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A Chlorate-Resistant Mutant Defective in the Regulation of Nitrate Reductase Gene Expression in Arabidopsis Defines a New *HY* Locus

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Light acts both directly as a signal and indirectly through photosynthesis to regulate the expression of genes encoding nitrate reductase (NR). Here, we report the isolation and characterization of a novel chlorate-resistant mutant that is defective in the regulation of *NR* gene expression. The response of *NR2*, but not *NR1* or the gene encoding nitrite reductase (NiR), to light signals was impaired in this Arabidopsis mutant, designated *cr88*. In addition to *NR2*, the light regulation of the genes encoding the chlorophyll *a/b* binding protein (CAB) and the small subunit of ribulose bisphosphate carboxylase (RBCS) was also impaired in this mutant. These results suggest that the pathway through which light regulates the expression of *NR2*, *CAB*, and *RBCS* genes is different from those that regulate the expression of *NR1* and *NiR*. An examination of the deetiolation process under different light spectra showed that *cr88* is defective in red light–mediated deetiolation. Complementation tests with various long hypocotyl (*hy*) mutants indicated that *CR88* identifies a new *HY* locus. The possible functions of CR88 are discussed.

INTRODUCTION

Nitrate assimilation is the major process by which the majority of the higher plant species obtain reduced nitrogen. In this process, nitrate, once taken up from the soil, is reduced to nitrite by nitrate reductase (NR) (EC 1.6.6.1). Nitrite is translocated to the chloroplasts, where it is further reduced to ammonium by nitrite reductase (NiR) (EC 1.7.7.1). NR reduces nitrate in the cytosol (Rufty et al., 1986; Vaughn and Campbell, 1988; Fedorova et al., 1994) by using NADH, which is generated by carbon catabolism. NiR reduces nitrite in the chloroplast (Back et al., 1988) by using reduced ferredoxin, which is generated during the light reactions of photosynthesis. These reduction processes require high energy input. As much as 25% of the energy generated by photosynthesis can be consumed in driving nitrate assimilation (Guerrero et al., 1981; Solomonson and Barber, 1990).

NR and NiR are expressed and function at low levels if there is no nitrate available to the plants. Once exposed to nitrate, levels of NR and NiR increase drastically (Rajasekhar and Oelmuller, 1987; Solomonson and Barber, 1990), at least partially because of increases in mRNA levels (for recent reviews, see Crawford et al., 1992; Crawford and Arst, 1993). This rapid response enables plants to assimilate nitrate whenever it becomes available. An unlimited reduction of nitrate, however, overtaxes the energy reserves of the plant. Therefore, energy needs to be partitioned properly between carbon fixation and nitrate assimilation. One effective means to achieve this balance is to place nitrate assimilation under the control of photosynthesis. Indeed, the products of photosynthesis, sugars, at once repress the expression of carbon fixation genes (Sheen, 1990; Cheng et al., 1992) and induce the expression of *NR* genes (Cheng et al., 1992; Vincentz et al., 1993).

Light affects plant growth and development in two profound ways: it is perceived as signals, and it is used as the energy source for photosynthesis. Light plays both roles in the regulation of *NR* gene expression. On the one hand, exogenous sucrose can at least partially replace white light to induce *NR1* gene transcription in etiolated Arabidopsis seedlings (Cheng et al., 1992) and in dark-adapted mature Arabidopsis (Cheng et al., 1992) and *Nicotiana plumbaginifolia* (Vincentz et al., 1993) plants. This suggests that white light induces *NR* gene transcription indirectly through photosynthates. On the other hand, the steady state *NR* mRNA levels of etiolated seedlings of various species increase in response to red (Rajasekhar et al., 1988; Melzer et al., 1989; Becker et al., 1992; Bergareche et al., 1994) and blue (Melzer et al., 1989) light. In the constitutive photomorphogenic mutant *cop1*, *NR2*, along with many other light-regulated genes, is derepressed in the dark (Deng et al., 1991). However, the relative contributions of the two roles that light plays on *NR* gene expression remain to be determined.

Similar to regulation of the *NR* gene, the steady state mRNA level of *NiR* is also induced by light signals (Schuster and Mohr, 1990a; Becker et al., 1992; Neininger et al., 1992). Sucrose, in contrast to its role in regulating *NR* gene expression, has no effect on the level of *NiR* mRNA in *N. plumbaginifolia* (Vincentz et al., 1993). In addition, a plastidic factor is suggested to be required for the expression of both

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NR and *NiR* genes (Oelmuller and Briggs, 1990; Schuster and Mohr, 1990b; Seith et al., 1991; Neininger et al., 1992).

Understanding the molecular mechanisms of light- and carbohydrate-mediated signaling and the integration of these signal transduction pathways upon the regulation of *NR* gene transcription is essential for understanding the regulation of nitrate assimilation. This understanding will also help to shed light on how the interactions of plant signal transduction pathways lead to regulated gene expression. *NR* offers a special opportunity for genetic dissection of the two roles light may play in gene regulation. Chlorate, acting as an analog of nitrate, can be reduced to chlorite by *NR*. Because chlorite is toxic to cells, mutants that are deficient in chlorate uptake or NR activity can be selected by using chlorate. Chlorate-resistant mutants of Arabidopsis have been isolated and characterized in several laboratories, and together they define seven loci. These loci include *CHL1*, encoding the low-affinity nitrate/chlorate transporter; *CHL3*, the *NR2* structural gene; and five genes, *CHL2*, *CHL4* (*RGN*), *CHL5*, *CHL6* (*CNX*), and *CHL7*, that are responsible for the synthesis of a molybdenum cofactor (MoCo). The MoCo is required for a functional *NR* (Braaksma and Feenstra, 1973, 1982; Wilkinson and Crawford, 1991; LaBrie et al., 1992; Tsay et al., 1993). In addition, a mutant of the second *NR* gene (*NR1*), initially identified by molecular cloning and restriction fragment length polymorphism mapping (Cheng et al., 1988), has been isolated in a *chl3* background (Wilkinson and Crawford, 1993). Despite extensive screening, to date no chlorate-resistant mutants have been isolated that are defective in the regulation of *NR* gene expression.

To identify *NR* regulatory mutants, we conducted chlorate selection under an optimized condition that allowed us to select weak chlorate-resistant mutants. In this report, we describe the isolation and characterization of an *NR* regulatory mutant, *cr88*. The nitrate and the sucrose responses of the *NR* genes were retained in *cr88*. Interestingly, only the light response of *NR2*, but not of *NR1* or *NiR*, was impaired in *cr88*, indicating that the light signal transduction pathway regulating *NR2* expression differs from those regulating the other two genes. Consistent with this finding, sucrose, although as effective in the increase of *NR2* mRNA levels in *cr88* seedlings as in the wild type, could not completely replace the function of CR88. The deetiolation process of *cr88* in red but not in far-red or blue light was impaired. Complementation tests with long hypocotyl mutants *hy1*, *hy2*, *hy3*, *hy5*, and *hy6* indicated that *CR88* defines a new *HY* locus.

RESULTS

Selection of *NR* Regulatory Mutants

NR genes are regulated by multiple factors. If the regulatory mechanism for the response to one factor is defective, the expression of *NR* genes may decrease only partially. Therefore, we reasoned that some of the regulatory mutants affecting *NR* gene expression may retain higher levels of NR activity relative to those *chl3* and MoCo mutants isolated previously. Based on this assumption, we optimized the selection conditions to increase the sensitivity for detecting mutants that may retain relatively high levels of NR activity.

Arabidopsis plants grown on ammonium as the sole nitrogen source are much more sensitive to chlorate than are those grown on nitrate (Braaksma, 1970; LaBrie et al., 1991). The toxic effect of chlorate, however, depends on NR activity. This paradox was explained by LaBrie et al. (1991). The K_m value of NR for chlorate is 50 to 100 times greater than for nitrate (Solomonson and Vennesland, 1972; Nakagawa and Yamashita, 1986). Thus, nitrate protects the plant by acting as a strong competitive inhibitor of chlorate reduction. Consistent with this explanation, we observed that increasing nitrate concentration correlates with increasing protection of plants from chlorate toxicity (data not shown). Therefore, nitrate acts as both an *NR* inducer and a chlorate competitor in the chlorate selection procedure. Because nitrate is required for selecting regulatory mutants, we determined a concentration of nitrate that minimizes its competition with chlorate yet still induces NR expression significantly. At 0.5 mM nitrate, a concentration lower than the published procedures of 3 mM (Braaksma and Feenstra, 1973) and 5 mM (Wilkinson and Crawford, 1991), we found that the *NR* activity of Arabidopsis seedlings was induced to 30 to 50% of that induced by 5 mM nitrate. Using this concentration of nitrate, we determined that 1 mM chlorate was the minimum concentration that allowed a clear display of a phenotypic difference between the wild type and known chlorate-resistant mutants.

