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Sucrose mimics the light induction of *Arabidopsis* nitrate reductase gene transcription

(gene regulation/metabolic regulation/photosynthesis/ribulose-bisphosphate carboxylase small subunit/transgenic plants)

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ABSTRACT **Nitrate reductase, the first enzyme in nitrate assimilation, is located at the crossroad of two energy-consuming pathways: nitrate assimilation and carbon fixation. Light, which regulates the expression of many higher-plant carbon fixation genes, also regulates nitrate reductase gene expression. Located in the cytosol, nitrate reductase obtains its reductant not from photosynthesis but from carbohydrate catabolism. This relationship prompted us to investigate the indirect role that light might play, via photosynthesis, in the regulation of nitrate reductase gene expression. We show that sucrose can replace light in eliciting an increase of nitrate reductase mRNA accumulation in dark-adapted green *Arabidopsis* plants. We show further that sucrose alone is sufficient for the full expression of nitrate reductase genes in etiolated *Arabidopsis* plants. Finally, using a reporter gene, we show that a 2.7-kilobase region of 5′ flanking sequence of the nitrate reductase gene is sufficient to confer the light or the sucrose response.**

Nitrate is the predominant form of soil nitrogen available to plants. Once taken up by plants, nitrate must be reduced to ammonia prior to incorporation into amino acids. The process of nitrate assimilation requires high energy input. As much as 25% of the energy generated by photosynthesis can be consumed in driving nitrate assimilation (1). Nitrate reductase (NR; NADH:nitrate oxidoreductase, EC 1.6.6.1), the first enzyme in nitrate assimilation, is located at the crossroad of two energy-consuming pathways: nitrate assimilation and carbon fixation. On one hand, the two pathways compete for electrons arising from the photosynthesis light reaction. On the other hand, nitrate reduction depends on energy generated from the products of carbon fixation. Thus, placing NR and photosynthetic genes under the control of photosynthesis would be an effective means of balancing these pathways. Indeed, NR activity has been correlated with environmental changes that affect photosynthesis (i.e., light, CO_2) (2–5). However, it is not clear whether these environmental factors affect NR gene expression directly or, rather, indirectly by affecting the rate of photosynthesis and hence the carbohydrate levels in plants.

Light regulates the expression of many photosynthetic genes and also regulates NR gene expression. Although it has been known for a long time that light enhances NR activity in higher plants (for a review, see ref. 5), the role of light on the induction of NR genes is far less understood than its effect on the induction of the photosynthetic genes such as those encoding the chlorophyll a/b-binding protein and the small subunit of ribulose-bisphosphate carboxylase (*rbcS*). By using NR cDNA clones, it has been demonstrated that light increases the steady-state levels of NR mRNA (1). In squash cotyledons and in etiolated barley seedlings, a red light pulse induces NR mRNA accumulation and a far-red light pulse reverses the induction (6, 7). Blue light also induces NR mRNA accumulation in barley seedlings (7). These limited examples suggest that light, acting via the photoreceptor phytochrome and perhaps the blue-light receptor, plays a direct role in NR gene regulation in etiolated plants.

The effect of light on green barley seedlings is quite different from that on the etiolated seedlings. Only white light, but not red or blue light, stimulates significant accumulation of NR mRNA (7). Using dark-adapted green plants, we have shown previously that *Arabidopsis* NR genes are induced by white light and that the induction kinetics are similar to those of the *rbcS* genes (8). The photon energy of the white light used for the induction of NR mRNA is sufficient to be captured by chlorophyll for photosynthesis (9). We now use this system to test the hypothesis that light plays an indirect role in regulating NR gene expression. Here we show that sucrose, the major sugar derived from photosynthesis and an energy source for nitrate reduction, can substitute for light to induce *NR1* mRNA accumulation. In contrast, *rbcS* mRNA can be induced by light but not by sucrose. Using transgenic plants in which transcription of a reporter gene is driven by the upstream region [≈2.7 kilobases (kb)] of the *NR1* gene, we demonstrate further that the induction is, at least in part, transcriptional.

MATERIALS AND METHODS

Plant Material and Growth Conditions. *Arabidopsis thaliana* (L.) Heynh line Columbia was used in all experiments. Plants were grown for 16 days in sterile hydroponic conditions with 0.5× Murashige and Skoog (MS) salts (10) and 2% sucrose, either in continuous white light [120 $\mu E \cdot m^{-2} \cdot s^{-1}$, obtained from cool white fluorescent lamps; 1 E (einstein) = 1 mol of photons] or in the dark. The same light conditions were used for induction. For carbohydrate depletion, plants were washed and grown in the dark for 3 days in 0.5× MS salts without sucrose. For sucrose induction, sucrose was added at 2% final concentration to depleted plants.

