Plant Physiol. (1991) 95, 281–285
0032-0889/91/95/0281/05/$01.00/0

Received for publication June 19, 1990
Accepted October 4, 1990

Effect of Light/Dark Cycles on Expression of Nitrate Assimilatory Genes in Maize Shoots and Roots[1]

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ABSTRACT

The level of nitrate reductase (NR) and nitrite reductase (NiR) varied in both shoot and root tissue from nitrate-fed *Zea mays* L. grown under a 16-hour light/8-hour dark regime over a 10-day period postgermination, with peak activity occurring in days 5 to 6. To study the effect of different light regimes on NR and NiR enzyme activity and mRNA levels, 6-day-old plants were grown in the presence of continuous KNO_3 (10 millimolar). Both shoot NRA and mRNA varied considerably, peaking 4 to 8 hours into the light period. Upon transferring plants to continuous light, the amplitude of the peaks increased, and the peaks moved closer together. In continuous darkness, no NR mRNA or NR enzyme activity could be detected by 8 hours and 12 hours, respectively. In either a light/dark or continuous light regime, root NRA and mRNA did not vary substantially. However, when plants were placed in continuous darkness, both declined steadily in the roots, although some remained after 48 hours. Although there was no obvious cycling of NiR enzyme activity in shoot tissue, changes in mRNA mimicked those seen for NR mRNA. The expression of NR and NiR genes is affected by the light regime adopted, but light does not have a direct effect on the expression of these genes.

In higher plants, the principal source of nitrogen under normal field conditions is nitrate. Depending on the concentration of nitrate in the medium and the plant species, a portion of the nitrate is transported via the xylem sap to the leaves (1). In both roots and leaves, nitrate is either stored in the vacuoles or reduced to nitrite by NR[2] (EC 1.6.6.1). Nitrite is further reduced to ammonia by the enzyme NiR (EC 1.6.6.4). The ammonia can then be used to form various amino acids via the action of the glutamine synthetase-glutamate synthase pathway (19).

It has been known for some time that both nitrate and light are required for the synthesis of NR (4, 27) and NiR (12) proteins. When the system is induced by nitrate, the activity of NR in higher plants is regulated by enzyme synthesis and/or degradation (24, 27, 30) rather than by the activation-inactivation mechanisms reported for algae (23). Recently, with the cloning of the NR (5–7, 10) and NiR (2, 14) genes, it has been possible to demonstrate that this induction by nitrate occurs at the level of transcription.

The induction of NR by a phytochrome response was first demonstrated by Jones and Sheard (13) in pea seedlings and has since been shown to occur in a number of other plant species (9). The expression of NR and NiR in photosynthetic tissue is not affected by light in the absence of nitrate. However, upon the addition of nitrate to plants grown in the dark, treatment with red light further increased levels by 78% and 51% for NR and NiR, respectively (26). Such an increase is reversible by far-red light, which suggests that phytochrome is involved at some level in controlling the expression of these genes. However, the phytochrome response is seen only with etiolated plants, suggesting that light has an effect at some other level (18). For instance, light may have an effect on the level of reductant, uptake of nitrate, transfer of nitrate to the xylem, or the release of nitrate from vacuoles in the leaves (9, 25).

In this paper, we report the results from a study on the expression of NR and NiR genes in maize (*Zea mays* L.) seedlings grown in the presence of nitrate. The amount of enzyme activity and the mRNA levels were analyzed in both root and shoot tissue at 4-h intervals during a 48-h period to determine the variability in their expression levels during the course of the day. The expression of these genes is shown to be affected by the light regime utilized. However, it does not appear that light directly controls the expression of NR and NiR.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Maize kernels (*Zea mays* cv W64A × W182E), purchased from the Wisconsin Seed Foundation, Madison, were grown in the dark at 28°C for 2 d in large Petri plates containing 1% agar. Seedlings were then inserted into slits cut into foam and grown hydroponically by floating the foam on the surface of the media. The media consisted of 0.1 strength Hoagland's solution modified to contain a final concentration of 10 mM KNO_3. Circulation in the tanks was provided by a submersible pump. The plants were grown under a 16-h light/8-h dark regime for a further 4 d. At this point one of the three following light regimes was adopted: (a) the light/dark regime continued, (b) continuous light, or (c) continuous darkness. The growth chamber contained fluorescent and incandescent

[1] Research was supported by operating grants from National Sciences and Engineering Research Council of Canada; D. M. L. was supported by an NSERC postgraduate scholarship.

[2] Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; NRA, nitrate reductase activity.

light bulbs which emitted a fluence rate of 250 μmol m^{-2} s^{-1} at the level of the plants.

Plants were harvested by removal of 25 plants per sample from the tank at random. Shoots and roots were excised separately, frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle. Samples were stored at −70°C, for further analysis.