Another way to decrease the competition between nitrate and chlorate is to couple the chlorate treatment to nitrate induction. Nitrate is actively taken up from the soil by plants, and it can accumulate to high concentrations in the plant (Melzer et al., 1989). To prevent excessive accumulation of nitrate, we grew the seedlings first on ammonium medium instead of growing them continuously on nitrate; then we exposed them to nitrate before chlorate treatment. Because a nitrate-induced increase of *NR* mRNA levels displays a transient pattern (Melzer et al., 1989; Cheng et al., 1991), coupling nitrate induction and chlorate treatment may have an additional advantage. It may accentuate the phenotypic difference between the wild type and those regulatory mutants that are defective in the nitrate induction process during selection. Under this optimized condition, 500,000 M_2 individuals of the Columbia *glabrous* (Col-*gl*) background, representing 25,000 M_1 parents, and 111,000 M_2 individuals of the Landsberg *erecta* (L*er*) background, representing 23,250 M_1 parents, were subjected to selection. Among 126 putative mutants, 45 were confirmed to be chlorate resistant in the M_3 generation. Genetic and biochemical analyses indicated that six of them were not *chl1*, *chl3*, or MoCo mutants (Y. Lin and C.-L. Cheng, manuscript in preparation) and thus were of potential interest. One of the six mutants, *cr88*, was characterized further.

Table 1. NR Activities of *cr88* and Known *chl3* and MoCo Mutants

Strains	NR Activity[a]		
	NH_4^+	24 Hr	48 Hr
WT Col[b]	4.2 ± 0.2	25 ± 5 (100%)	31 ± 5 (100%)
cr88[c]	3.8 ± 0.2	9.7 ± 0.4 (38%)	16 ± 0.6 (52%)
WT Ler[d]	4.5 ± 0.3	32 ± 4 (100%)	39 ± 6 (100%)
chl3 (B29)[e]	4.0 ± 0.2	5.8 ± 0.3 (18%)	6.4 ± 0.3 (16%)
chl2[e]	4.2 ± 0.2	5.4 ± 0.3 (17%)	5.7 ± 0.4 (15%)
chl4 (B25)[e]	3.9 ± 0.2	5.2 ± 0.2 (16%)	5.4 ± 0.2 (14%)

[a] Plants were germinated and grown on a perlite/vermiculite mixture containing ammonium medium under continuous light for 14 days. NR activities were assayed before and 24 or 48 hr after induction with 0.5 mM KNO_3. NR activity is expressed as nanomoles of nitrite produced per minute per gram fresh weight of tissue at 30°C. Numbers are means ± SE of three assays with 20 to 25 seedlings each. Percentage activities of the wild type are given in parentheses. For more details, see Methods.

[b] WT Col, wild-type Col-*gl*.

[c] *cr88* is in the Col-*gl* background.

[d] WT L*er*, wild-type L*er*.

[e] *chl3* (B29), *chl2*, and *chl4* (B25) are in the L*er* background.

cr88 Has Lower Than Wild-Type NR Activity under the Selection Condition

Wild-type and *cr88* seedlings were grown under conditions similar to those used for chlorate selection (without adding chlorate). Their NR activities before and after nitrate induction were measured (Table 1). NR activities in *cr88* after 24 and 48 hr of nitrate induction were 38 and 52%, respectively, of the levels in wild-type seedlings. At lower levels, however, NR activity increased in response to nitrate induction. NR activity in *cr88* was also higher than that in *chl2* and *chl4* (both are MoCo mutants) and in *chl3* (Table 1). These results suggest that the chlorate resistance of *cr88* is indeed due to lower levels of NR activity and that the optimized selection condition is sufficiently sensitive to allow the selection of mutants having intermediate NR activity levels.

Nitrate and Sucrose Induction Processes Are Retained in *cr88*

In most higher plant species analyzed, both nitrate and white light are required for full levels of steady state *NR* mRNA expression (Solomonson and Barber, 1990). We have shown previously that exogenous sucrose (2% in the medium) can at least partially replace white light and induce *NR1* gene transcription in both dark-grown (yellow) and dark-adapted (green) 3-week-old Arabidopsis plants (Cheng et al., 1992). A simple interpretation of these results is that, at this developmental stage, white light increases *NR1* transcription mainly through the accumulation of photosynthates. If this indeed is true, the levels of carbohydrates and nitrate are the two critical factors regulating *NR1* expression.

To investigate whether nitrate or sucrose properly regulates the expression of *NR* genes in *cr88*, we compared the steady state mRNA levels of the two *NR* genes in response to these factors. Previous time-course studies of the two *NR* genes in Arabidopsis showed that the nitrate induction kinetics exhibit a transient pattern and the sucrose induction kinetics reach a plateau after 4 to 8 hr of treatment (Cheng et al., 1991). Therefore, a comparison of the nitrate induction kinetics of *NR1* and *NR2* steady state mRNA levels in the presence and absence of exogenous sucrose would be most informative. To avoid potential physiological differences caused by prolonged growth in the presence and absence of sucrose, we grew seedlings for 7 days in liquid medium with ammonium as the sole nitrogen source. Sucrose was added only 24 hr before nitrate induction. To sustain growth for 7 days in the absence of sucrose, we used a light condition (120 μmol m^{-2} sec^{-1}) much lower than optimum (800 μmol m^{-2} sec^{-1}) for soil-grown plants. It is apparent in Figures 1A and 1B that exogenous sucrose further increased the levels of *NR1* and *NR2* mRNA in both wild-type and *cr88*

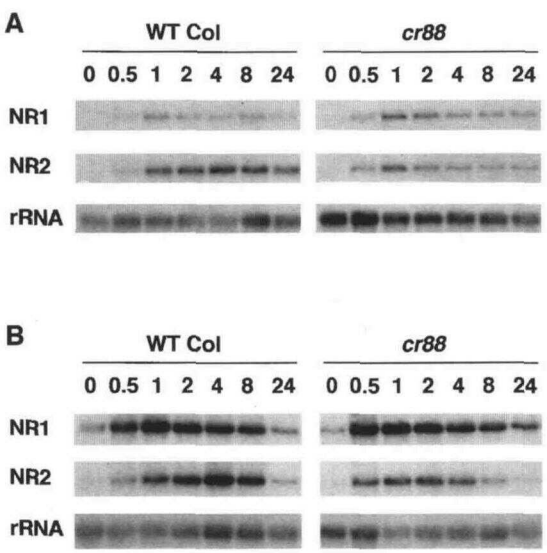

Figure 1. RNA Gel Blot Analysis of Nitrate Induction of *NR1* and *NR2* mRNA in Wild-Type Col-*gl* and *cr88* Seedlings.

The number above each lane indicates the hours of nitrate induction. At left, *NR1*, *NR2*, and rRNA indicate the RNAs hybridizing with their respective probes. WT Col, wild-type Col-*gl*.

(A) Nitrate induction in the absence of sucrose. Seedlings were grown in nitrate-free medium for 8 days before the addition of 5 mM KNO_3.

(B) Nitrate induction in the presence of sucrose. Seedlings were grown in nitrate-free medium for 7 days before the addition of 1% sucrose. Twenty-four hours later, 5 mM KNO_3 was added to the medium.

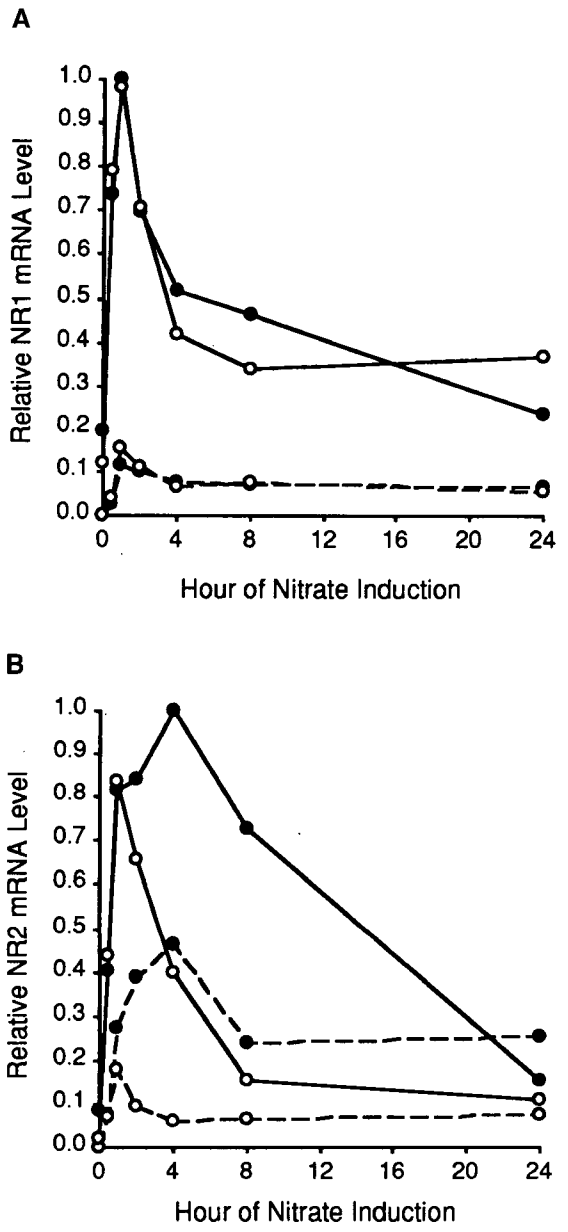

Figure 2. Nitrate Induction Kinetics of *NR1* and *NR2* mRNA in the Wild-Type Col-*gl* and *cr88* Seedlings in the Presence and Absence of 1% Sucrose.