***NR* Promoter–Chloramphenicol Acetyltransferase (CAT) Gene Chimeric Constructs.** Fig. 1*A* illustrates pB10, a binary *Agrobacterium* vector containing an *NR1* promoter–CAT gene chimeric construct. Briefly, the *Nco* I site at the translation start site of the *NR1* gene, with about 2.7 kb of 5′ flanking region (NP1), was fused to BlueCATKS. BlueCATKS is a Bluescript-based CAT–nos3′ plasmid (11) with an *Nco* I site introduced at the start codon of CAT (12). The *Hin*dIII–*Eco*RI fragment containing the NP1–CAT–nos3′

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Abbreviations: CAT, chloramphenicol acetyltransferase; GUS, β-glucuronidase; Kan, kanamycin; NR, nitrate reductase; CaMV, cauliflower mosaic virus; nos, nopaline synthase.

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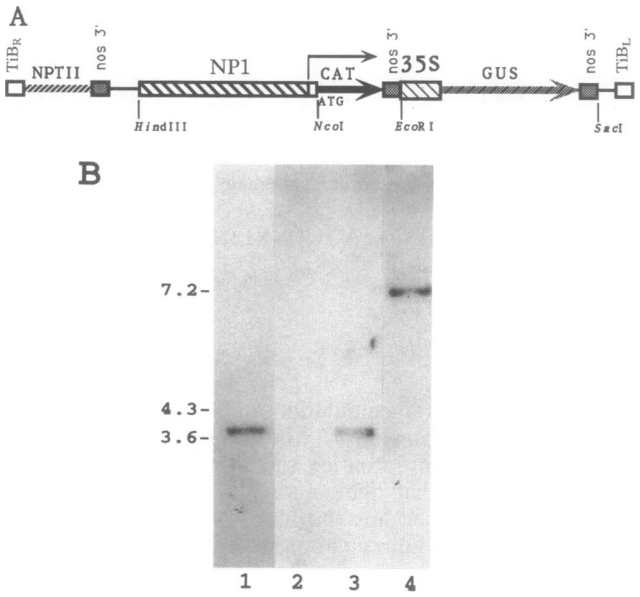

FIG. 1. (*A*) NR1 promoter–CAT gene chimeric construct. The region between the Ti plasmid right and left borders (TiB_R and TiB_L) of pB10 is shown. The neomycin phosphotransferase (NPTII) gene is used as the selectable marker when transformed into plants. The direction of transcription for the CAT and β-glucuronidase (GUS) genes is indicated by arrows. NP1, *NR1* promoter region; 35S, cauliflower mosaic virus (CaMV) 35S promoter; nos3′, 3′ flanking region of nopaline synthase gene. (*B*) DNA blot analysis of transgenic plants. One picogram of pB10 DNA (lane 1, digested with *Hin*dIII and *Eco*RI) or 1 μg of genomic DNA from wild-type plants (lane 2, digested with *Hin*dIII and *Eco*RI) or from transgenic plants homozygous for the transgenes (lane 3, digested with *Hin*dIII and *Eco*RI; lane 4, digested with *Eco*RI) was loaded in each lane. After DNA transfer, the membrane was hybridized to CAT probe (5×10^6 cpm/ml). Molecular size markers are in kilobases.

