domains of the protein or expression of isolated domains. It will

also be interesting to examine the functionality of N282 in a null *cop1* mutant background. This is because our current interpretations are based on the assumption that the phenotype of N282 expression is a result of its interference with the endogenous COP1 function. It is formally possible that high levels of N282 itself may cause the observed phenotypic effects independent of the presence of wild-type COP1. Continuous investigation along those lines, in conjunction with isolation of interacting partners of COP1, may reveal the mode of action of COP1 in regulating Arabidopsis development.

METHODS

Construction of Expression Cassettes, Stable Transformation, and Growth Conditions

For the N282 construct, the start codon of the cloned *cop1-4* mutant cDNA was mutated to create an NcoI site, and the N-terminal half of the *cop1-4* cDNA (McNellis et al., 1994a) was excised as an NcoI-HincII fragment and used to replace the β-glucuronidase (*GUS*) gene in the pRTL2-GUS plasmid (Restrepo et al., 1990). To excise the *GUS* gene from pRTL2-GUS, the plasmid was cut with BamHI, blunt ended with the Klenow fragment of DNA polymerase I, and then cut with NcoI. The resultant N282 expression construct was ligated as HindIII fragments into the binary plant transformation vector pBIN19 (Bevan, 1984). Arabidopsis plants of the Nossen ecotype were transformed according to a published procedure (McNellis et al., 1994b). All wild-type plants were thus of the Nossen ecotype unless specified otherwise. Plant germination and growth conditions and light sources were identical to those described previously (McNellis et al., 1994b).

Protein Gel Blot and RNA Gel Blot Analysis

Protein immunoblot analysis was performed exactly as described previously (McNellis et al., 1994a). For RNA analysis, wild-type and N282 (L2) overexpressing seedlings were grown in darkness or cycling white light for 6 days. The seedlings were then harvested in darkness or room light, and total RNA was isolated (Deng et al., 1991; Torii et al., 1996). Equal amounts of RNA (2.5 μg per lane) were subjected to RNA gel blot hybridization analysis, as described previously (Torii et al., 1996). The RNA gel blots were hybridized with probes for light-regulated genes; later, they were reprobed with a probe for the 18S rRNA, which served as an equal loading control (Deng et al., 1991).

For the pathogenesis-related (PR) genes, the seedlings were grown in GM medium (McNellis et al., 1994b) with or without 0.1 mM 2,6-dichloroisonicotinic acid (INA) for 10 days under 16 hr of white light and 8 hr of darkness. Whole seedlings were harvested and used for RNA extraction. Equal amounts of total RNA (20 μg per lane) were used for RNA blot analysis.

Light and Electron Microscopy

The light and scanning electron microscopy procedures were performed as described previously (Hou et al., 1993). Transmission electron microscopy was performed as previously described (Wei et al., 1994a).

Yeast Two-Hybrid Assay

The pTA1-4, a pCR II clone (Invitrogen, San Diego, CA) with a cDNA insert corresponding to *cop1-4* (McNellis et al., 1994a), was cut with BamHI and XhoI and inserted into a vector portion of pKS-COP1 (Deng et al., 1992) to generate pKS-N282. pKS-N282 was cleaved with EcoRI and cloned into pEG202 and pJG4-5 (Ausubel et al., 1994) to make in-frame fusions with LexA and an activation domain, respectively. The generated plasmids were designated as pEG-N282 and pJG-N282, respectively. To construct pJG-COP1, which contains the full-length COP1 fused to an activation domain, pKS-COP1 was cleaved with EcoRI, and the released insert was cloned into an EcoRI-digested pJG4-5 vector.

Yeast strain EGY48-0 (Ausubel et al., 1994) was transformed with a combination of three plasmids: a bait (pEG-N282), a prey (pJG-N282 or pJG-COP1), and a reporter (pSH18-34), according to Chen et al. (1992). Colonies were selected on synthetic complete media without histidine, tryptophan, and uracil. The following combinations were transformed as controls: pEG-N282, pJG4-5 (activation domain only), and pSH18-34; pEG202 (LexA only), pJG-N282, and pSH18-34. Expression of N282 and COP1 in yeast transformants was confirmed by protein gel blots using anti-COP1 and/or anti-LexA antibodies (data not shown).

A β-galactosidase activity assay of the transformants was performed using o-nitrophenyl-β-D-galactoside, according to Guarente (1983), with the following modifications. Yeast strains were cultured overnight in liquid media supplemented with 2% (w/v) glucose, and then aliquots were transferred to media supplemented with 2% (w/v) galactose and 1% (w/v) raffinose to induce the expression of preys, which are under the control of *GAL1* promoter (Ausubel et al., 1994). Relative activity units were calculated according to Ausubel et al. (1994).

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