The amounts of *NR1* and *NR2* transcripts for each lane shown in Figure 1 were normalized to that of the corresponding rRNA. Those values were then used to calculate the relative levels with the highest values of either *NR1* or *NR2*, with each being set at 1.0. Closed circles (●), wild-type Col-*gl;* open circles (○), *cr88*; dashed lines, kinetics in the absence of 1% sucrose; solid lines, kinetics in the presence of 1% sucrose.

(A) Nitrate induction kinetics of *NR1* mRNA.

(B) Nitrate induction kinetics of *NR2* mRNA.

seedlings and that only the nitrate induction patterns of *NR2* were different between the wild-type and *cr88* seedlings.

To visualize these results better, we show the nitrate induction kinetics in Figures 2A and 2B. Both the relative levels and the nitrate induction kinetics of *NR1* mRNA in *cr88* were similar to those of the wild type, either in the absence or in the presence of sucrose (Figure 2A). In contrast, the relative levels of *NR2* mRNA were lower in *cr88* than in wild-type seedlings, both in the presence and absence of sucrose. Also, the nitrate induction kinetics of *NR2* mRNA in *cr88* were different from those in the wild-type seedlings (Figure 2B). In the absence of sucrose, *NR2* mRNA reached a peak in *cr88* 1 hr after nitrate induction and then declined rapidly. At this time (1 hr after induction), the *NR2* mRNA level in the wild type already exceeded that in *cr88* and continued to increase, peaking 3 hr later. Similar nitrate induction kinetics of *NR2* mRNA were observed in the presence of sucrose, except that the relative levels were much higher both in *cr88* and the wild-type seedlings. The magnitude of *NR2* mRNA induction by sucrose in *cr88* was at least as high as in the wild-type seedlings. This can be seen either by comparing the peak levels or by comparing the average levels of the curves (Figure 2B).

These results show that the two *NR* genes are differentially regulated by CR88. In addition, both the nitrate induction and the sucrose induction processes were retained in *cr88*, indicating that the lower levels of *NR2* expression are likely to be due to a defect in other processes. Finally, 1% sucrose was sufficient to cause the induction magnitudes of *NR2* mRNA in *cr88* to be as high as those in the wild-type seedlings. This increase, however, was not sufficient to replace the function of CR88 at this stage of the plant development.

cr88 Is Yellow Green and Contains Underdeveloped Chloroplasts

The greening process in *cr88* is slower than in the wild type (Col-*gl* background) throughout the life span of the plant. Newly emerged cotyledons, rosette leaves, cauline leaves, stems, and flower buds are yellow green. A 3-day-old *cr88* seedling grown in white light displayed yellow-green cotyledons, whereas wild-type cotyledons at the same stage were green (Figure 3A). A 4-week-old rosette plant of *cr88* displayed a yellow-green center, whereas that of the wild type was green (Figure 3B). For clearer visualization, detached rosette leaves from 4-week-old *cr88* and wild-type plants were compared. The terminal bud and all of the leaves of the wild-type plant were green, whereas at least the youngest four leaves from *cr88* were yellow green. Even the oldest rosette leaf of *cr88* was slightly paler than that of the wild-type plant (Figure 3C).

Segregation analysis of *cr88* was performed. Among 540 seedlings from the F_2 generation of a cross between *cr88* and the wild type, all 409 chlorate-sensitive plants were green and all 131 chlorate-resistant plants were yellow

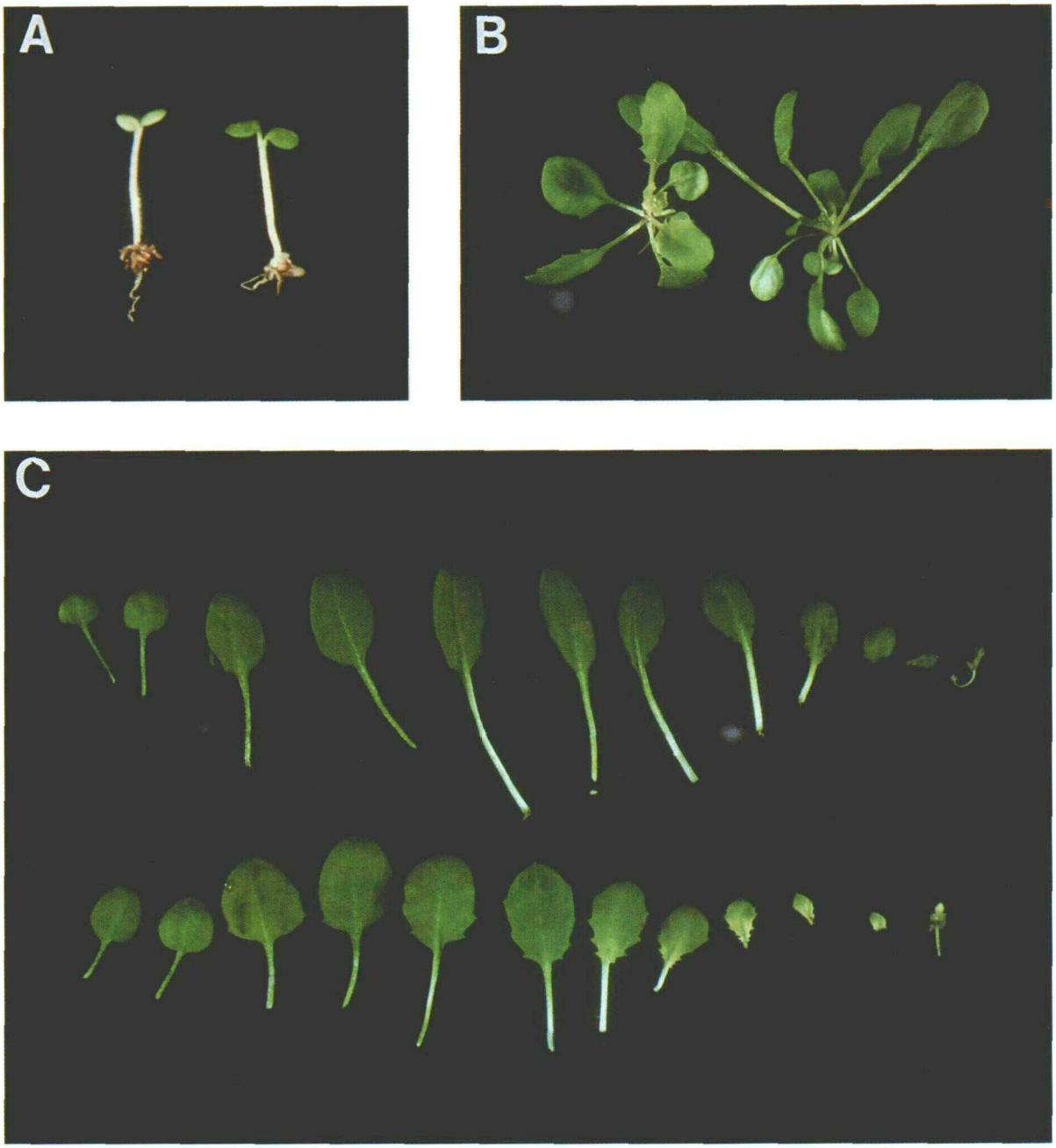

Figure 3. Yellow-Green Phenotype of *cr88* Seedlings and Mature Plants.