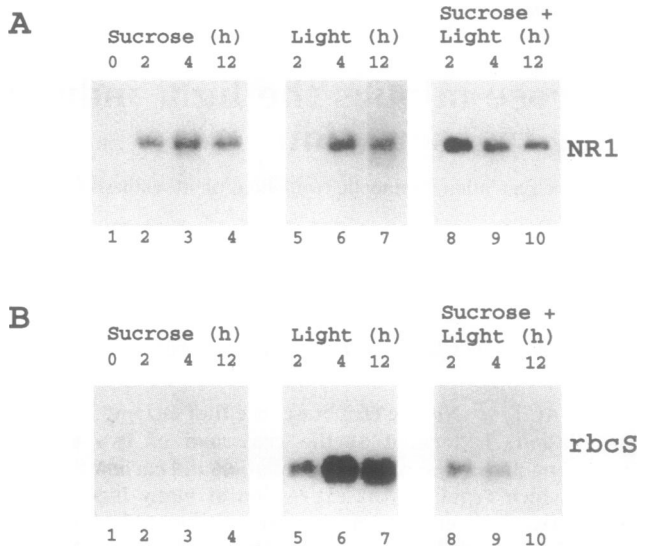

FIG. 2. RNA blots of light- and sucrose-induced dark-adapted green plants. Sixteen-day-old green plants were transferred to fresh medium lacking sucrose and placed in the dark for 3 days to deplete carbohydrates. These plants were then treated with sucrose, light, or sucrose plus light. Total RNA was extracted from leaves at 0, 2, 4, and 12 hr, and 5 μg was loaded in each lane. After RNA transfer, the membranes were hybridized to *NR1* (*A*) or *rbcS* (*B*) probe (5×10^6 cpm/ml).

gene was subcloned in pBI121-R, a pBI121 (13) derivative with the *Eco*RI site removed and the CaMV 35S promoter shortened to about 400 base pairs (J. Sheen, personal communication).

Plant Transformation and Genetic Analysis. pB10 was introduced into wild-type Columbia plants by way of the *Agrobacterium*-mediated root transformation system (14). To determine the genotypes of the transgenic progeny, selfed seeds were plated on 0.5× MS plates containing kanamycin (Kan) at 50 μg/ml, and the segregation of the Kan-resistance (Kan^r) trait was scored.

Nucleic Acid Analyses. For RNA and DNA analyses, leaves were harvested, immediately frozen in liquid nitrogen, and stored at −70°C. Genomic DNA was isolated by the method of Dellaporta *et al.* (15). RNA was isolated by guanidine/phenol extraction (16) followed by LiCl precipitation to remove double-stranded nucleic acid (17). RNA blot analyses were performed as described (9). The same hybridization conditions were used in DNA blot analysis, except that washing was at 50°C. Probes used in DNA and RNA blot analyses were *Arabidopsis NR1* cDNA (18), *rbcS* cDNA (9), CAT coding region (11), and GUS coding region (19).

RESULTS

Kinetics of Light and Sucrose Induction in Dark-Adapted Green Plants. Sixteen-day-old green plants, grown in sucrose under light, were placed for 3 days in the dark without sucrose, to deplete their carbohydrate reserves. *NR1* mRNA was not detectable in these plants (Fig. 2*A*, lane 1). The starved plants were then exposed to light. *NR1* mRNA could be detected 4 hr after light exposure (Fig. 2*A*, lane 6). When sucrose was given to the starved plants and the plants were kept in the dark, *NR1* mRNA appeared 2 hr after sucrose addition (Fig. 2*A*, lane 2). Thus, *NR1* mRNA accumulation initiated earlier in plants given sucrose than in plants exposed to light. When the plants were exposed simultaneously to sucrose and light, *NR1* mRNA accumulated to significant amounts after 2 hr (Fig. 2*A*, lane 8), similar to that in plants exposed to sucrose alone (lane 2).

rbcS, one of the carbon fixation genes, is regulated by light (20). The steady-state level of *rbcS* mRNA was examined for its response to light and sucrose in parallel to *NR1* mRNA. As predicted, the level of *rbcS* mRNA increased dramatically in plants exposed to light (Fig. 2*B*, lane 2). In contrast to *NR1* mRNA, sucrose without light did not induce *rbcS* expression (Fig. 2*B*, lanes 5–7). Moreover, when both light and sucrose were present, the induction of *rbcS* mRNA was inhibited (Fig. 2*B*, lanes 8–10).

High-Level Expression of *NR1* mRNA in the Absence of Light. To determine whether *NR1* mRNA accumulated in plants that were never exposed to light, we grew plants in the dark for 16 days supplemented with sucrose to sustain growth. *NR1* mRNA accumulated to high levels in these plants (Fig. 3*A*, lane 1). We further examined the response of these etiolated plants to light. After 12 hr of light treatment, no increase in *NR1* mRNA was observed (Fig. 3*A*, lanes 2–4). Light showed some enhancement after 24 hr (Fig. 3*A*, lane 5). In contrast, dark-grown plants accumulated insignificant amounts of *rbcS* mRNA (Fig. 3*B*, lane 1). Upon light induction, *rbcS* mRNA accumulated to appreciable levels by 12 hr (Fig. 3*B*, lane 4) and continued to increase during the next 12 hr (lane 5). The light response (in the presence of sucrose) of *rbcS* mRNA accumulation in etiolated plants was different from that of the green plants, where a 12-hr light treatment did not increase the level of *rbcS* mRNA (Fig. 2*B*, lane 10).