RNA Isolation and Hybridization

Total RNA was extracted from 3 g of shoot or root tissue by a procedure derived from Lahners *et al.* (14). For RNA blot hybridization, 30 μg of total leaf RNA and 20 μg of total root RNA were denatured with formaldehyde and subjected to electrophoresis through a 1.2% agarose, 2.2 M formaldehyde gel, and the RNA was blotted onto nitrocellulose (17). The probe used for the hybridization was either an NiR cDNA insert from the plasmid pCIB808 (14), an NR cDNA insert from the plasmid pCIB831, or a wheat rRNA cDNA clone. In all cases, DNA probes were radiolabelled with [α-^{32}P]dCTP (Amersham) using a nick translation kit (Amersham International) to a specific activity of 1 to 4 × 10^8 cpm/μg. The filters were prehybridized and hybridized with a radioactively labeled denatured DNA probe according to Maniatis *et al.* (17). Filters were washed, dried, and subjected to autoradiography on Kodak XOM films as previously described (14). The blot was reprobed with the wheat rRNA cDNA clone to confirm that equal amounts of RNA were loaded on each lane (data not shown). To allow reprobing of the filters with the different probes, radiolabel on the filters was allowed to decay, and the filters were rehybridized with nick translated probe.

Autoradiograms of different exposure times were scanned with a Bio-Rad 620 video densitometer. Relative amounts of mRNAs were determined by measurements of the peak areas.

Enzyme Assays

Frozen tissue samples were ground at a ratio of 1 g frozen tissue to 4 mL extraction buffer and assayed for NR activity as described by Long and Oaks (15). NiR was assayed in the same extract by the method of Losada and Paneque (16) with methyl viologen reduced by dithionite as the electron donor. All assays were carried out in duplicate.

RESULTS

Effect of Plant age on NR and NiR Activities

There is some evidence that seedling age affects the expression of NR (15, 29). In order to determine the effect of seedling age on NR and NiR enzyme activity levels, seedlings were germinated on plates and then transferred to a hydroponic system with a 16-h light/8-h dark cycle. Twenty-four hours before harvest the growth medium was adjusted to a final concentration of 10 mM KNO_3. During each of the next 5 d, plant tissue was harvested 7 h into the light period. Roots and shoots were assayed separately for NR and NiR activities. Both of these activities were maximal at either d 5 or 6 in both tissue types (Fig. 1). Based on these results, the sampling period for subsequent experiments began on d 6. Any alterations in the light regime were also done at this time.

Effect of Altered Light Regimes on NR Activity and mRNA in Shoots

NR activity and mRNA levels were measured in hydroponically grown maize shoot tissue over the course of the 16-h light/8-h dark cycle. In seedlings grown under these light conditions, NR activity was found to vary considerably during the time-course (Fig. 2a). Maximal activity was attained 4 to 8 h into the light period and then declined gradually. As expected, given the fact that seedling age affects the level of activity (Fig. 1), the peak activity was lower on the second day in comparison with the first.

Total RNA was probed for the level of NR mRNA by RNA blot hybridization. Similar results to those found for enzyme activity were obtained (Fig. 2a). Two aspects of these results should be noted. The first is that the fluctuations in both the enzyme activity and the mRNA levels were not necessarily directly correlated with the onset of the light and dark periods. Both the NR activity and the mRNA level started to decrease approximately half-way through the light cycle. On the other hand, they began to increase again with the onset of the light cycle. The second point is that the fluctuation of NR activity with seedling age was correlated with the level of NR mRNA. Therefore, it appears that these differences in NR activity are primarily due to differences in the level of NR mRNA present.