(A) A 3-day-old soil-grown *cr88* seedling (left) and wild-type Col-*gl* seedling (right).
(B) A 4-week-old soil-grown *cr88* plant (left) and wild-type Col-*gl* plant (right).
(C) Rosette leaves of 4-week-old soil-grown wild-type Col-*gl* (top) and *cr88* (bottom) plants were detached and arranged from the oldest (leftmost) to the youngest (second from the right). The rightmost are the terminal buds.

green. The 3:1 ratios ($\chi^2 = 0.158$) of chlorate-sensitive to chlorate-resistant and green to yellow-green phenotypes indicate that both chlorate resistance and yellow-green phenotypes are monogenic recessive traits. In addition, the co-segregation of these two traits indicates that they are most likely caused by a mutation in a single gene.

The delayed greening of *cr88* is reflected in its chloroplast development. In Figures 4A and 4B, respectively, the

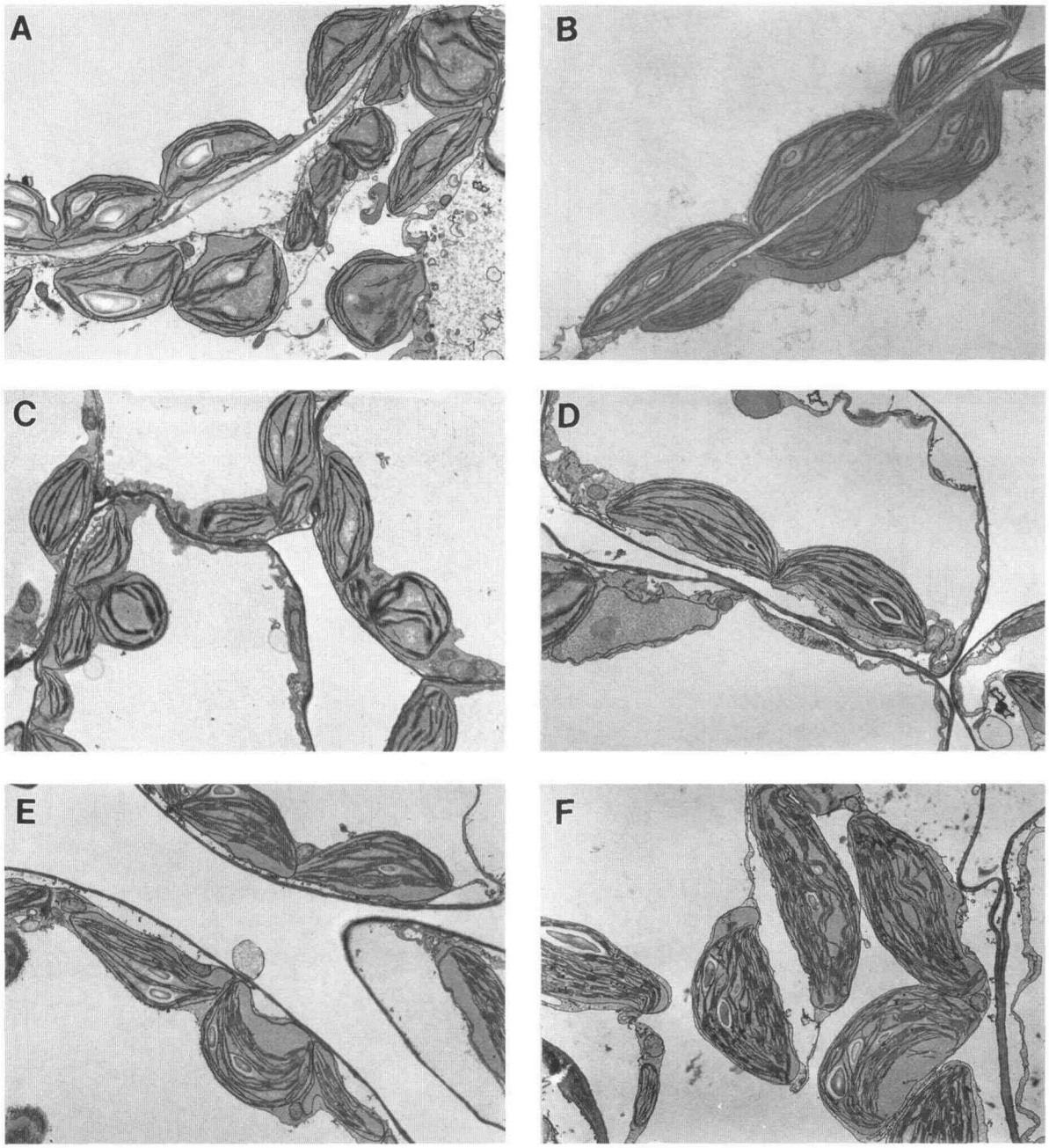

Figure 4. Chloroplast Development in 3-Day-Old and 4-Week-Old *cr88* and Wild-Type Col-*gl* Plants.

Representative chloroplasts are from cotyledons and young and mature leaves. Magnification is ×5000.
(A) Cotyledons of a 3-day-old *cr88* seedling.
(B) Cotyledons of a 3-day-old wild-type Col-*gl* seedling.
(C) Young leaves of a 4-week-old *cr88* plant. The leaves correspond to the ninth oldest leaf shown in Figure 3C.
(D) Young leaves of a 4-week-old wild-type Col-*gl* plant. The leaves correspond to the ninth oldest leaf shown in Figure 3C.
(E) Mature leaves of a 4-week-old *cr88* plant. The leaves correspond to the fifth oldest leaf shown in Figure 3C.
(F) Mature leaves of a 4-week-old wild-type Col-*gl* plant. The leaves correspond to the fifth oldest leaf shown in Figure 3C.

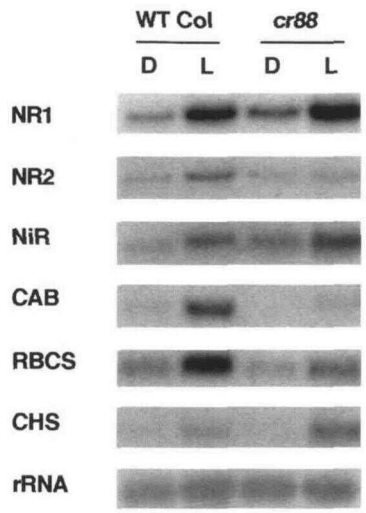

Figure 5. RNA Gel Blot Analysis of Light Induction of Steady State mRNA of *NR1*, *NR2*, *NiR*, and Representative Light-Regulated Genes in Etiolated Wild-Type Col-*gl* and *cr88* Seedlings.

Seeds were sown in liquid medium and cold treated, as described in Methods. Seedlings were grown in the dark for 8 days and harvested before (D) or after (L) 8 hr of exposure to white light. The cDNA probes used in the hybridization analysis were *NR1*, *NR2*, *NiR*, *CAB*, *RBCS*, and *CHS*. The gene probe for rRNA is from a soybean gene encoding 28S rRNA. WT Col, Col-*gl*.

chloroplasts in cotyledons from 3-day-old seedlings of *cr88* are compared with those of the wild type. The chloroplasts in *cr88* are smaller than those of the wild type, and they are round. They also show less thylakoid stacking than do the wild-type chloroplasts. In mature rosettes, the chloroplasts in the young leaves (yellow green) in *cr88* (Figure 4C) exhibited less development than did those of the wild type (Figure 4D). When leaves became older and greener, the chloroplasts in *cr88* (Figure 4E) were more similar to wild-type leaves (Figure 4F) but still not as well developed.

Light Induction of Genes Encoding NR2, the Chlorophyll *a/b* Binding Protein, and the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase Are Impaired in *cr88*

Light signals induce *NR* gene expression in etiolated seedlings (Rajasekhar et al., 1988; Melzer et al., 1989; Becker et al., 1992; Bergareche et al., 1994). The phenotypes of slow greening and underdeveloped chloroplasts suggest the possibility that *cr88* is defective in light-regulated processes. Time-course studies demonstrated that *cr88* still retains nitrate and sucrose responses (Figures 1 and 2); however, both the levels and nitrate induction kinetics of steady state *NR2* mRNA were abnormal in *cr88*. Could this abnormality be caused by a defect in light regulation of the *NR2* gene? To answer this question, we grew seedlings for 8 days in the dark in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 1% sucrose. They were then exposed to 120 μmol m^{-2} sec^{-1} white light for 8 hr. The steady state mRNA levels of *NR1*, *NR2*, and *NiR* before and after light induction were compared between *cr88* and the wild-type seedlings. Consistent with the results obtained with sucrose and nitrate induction, the light-induced expression of *NR1* in *cr88* was indistinguishable from that of the wild type, but the light induction of *NR2* was defective in *cr88*. Like *NR1*, expression of *NiR* in *cr88* was also indistinguishable from that of the wild type (Figure 5).