Transgenic Plants Containing the *NR1* Upstream Region Fused to a CAT Reporter Gene. A previous report (18) described the cloning of the *NR1* cDNA and the *NR1* gene. Approximately 2.7 kb of DNA immediately 5′ of the translational start site of the *NR1* gene was fused to the CAT coding region (see *Materials and Methods*, Fig. 1*A*). This

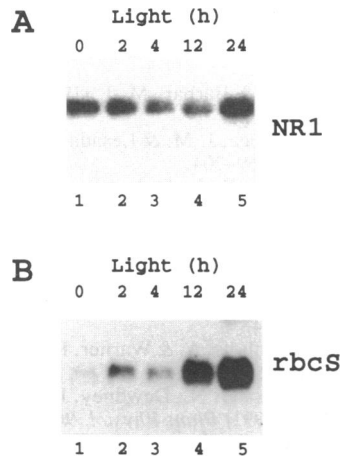

FIG. 3. RNA blots of dark-grown plants and the effect of light. Sixteen-day-old etiolated plants were transferred to fresh medium containing sucrose. These plants were then exposed to light. Total RNA was extracted from leaves at 0, 2, 4, 12, and 24 hr, and 5 μg was loaded in each lane. After RNA transfer, the membranes were hybridized to *NR1* (*A*) or *rbcS* (*B*) probe (5×10^6 cpm/ml).

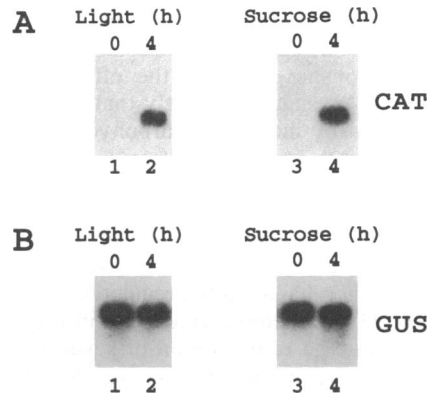

FIG. 4. RNA blots of sucrose- and light-induced transgenic plants. Transgenic plants were grown for 16 days in light and sucrose. These plants were transferred to fresh medium without sucrose and placed in the dark for 3 days to deplete carbohydrates. The plants were then treated with either sucrose or light for 4 hr. Total RNA was extracted from leaves before and after treatment, and 5 μg was loaded in each lane. After RNA transfer, the membranes were hybridized to CAT (*A*) or GUS (*B*) probe (5×10^6 cpm/ml).

construct, pB10, was transformed into *Arabidopsis* by *Agrobacterium*-mediated transformation (14). The transformant used in this study was shown to contain insertions at a single locus by segregation of the Kan^r selectable marker. T_2 seeds grown on Kan plates exhibited a Kan^r/Kan^s ratio of 65:20, close to the 3:1 ratio predicted for segregation of a single locus. Twelve of the T_2 Kan^r plants were grown to maturity and allowed to self. Bulked seeds from each of the individual plants were screened for the segregation of Kan^r. Progeny of 4 of the 12 individuals showed no Kan^s progeny, suggesting that they were homozygous for Kan^r. Progeny of the remaining 8 individuals segregated in the predicted 3:1 ratio. Homozygous T_2 seeds were used in subsequent experiments. Both genomic DNA from transgenic plants and DNA from pB10, when digested with *Hin*dIII and *Eco*RI (excising the NP1–CAT–nos3′ fragment), exhibited the predicted ≈3.7-kb band when hybridized to a CAT probe (Fig. 1*B*, lanes 1 and 3). This result showed that no detectable rearrangements of the NP1–CAT–nos3′ region occurred during transformation. Genomic DNA digested with *Eco*RI exhibited a single band when hybridized to a CAT probe (Fig. 1*B*, lane 4). This result confirmed the genetic analysis and showed further that the T_1 parent was transformed by a single insertion.

Light and Sucrose Increase *NR1* Gene Transcription. Transgenic plants were used to investigate whether the light- and sucrose-dependent accumulation of *NR1* mRNA was regulated transcriptionally. In dark-adapted sucrose-depleted plants, neither CAT mRNA was detected (Fig. 4*A*, lane 1), as was true for *NR1* mRNA (Fig. 2*A*, lane 1). Under the same conditions, high levels of GUS mRNA under the control of the CaMV 35S promoter could be detected (Fig. 4*B*, lane 1). When these plants were given either light or sucrose for 4 hr, both CAT mRNA (Fig. 4*A*, lanes 2 and 4) and *NR1* mRNA (Fig. 2*A*, lanes 4 and 7) accumulated to significant levels. GUS mRNA (Fig. 4*B*, lanes 2 and 4), however, remained at approximately the same level as before the treatments. Thus, the 5′ flanking region of the *NR1* gene conferred light- and sucrose-regulated expression upon a reporter gene that paralleled the expression of the endogenous *NR1* gene. Therefore, light and sucrose induced *NR1* gene transcription.