If *cr88* is defective in light perception or signal transduction, the expression of some other light-regulated genes may also be affected. As shown in Figure 5, the same blot was hybridized with nuclear-encoded photosynthetic gene probes, the light-harvesting chlorophyll *a/b* binding protein (*CAB*) probe, the small subunit of ribulose-1,5-bisphosphate carboxylase (*RBCS*) probe, and a nonphotosynthetic gene probe, chalcone synthase (*CHS*). The light-induced expressions of *CAB* and *RBCS* in *cr88* were at levels lower than those in the wild type. In contrast, the light-induced expression of *CHS* in *cr88* was at a level similar to, if not higher than, that in the wild-type seedlings. These results suggest that CR88 is a component of a common light perception or signal transduction pathway that regulates *NR2* and the photosynthetic genes *CAB* and *RBCS* and that a different light signal transduction pathway(s) regulates the light response of *NR1* and *NiR* genes.

cr88 Is Defective in the Deetiolation Process and Defines a New *HY* Locus

The decreased expression of photosynthetic genes and the yellow-green phenotype of *cr88* raised the possibility that *cr88* might be defective in deetiolation. In white light, *cr88* exhibited similar to wild-type–length hypocotyls (Figures 3A and 6, and Table 2) and opened cotyledons (Figure 3A). When the deetiolation process was examined in continuous red light, however, *cr88* (Figures 6 and 7H) exhibited longer than wild-type–length hypocotyls (Figures 6 and 7A) and closed but partially expanded cotyledons (Figure 7H). To test whether the two phenotypes, yellow green and defective deetiolation in red light, were caused by the same mutation, we analyzed segregation of these two phenotypes in the F_2 generation of a cross between *cr88* and the wild type. Among 406 seedlings examined, the two traits always cosegregated. The cotyledons of all of the 304 seedlings that exhibited normal deetiolation in red light were green; the cotyledons of all of the 102 etiolated seedlings were yellow green. Thus, these two phenotypes are most likely pleiotropic traits caused by a single gene mutation.

At least nine *HY* loci have been defined in Arabidopsis. They include light-stable phytochrome B (PHYB) locus *HY3* (Koornneef et al., 1980; Somers et al., 1991); light-labile phytochrome A (PHYA) locus *HY8* (Dehesh et al., 1993; Nagatani

Table 2. Allelism Tests of *cr88* with Known *hy* Mutants

Strains or Crosses	No.[a]	Hypocotyl Length (mm)[b]
Col-*gl*	9	5.5 ± 0.1
Ler	11	4.8 ± 0.2
cr88[c]	15	7.6 ± 0.1
hy1[d]	8	10.5 ± 0.1
hy2[d]	7	9.8 ± 0.2
hy3[d]	9	9.1 ± 0.3
hy5[d]	7	9.0 ± 0.3
hy6[d]	10	9.8 ± 0.3
Col-*gl* × *Ler* (F_1)	7	5.8 ± 0.1
cr88 × *hy1* (F_1)	7	5.2 ± 0.3
cr88 × *hy2* (F_1)	7	4.7 ± 0.2
cr88 × *hy3* (F_1)	9	5.1 ± 0.3
cr88 × *hy5* (F_1)	7	4.5 ± 0.3
cr88 × *hy6* (F_1)	8	5.1 ± 0.2

[a] No., number of seedlings examined.

[b] Seedlings were grown under continuous red light. Hypocotyl lengths were measured on the third day of germination. Numbers are means ± SE.

[c] *cr88* is in the Col-*gl* background.

[d] The known *hy* mutants are in the *Ler* background.

et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993); phytochrome chromophore biosynthesis loci *HY1*, *HY2*, and *HY6* (Koornneef et al., 1980; Chory et al., 1989; Parks and Quail, 1991); blue light receptor CRY1 locus *HY4;* a component in the signal tranduction pathway downstream of both phytochromes and CRY1, *HY5* (Koornneef et al., 1980; Ang and Deng, 1994); and two loci probably acting in the pathway downstream of PHYA, *FHY1* and *FHY3* (Whitelam et al., 1993). Among these *hy* mutants, *hy4*, *hy8*, *fhy1*, and *fhy3* seedlings are deetiolated in continuous red light; *hy4* is etiolated in continuous blue light (Ahmad and Cashmore, 1993), and the other three are etiolated in continuous far-red light (Koornneef et al., 1980; Chory et al., 1989; Parks and Quail, 1991). Thus, the possibility of *cr88* being allelic to *hy4*, *hy8*, *fhy1*, or *fhy3* is ruled out.

Like *cr88*, the remainder of the *hy* mutants *hy1*, *hy2*, *hy3*, *hy5*, and *hy6* all exhibited the etiolated phenotype in continuous red light. Therefore, the deetiolation of *cr88* seedlings was examined in various light spectra, and the hypocotyl lengths of *cr88* seedlings were compared with those of the wild-type, *hy1*, *hy2*, *hy3*, *hy5*, and *hy6* seedlings. Because similar results were obtained with chromophore mutants *hy1*, *hy2*, and *hy6*, only the hypocotyl lengths of *hy1*, *hy3*, and *hy5* are shown in Figure 6. In continuous light of the blue and far-red spectra, *cr88* exhibited wild-type–length hypocotyls (Figure 6) and opened cotyledons. In continuous white light (100 μmol m^{-2} sec^{-1}), *cr88* exhibited slightly longer than wild-type–length hypocotyls (Figure 6) and opened cotyledons (data not shown; see Figure 3A for soil-grown seedlings). In continuous red light, however, *cr88* exhibited hypocotyls longer than those of the wild type (Figures 6, 7A, and 7H) and closed but partially expanded cotyledons (Figure 7H). Because far-red light inhibited *cr88* hypocotyl elongation completely (Figure 6), *cr88* is unlikely to be an allele of the chromophore biosynthesis mutants *hy1*, *hy2*, and *hy6.* Because both blue and far-red light inhibited *cr88* hypocotyl elongation, *cr88* is unlikely to be an allele of *hy5*. These results, however, do not exclude the possibility that *cr88* is an allele of *hy3.* To consider *cr88* as an allele of *hy3*, one must reconcile the contradiction that, in comparison with *hy3*, *cr88* caused a weaker defect in light inhibition of hypocotyl elongation yet a more severe defect in greening.

To investigate the possibility that *cr88* defines a new chromophore mutant locus, we tested whether the chromophore precursor biliverdin would inhibit hypocotyl elongation in continuous red light. Similar to previous observations (Parks and Quail, 1991), biliverdin at 0.005 to 0.01 mM was effective in inhibiting the hypocotyl elongation of *hy1* (Figure 8) and other chromophore mutants *hy2* and *hy6* (data not shown). In contrast, the hypocotyl lengths of *cr88* and the wild-type seedlings did not change significantly in response to biliverdin (Figure 8). These results rule out the possibility that *cr88* is defective in the biosynthetic steps before the addition of biliverdin. *cr88*, however, still could be a chromophore mutant that cannot be rescued by biliverdin, as is the case of the tomato *aurea* mutant (van Tuinen et al., 1996).

To confirm further that *cr88* is not an allele of any of the five relevant *hy* mutants, we conducted complementation tests between *cr88* (Figure 7H), which is in the Col-*gl* background (Figure 7A), and *hy1*, *hy2*, *hy3*, *hy5*, and *hy6* mutants (Figures 7C to 7G), which are in the *Ler* background (Figure

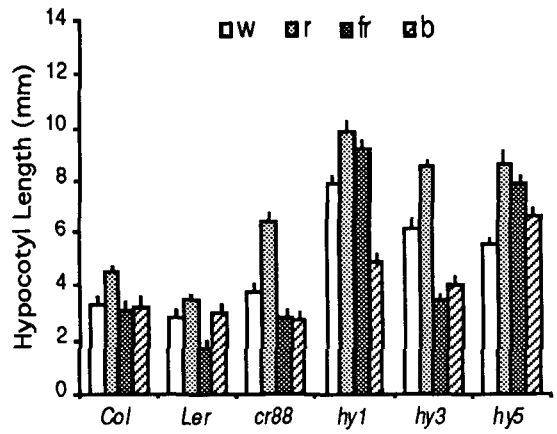

Figure 6. Hypocotyl Lengths of Wild-Type, *cr88*, *hy1*, *hy3*, and *hy5* Seedlings in Different Light Spectra.

The seeds of mutants and their corresponding wild types (*Col*, wild-type Col-*gl* for *cr88*; *Ler*, wild-type *Ler* for *hy1*, *hy3*, and *hy5*) were sown onto agar plates and germinated, as described in Methods. Seedlings were then grown for an additional 3 days under continuous white (w), red (r), far-red (fr), and blue (b) light. Bars represent mean hypocotyl lengths of 10 to 15 seedlings, and vertical lines represent standard errors.

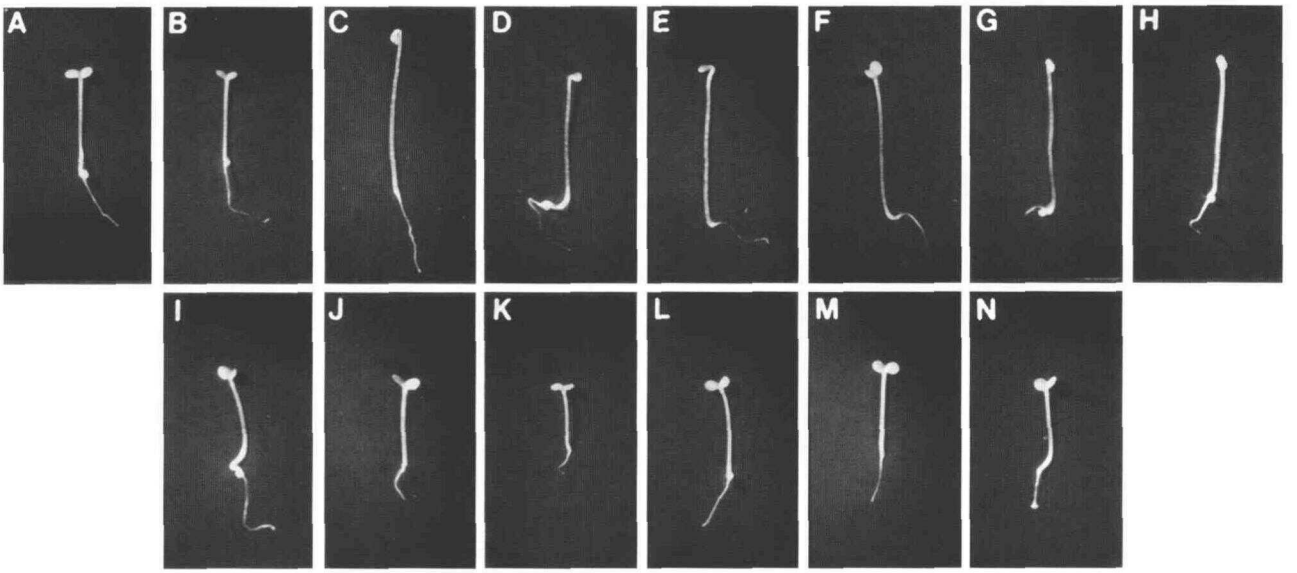

Figure 7. Allelism Test of *cr88* with *hy1*, *hy2*, *hy3*, *hy5*, and *hy6*.

The seeds of mutants, their corresponding wild types (wild-type Col-*gl* for *cr88*; wild-type L*er* for *hy1*, *hy2*, *hy3*, *hy5*, and *hy6*), and the F_1 progeny were sown on agar plates and germinated, as described in Methods. Seedlings were then grown for an additional 3 days in continuous red light.
(A) Wild-type Col-*gl*.
(B) Wild-type L*er*.
(C) *hy1*.
(D) *hy2*.
(E) *hy3*.
(F) *hy5*.
(G) *hy6*.
(H) *cr88*.
(I) F_1 seedling from a cross between Col-*gl* and L*er*.
(J) F_1 seedling from a cross between *cr88* and *hy1*.
(K) F_1 seedling from a cross between *cr88* and *hy2*.
(L) F_1 seedling from a cross between *cr88* and *hy3*.
(M) F_1 seedling from a cross between cr88 and *hy5*.
(N) F_1 seedling from a cross between *cr88* and *hy6*.

7B). F_1 seedlings from these crosses (Figures 7J to 7N) all exhibited short hypocotyls and opened cotyledons in red light, similar to F_1 seedlings from a cross between Col-*gl* and L*er* (Figure 7I). These results indicate strongly that *cr88* is not an allele of any of these five *hy* mutants.

cr88 Exhibits Low NR Activity Only at the Seedling Stage but Not in Mature Plants

All of the studies on photoreceptor-mediated light induction of *NR* gene expression were performed with seedlings (Rajasekhar et al., 1988; Melzer et al., 1989; Becker et al., 1992; Bergareche et al., 1994). In addition, *cr88* was selected for chlorate resistance during the seedling stage. Therefore, it was of interest to determine whether the defect of light response in *cr88* that affected *NR2* gene expression is restricted to the seedling stage or also has an effect in the mature plant. For *cr88* and wild-type plants, the NR activities of soil-grown 10-day-old seedlings and 4-week-old rosettes were compared. At the seedling stage, *cr88* possessed 45% of the wild-type level of NR activity (Table 3). In mature plants, however, both young (yellow green) and old (green) rosette leaves of *cr88* exhibited levels of NR activities similar to those of wild-type plants (Table 3). These results indicate that the function of CR88 is required at the seedling stage for full expression of *NR* genes but is dispensable in mature plants.

DISCUSSION

Identification of the *NR* Regulatory Mutant *cr88*

Nitrate assimilation provides plants with reduced nitrogen at the expense of energy. To maintain optimum growth, the rate of this process must be coordinated with the plant's demand for nitrogen and the supply of substrate (nitrate) and energy (carbohydrates and photosynthesis). Therefore, it is

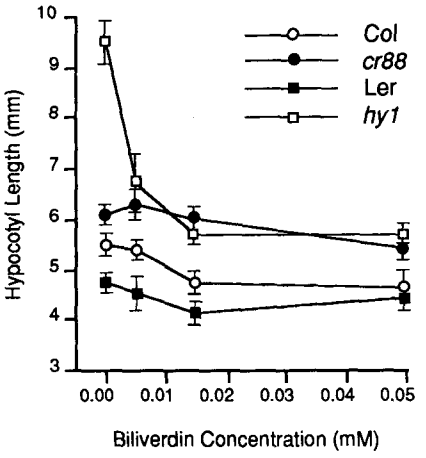

Figure 8. Dose–Response Curves for the Effect of Biliverdin on Hypocotyl Elongation of Red Light–Grown *cr88*, *hy1*, and Wild-Type Seedlings.

The seeds of *cr88*, *hy1*, and their corresponding wild-type plants (Col, wild-type Col-*gl* for *cr88*; Ler, wild-type Ler for *hy1*) were sown on agar plates and germinated, as described in Methods. Seedlings were then grown for an additional 3 days in continuous red light. The mean values of 10 seedlings were plotted. Vertical lines represent the standard errors.

not surprising that NR, which catalyzes the first committed step in nitrate assimilation, is regulated by nitrate, light, and the level of carbohydrates. To understand such a complex regulatory system, a genetic approach would be informative. *NR* regulatory mutants, which show lower levels of NR activity, theoretically can be selected by using chlorate resistance as a phenotype. Despite the extensive selection of Arabidopsis chlorate-resistant mutants, *NR* regulatory mutants proved to be elusive (Braaksma and Feenstra, 1973, 1982; Wilkinson and Crawford, 1991; LaBrie et al., 1992; Tsay et al., 1993).

In this study, we selected chlorate-resistant mutants by using an optimized condition that allows regulatory mutants to be identified. One of these chlorate-resistant mutants, *cr88*, retained higher NR activity (up to 50% of wild-type levels) in comparison with known chlorate-resistant mutants of Arabidopsis. Biochemical and genetic analyses eliminated the possibility that *cr88* is defective in nitrate uptake or in the enzymatic function of NR. Further characterization of *cr88* showed that it is indeed a regulatory mutant affecting the expression of the *NR2* gene. Specifically, the light-induced gene expression of *NR2*, but not *NR1* or *NiR*, is impared in *cr88*.

cr88 Is Defective in the Light Induction of *NR2* and Photosynthetic Genes

Although expression of the *NR2* gene in *cr88* seedlings was induced by sucrose and nitrate (Figures 1 and 2), it barely responded to light (Figure 5). A relatively low fluence of light (120 μmol m^{-2} sec^{-1}) was used in this experiment to prevent accumulation of photosynthates. Under this condition, etiolated seedlings did not turn green after 8 hr of illumination by white light, suggesting that little if any photosynthesis occurred. Therefore, the light-induced gene expression shown in Figure 5 is most likely due to the response to light signals, not to the products of photosynthesis. This conclusion is consistent with previous observations in which light acts as a signal to induce the expression of *NR* genes in etiolated seedlings (Rajasekhar et al., 1988; Melzer et al., 1989; Becker et al., 1992; Bergareche et al., 1994). In addition to *NR2*, the mRNA levels of light-regulated nuclear-encoded photosynthetic genes *CAB* and *RBCS* were also reduced in *cr88*, further supporting the conclusion that *cr88* is defective in the direct role that light plays in the regulation of *NR2*.