DISCUSSION

Light profoundly influences plant growth and development in two different ways: (*i*) at low intensity and particular wavelengths, light can be perceived by photoreceptors such as phytochrome, with the result of altered gene expression leading to various morphological changes; (*ii*) at higher intensity, light energy can be captured during photosynthesis to provide plants with carbohydrates. Many genes of diverse functions are regulated by light via the phytochrome photoreceptor. The *rbcS* gene, a nuclear gene whose product functions in the chloroplast to fix CO_2, is one of the best studied of such genes. Red light induces *rbcS* transcription in etiolated or dark-adapted plants, and the induction may be reversed by a far-red pulse, the hallmark of phytochrome control (21). Similarly, NR mRNA has been shown to accumulate in a phytochrome-dependent fashion (6, 7). Blue light also induces NR mRNA accumulation in barley seedlings (8). These data suggest a direct role of light in regulating NR gene expression in etiolated plants. Although the importance of this form of light regulation on NR gene expression is unclear, one can assume that it may play some part in the early stages of plant development.

In examining further the light requirement of NR induction, we discovered that dark-grown etiolated *Arabidopsis*, in the presence of sucrose, accumulated maximal levels of *NR1* mRNA. Light showed no further enhancement (Fig. 3*A*). Gowri and Campbell (22) observed that maize seedlings grown in the dark accumulated significant amounts of NR mRNA (38% of the amount found in green seedlings). Presumably these maize seedlings were nurtured by their endosperm carbohydrate reserves. These data suggest that light is not essential for NR gene expression as long as sufficient carbohydrate is available.

NR, a cytosolic enzyme, requires reducing power from NADH to reduce nitrate. In turn, NADH is derived from carbohydrate catabolism. Our results show clearly that in dark-adapted green plants the availability of sucrose can replace light to induce the accumulation of *NR1* mRNA. Induction by sucrose preceded that by light (Fig. 2*A*). The longer lag time observed with light induction was most likely due to the time needed for the plants to produce sufficient photosynthates for induction. In dark-adapted green barley seedlings, Melzer *et al.* (7) showed that white light stimulated NR mRNA accumulation in an intensity-dependent manner. This observation also suggests that photosynthesis may be involved. In experiments in which the *NR1* promoter was fused to a CAT reporter gene, the addition of sucrose increased the accumulation of CAT mRNA, demonstrating that sucrose regulated NR gene expression, at least in part,

transcriptionally. Similarly, light increased the accumulation of CAT mRNA. While it is difficult to establish unequivocally the causal relationship between the effects of light and sucrose, our results suggest strongly that light induces NR gene transcription by increasing carbohydrate levels in the cells.

Sucrose up-regulates NR gene expression, so that plants will reduce nitrate only when sufficient carbohydrates are present in the cells. This may preclude plants from overtaxing the carbohydrate reserves by excess nitrate reduction. Sucrose, on the other hand, down-regulates carbon fixation genes. Sheen (23) has demonstrated that in protoplasts isolated from leaves, the transcription of seven maize carbon fixation genes is repressed by sucrose and other carbon sources (23). Our results illustrate clearly that in a whole plant system, sugars can exert two opposite modes of regulation: the up-regulation of the NR gene and the down-regulation of the *rbcS* gene. We have also used glucose to replace sucrose and obtained similar effects (data not shown). It is difficult to ascertain whether it is sugars or other metabolites, such as phosphorylated sugars, that are the molecules involved directly in the pathway controlling NR gene transcription. The NR gene is probably only one of many genes whose expression is up-regulated by sugars (24). Nonetheless, the increase of NR gene transcription is not indiscriminate. CAT expression under the control of the *NR1* promoter was regulated by sucrose similarly to the endogenous *NR1* gene, whereas the adjacent GUS gene under the control of the CaMV 35S promoter (Fig. 1*A*) exhibited no change in its mRNA accumulation.

In conclusion, we have demonstrated that light can stimulate the transcription of a higher plant NR gene and that the light regulation is likely to be mediated by sugars produced by photosynthesis. The involvement of sugars in prokaryote gene transcription is now an old tale. It may yet yield new stories of gene regulation in higher plants.

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