The demonstration that sugars can substitute for white light to increase *NR* gene transcription indicates that in addition to acting as a signal, light regulates *NR* gene expression through the accumulation of photosynthates (Cheng et al., 1992; Vincentz et al., 1993). Because it is difficult to separate the two effects of light, it is unclear to what degree light signals and photosynthesis contribute to *NR* gene expression. *cr88* offers an opportunity to dissect the two roles that light plays in *NR* gene expression. The steady state levels of *NR2* mRNA were lower in *cr88* than in the wild-type seedlings, even in the presence of exogenous sucrose (Figures 1B and 2B). To determine whether 1% sucrose was insufficient to compensate for the defect in *cr88*, we examined nitrate induction kinetics in the presence of 3% sucrose. Similar results were obtained (data not shown). These results suggest that at the seedling stage, light acts as a signal to regulate *NR2* gene expression, and its role cannot be re-

Table 3. NR Activities of Soil-Grown *cr88* Seedlings and Mature Plants

	NR Activity[a]	
Developmental Stage	*Col-gl*	*cr88*
Seedlings[b]	53 ± 3	24 ± 3 (45%)
Mature plants[c]		
Young leaves[d]	126 ± 4	134 ± 3 (106%)
Mature leaves[e]	108 ± 5	98 ± 3 (91%)

[a] NR activity is expressed as nanomoles of nitrite produced per minute per gram fresh weight of tissue at 30°C. Numbers are means ± SE of three assays with five leaves each. Percentage activities of the wild type are shown in parentheses.

[b] Whole 10-day-old seedlings grown on soil were assayed. For more details, see Methods.

[c] Rosette leaves of 4-week-old soil-grown plants were assayed. For more details, see Methods.

[d] Young leaves of *cr88* correspond to the green leaves shown in Figure 3C.

[e] Mature leaves of *cr88* correspond to the yellow-green leaves shown in Figure 3C.

placed completely by sucrose. This conclusion is consistent with the result that the NR activity in 10-day-old seedlings of *cr88* is lower than that of the wild type (Table 3). Different results were obtained with mature plants. In 4-week-old rosettes, the young (yellow green) leaves of *cr88* exhibited the same level of NR activity as the comparable leaves (green) of the wild type (Table 3). The young leaves of *cr88* were yellow green, suggesting that CR88 is required for greening in the mature plant just as in seedlings. The role of CR88 in the regulation of the *NR2* gene, however, is dispensable in mature plants.

A plastidic factor has been proposed to be required for nitrate- and light-induced *NR* gene expression (Oelmuller et al., 1988; Oelmuller and Briggs, 1990). In these experiments, the plastids of seedlings were destroyed by norflurazon treatment followed by photooxidation. These seedlings no longer exhibited nitrate- or light-induced expression of *NR* genes. Our results suggest that *cr88* is unlikely to be defective in this plastidic factor. The expression of both *NR* genes still responded to nitrate induction, and the expression of *NR1* still responded to light. In addition, the fairly advanced degree of chloroplast development (Figure 4) and the vigorous growth further suggest that *cr88* is unlikely to be deficient in this factor.

Although we observed previously that the two Arabidopsis *NR* genes, *NR1* and *NR2*, were differentially regulated (Cheng et al., 1991), the differences were subtle. Now, by studying the expression of these two *NR* genes and the *NiR* gene in the *cr88* background, the otherwise subtle differences between the regulation patterns of these genes have become better resolved. Most strikingly, *cr88* is defective in the light signal transduction pathway leading to *NR2* gene expression, thus uncoupling the light regulation of *NR2* from that of *NR1* and *NiR*. The *cr88* mutant was isolated from ethyl methanesulfonate–mutagenized seed stock. It may or may not be a null allele. If it is a leaky mutant, we cannot rule out the possibility that CR88 affects the expression of all three nitrate assimilation genes, and *NR2* is most sensitive to the activity of CR88.

Comparison of *cr88* with Other *hy* Mutants

Because of the deficiency of light-regulated gene expression and the slow greening of *cr88*, we decided to analyze the deetiolation process of *cr88* in different light spectra. In continuous white light, the hypocotyls of *cr88* seedlings were slightly longer than those of the wild type, and the cotyledons were as open as those of the wild type (Figure 3A). In continuous red light, *cr88* seedlings exhibited much longer hypocotyls than did those of the wild type, with no apical hooks and closed but partially expanded cotyledons (Figure 6). In continuous far-red and blue light, *cr88* seedlings displayed short hypocotyls and opened cotyledons similar to those of wild-type seedlings. These results rule out the possibility that *cr88* is an allele of *hy4*, *hy8*, *fhy1*, or *fhy3*. They also suggest that *cr88* is unlikely to be an allele of *hy1*, *hy2*, *hy5*, or *hy6*. In fact, the phenotype of *cr88* does not resemble any of the known Arabidopsis *hy* mutants. When compared with *hy1*, *hy2*, *hy3*, *hy5*, and *hy6*, *cr88* exhibited the mildest defect in the light inhibition of hypocotyl elongation. In contrast, the greening process in *cr88* is delayed the longest. Three days after germination, all five *hy* mutants displayed normal green cotyledons, whereas the cotyledons of *cr88* appeared yellow green. Throughout its life, newly initiated leaves, stems, and flower buds of *cr88* are yellow green; during maturation, the leaves and stems become greener but never as green as those of wild-type plants. In spite of its yellow-green leaves, *cr88* plants grew vigorously, unlike the *hy*6 mutant, which is pale and small. Consistent with the phenotypic discrepancies observed between *cr88* and the *hy1*, *hy2*, *hy3*, *hy5*, and *hy6* mutants, complementation between *cr88* and each of the *hy* mutants indicates that *CR88* defines a new *HY* locus.

Possible Functions of CR88 in Light Signal Transduction

Compared with other *hy* mutants, *cr88* displays two seemingly contradictory phenotypes. The greening of *cr88* is delayed more severely than it is for any of the *hy* mutants, yet the impaired inhibition of hypocotyl elongation is less severe than it is for any of the *hy* mutants. We propose three hypotheses for the possible functioning of CR88.

In the first hypothesis, *CR88* encodes one of the less understood light-stable phytochromes, PHYC, PHYD, or PHYE (Sharrock and Quail, 1989; Clark et al., 1994), or a component downstream of these three phytochromes. Extensive screening for long hypocotyl mutants in white light has allowed only the identification of the *PHYB* mutant *hy3* (Koornneef et al., 1980; Chory et al., 1989), suggesting that PHYB is the major phytochrome that mediates inhibition of hypocotyl elongation in white light. Therefore, the other three light-stable phytochromes may play more important roles in other aspects of light regulation. One of these roles could be to regulate a subset of light-regulated genes (such as *NR2*), some of which may be important for chloroplast development. One problem with this hypothesis is that although these chromophore-deficient mutants (*hy1*, *hy2*, and *hy6*) contain no spectrophotometrically detectable phytochrome (Chory et al., 1989; Parks et al., 1989), none of them displays as severely delayed greening as *cr88*. Maybe these chromophore-deficient mutants are leaky, and the amount of chromophore, which is below the level detectable spectrophotometrically, is sufficient to constitute biologically active PHYC, PHYD, or PHYE. *hy1*, *hy2*, and *hy6* mutants all exhibit red light–inducible and far-red light–reversible *CAB* mRNA expression (Chory et al., 1989), indicating the existence of biologically active phytochrome in these chromophore mutants.

Alternatively, *CR88* is a new chromophore biosynthesis or attachment locus. Recently, the *aurea* mutant of tomato has been shown to be a phytochrome chromophore biosynthesis

mutant (van Tuinen et al.,1996). The phenotype of the mature plants of *cr88* resembles the *aurea* mutant in that the newly initiated leaves are yellow green and the older leaves turn greener. In addition, *aurea* seedlings, like *cr88* seedlings, exhibit near wild-type–length hypocotyls in continuous white light and much longer than wild-type–length hypocotyls in continuous red light. Finally, as with *cr88*, the lack of inhibition of hypocotyl elongation in *aurea* seedlings cannot be restored by biliverdin. There is, however, a major difference between the two mutants. The deetiolation process of *cr88* is normal in continuous far-red light, indicating an active PHYA pool, whereas *aurea* has a severely reduced PHYA pool. It is possible that the active PHYA pool in *cr88* is sufficient to mediate the response to continuous far-red light, even though it is reduced.

The final hypothesis predicts that CR88 is a component in the signal transduction pathway downstream of photoreceptors. It performs a major function in regulating the expression of a subset of light-regulated genes, including those required for greening. It has a minor function in inhibiting hypocotyl elongation. Another mutant defective in the light signal transduction pathway downstream of photoreceptors that exhibits the *hy* phenotype is *hy5.* COP1 interacts directly or close to HY5 to suppress the deetiolation process in the dark (Ang and Deng, 1994). These authors also showed that *CAB* and *RBCS* mRNAs are expressed at wild-type levels and are light inducible in the *hy5* mutant. This finding suggests that the interaction of COP1 and HY5, although critical for the control of hypocotyl elongation, is not required for the regulation of *CAB* and *RBCS* expression. In *cop1*, on the other hand, many light-regulated genes, including *CAB*, *RBCS*, and *NR2*, are expressed constitutively in the dark (Deng et al., 1991). This indicates that COP1 suppresses the expression of these genes through another branch of the signal transduction pathway, of which HY5 is not a component. CR88 fits well as one of the components in this branch of the pathway. The deetiolation process involving COP1 is sensitive to light signals perceived by both phytochrome and blue light photoreceptors (McNellis et al., 1994). To explain why CR88 is required to inhibit hypocotyl elongation in continuous red but not in blue or far-red light, we have to take into account that red light is less efficient than blue and far-red light at inhibiting hypocotyl elongation (McNellis and Deng, 1995). If in far-red or blue light the removal of COP1 from the nucleus is more complete, this allows HY5 to fulfill its function in the inhibition of hypocotyl elongation. In red light, a higher concentration of COP1 may be retained in the nucleus, causing partial suppression of HY5 function. Under this condition, CR88 would become indispensable.

In summary, we have demonstrated that an optimized chlorate selection condition allowed us to isolate regulatory mutants defective in the regulation of *NR* gene expression. One of the mutants, *cr88*, specifically affected the light-induced expression of the *NR2* gene but not *NR1* or *NiR.* In addition, the light induction of photosynthetic genes *CAB* and *RBCS* was also affected in *cr88.* Physiological and genetic analyses showed that *CR88* identifies a new *HY* locus. The isolation of a chlorate-resistant mutant that is defective in the light regulation of both a nitrate assimilation gene and photosynthetic genes demonstrates that the regulation of nitrate assimilation is coupled genetically to photosynthesis. To place *NR* gene regulation under the same pathways that regulate the expression of photosynthetic genes is an effective means to coordinate nitrate assimilation and photosynthesis.

METHODS

Light Sources

Cool-white fluorescent lamps were used to provide continuous white light. Light sources for other continuous light irradiation are as follows: red (19 μmol m^{-2} sec^{-1}), under four F20T12/CW fluorescent lamps (Sylvania, Danvers, MA) with a red plexiglass 3-mm filter (model 2423; Rohm and Haas, Philadelphia, PA); blue (11 μmol m^{-2} sec^{-1}), under four F20T12/WW fluorescent lamps (Sylvania) with a blue plexiglass 3-mm filter (model 2424; Rohm and Haas); far red (10 μmol m^{-2} sec^{-1}), under a 120-W General Electric floodlamp with a 730-nm (one-half bandwidth of 12.6 nm) filter (Ditric Optics, Hudson, MA).

Plant Material, Seed Sterilization, and Cold Treatment

Arabidopsis thaliana Columbia *glabrous1* (Col-*gl*) seeds and ethyl methanesulfonate–mutagenized M_2 seeds from plants in the Col-*gl* and Landsberg *erecta* (*Ler*) backgrounds were obtained from Lehle Seeds (Round Rock, TX). Seed sources of the long hypocotyl *hy* mutants are *hy1* (21.84N) and *hy3* (Bo64) descended from the Koornneef et al. (1980) isolates; *hy2* (*hy2-2*) descended from the Chory et al. (1989) isolates; and *hy5* (*hy5-1*) and *hy6* from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). Seed sources of the chlorate-resistant mutants are *chl1*, *chl2*, *chl3* (B29), and *chl4* (B25), which are descendants of the Braaksma and Feenstra (1982) isolates.

Except for soil-grown plants, seeds were surface sterilized with 95% ethanol for 5 min and 1.5% sodium hypochlorite for 5 min. Seeds were rinsed five times with sterile water.

Seeds were treated under various conditions to achieve uniform germination. Seeds in liquid media were incubated in the dark at 4°C for 1 day. Seeds plated on solid medium for observation of deetiolation were incubated in the dark at room temperature for 1 day and at 4°C for 2 days before light exposure. For soil-grown plants, seeds were imbibed in 20 mM KNO_3 in the dark at 4°C for 2 days before sowing. For seedlings grown on a vermiculite/perlite mixture after sowing, the pots were incubated in the dark at room temperature for 1 day, at 4°C for 3 days, and at room temperature for 1 day.

Chlorate Selection Conditions

Vermiculite/perlite mixed at a 1:1 ratio was saturated with an ammonium medium (1.5 mM $[NH_4]_2SO_4$, 4.2 mM $MgSO_4$, 5.5 mM KH_2PO_4, pH 5.7, and 17 mM $CaCO_3$, and the micronutrients were the same as those in MS salts) (Murashige and Skoog, 1962). Plant growth was at 21°C.

Seeds were allowed to germinate in 50 to 100 μmol m^{-2} sec^{-1} continuous white light. After germination, seedlings were grown under 350 μmol m^{-2} sec^{-1} continuous white light and were irrigated with the ammonium medium every other day. Eight days after germination, seedlings were irrigated with the ammonium medium supplemented with 0.5 mM KNO_3 (nitrate medium). Ten (for the *Ler* background) or 21 (for the Col-*gl* background) hr later, they were treated with nitrate medium supplemented with 1 mM $KClO_3$. Subsequently, seedlings were irrigated with nitrate medium every other day. Two to 7 days after chlorate treatment, seedlings were analyzed for symptoms of necrosis. Those seedlings with few or no symptoms were transferred to soil and allowed to set seeds. Chlorate resistance was analyzed similarly in the M_3 generation.

Growth Conditions for Other Analyses

All plants were grown at 21°C. For soil-grown plants, seeds were sowed on Jiffy Mix Plus (Jiffy Products of America, Inc., Batavia, IL). Plants were grown under a 16-hr-light and 8-hr-dark cycle in 800 μmol m^{-2} sec^{-1} for the periods of time indicated.

For light induction of gene expression experiments, seedlings were grown in the dark in liquid medium containing one-quarter–strength MS salts and 1% sucrose, with constant shaking at 100 rpm. On day 7 after germination, seedlings were either exposed to 120 μmol m^{-2} sec^{-1} white light or were kept in the dark. Seedlings were harvested 8 hr later.

For nitrate induction of nitrate reductase (*NR*) gene expression experiments, seedlings were grown in nitrate-free medium as described by Cheng et al. (1991), except that one-half–strength macronutrients was used. Flasks containing the seedlings and medium were constantly shaken at 100 rpm in 120 μmol m^{-2} sec^{-1} continuous white light for 7 days before 1% sucrose was added to half of the samples. Twenty-four hours after the addition of sucrose, KNO_3 was added to the media to a final concentration of 5 mM, and seedlings were harvested at various time points.

For deetiolation experiments, seedlings were grown on Petri dishes containing one-half–strength MS salts and 0.8% agar. For studies of biliverdin inhibition of hypocotyl elongation, biliverdin was dissolved in methanol (Parks and Quail, 1991) and added to the sterile media to the final concentrations indicated. To promote uniform germination, a 15-min red light treatment was given to the seeds before growth in different light spectra. On day 3 after germination, the lengths of the hypocotyls were measured.

RNA Analysis

Procedures for RNA gel blot analysis and the sources for Arabidopsis *NR1*, *NR2*, and the small subunit of ribulose-1,5-bisphosphate carboxylase (*RBCS*) were described by Cheng et al. (1991). The cDNAs of Arabidopsis chalcone synthase (*CHS*) were obtained from the Arabidopsis Biological Resource Center (stock number ATTS0223). The light-harvesting chlorophyll *a/b* binding protein (*CAB*) (pAB180) cDNA was provided by E.M. Tobin (University of California, Los Angeles). The soybean 28S rRNA genomic clone was provided by J.L. Key (University of Georgia, Athens). RNA quantification was performed by using either a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or scanning densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, CA).

Enzyme Assay

NR activity assay was performed as described by Warner and Kleinhofs (1981).

Transmission Electron Microscopy

Cotyledons from 3-day-old soil-grown seedlings and rosette leaves from 4-week-old soil-grown plants were sliced and fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.2, kept at room temperature for 1 hr, and then transferred to 4°C overnight. After post-fixation in 1% osmium tetroxide for 1 hr, samples were stained with 2.5% uranyl acetate for 20 min. Samples were then dehydrated in acetone and embedded in Spurr's resin (Polysciences Inc., Warrington, PA). After microtomy, sections were poststained with 5% uranyl acetate for 8 min followed by lead citrate for 7 min. Specimens were observed with a transmission electron microscope (model H7000; Hitachi Scientific Instruments, Mountain View, CA). Five leaves (or cotyledons) were observed for each experiment.

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