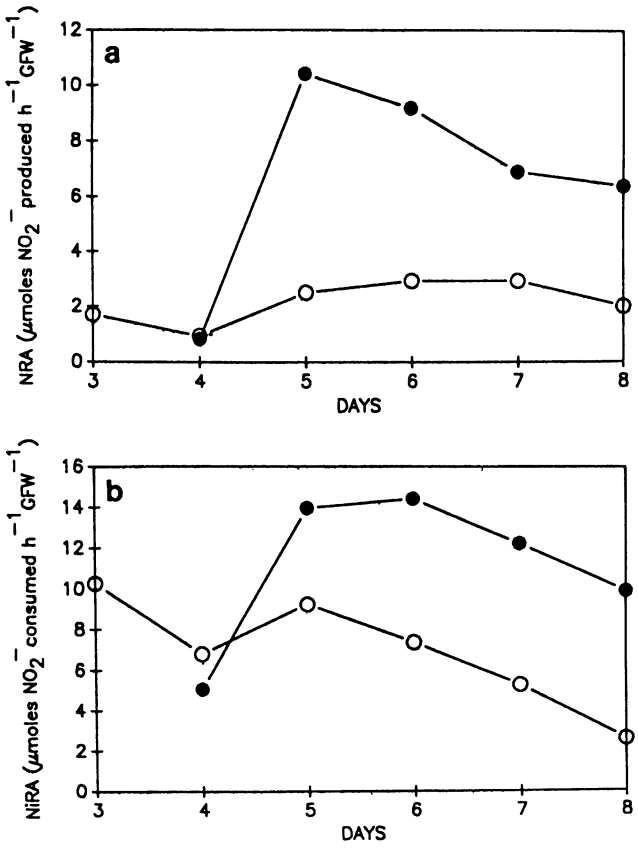

Figure 1. Ontogeny of nitrate reductase activity (a) and nitrite reductase activity (b) in maize shoots (●) and roots (○). Plants were grown on 0.1 strength Hoagland nutrient solution and induced with 10 mM KNO_3 24 h before harvesting as described in "Results" (representative data from two experiments).

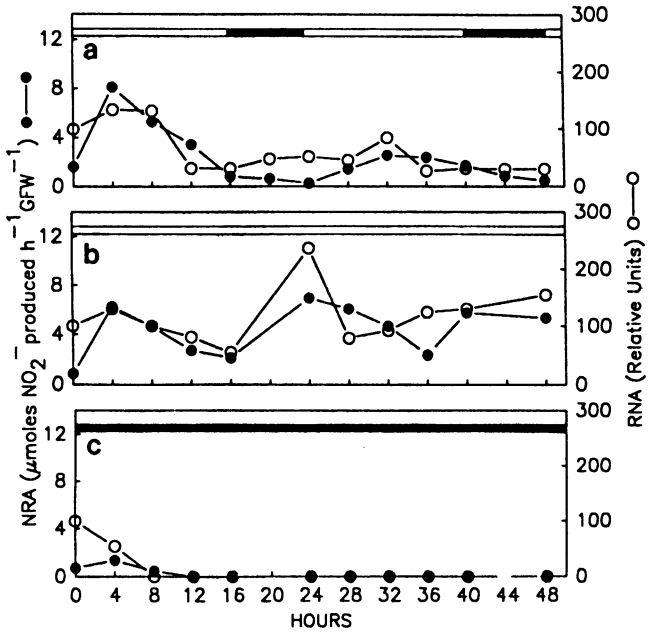

Figure 2. Variations in the amount of NR activity (●) and mRNA levels (○) in the shoots of 6-d-old maize plants either (a) maintained in a 16 h light/8 h dark regime, (b) transferred at time 0 h to continuous light, or (c) transferred at time 0 h to continuous darkness. The light conditions are indicated in each figure by an open bar (light period) and closed bar (dark period). Experiments were repeated twice and representative data are shown.

To help delineate the role of light in controlling expression of these genes, maize seedlings were grown under a 16-h light/8-h dark regime and then either grown in continuous light or continuous darkness. Plants placed into continuous light on d 6 had the same initial pattern for NRA on the first day of the experiment as was seen for plants grown under the 16-h light/8-h dark cycle. This was expected, since the light conditions during this time period are the same for the two experiments. However, the length of time in which NR activity was low in these plants was shortened considerably in the absence of a dark period (Fig. 2b). Although plants grown under 16-h light/8-h dark conditions had far less NR activity in the second 24-h period (Fig. 2a), the plants grown in continuous light had an NR activity peak on the second day equal to that found for the first. Furthermore, the maximum level of mRNA present on the second day was increased fourfold under these conditions, when compared with plants grown on a 16-h light/8-h dark cycle (Fig. 2, a and b).

To further test the effect of different light regimes, maize seedlings were grown under a normal 16-h light/8-h dark cycle until d 6. At this time, the plants were maintained in continuous darkness, with tissue samples removed and assayed during the following 48 h. There was a marked effect of continuous darkness on the expression of NR (Fig. 2c). The initial activity was the same as in the previous experiments, as expected given that the initial time-point is equivalent in each case. Afterward, NR activity did not increase nearly as rapidly, with the activity being fourfold less after 4 h than was found for plants grown in the light. By 12 h no NR activity was detectable. The level of NR mRNA in these plants was also found to decline, with none detectable at the 8-h time point. Therefore, the decrease in NR activity is concurrent with the decrease in the level of NR mRNA.

Effect of Different Light Regimes on Expression of Root NR

In the root tissue of maize, both NADH and NAD(P)H:NR activities are expressed (15). Because their pattern of expression was similar (except that the NADH activity values were approximately twofold greater than the NAD(P)H:NRA values), only NADH activity was plotted (Fig. 3). Unlike the shoot tissue, root tissue from seedlings grown either under a light/dark or a continuous light treatment showed little if any variation in the NR activity. The level of NR mRNA varied somewhat through the course of the first 24 h after day 6. It decreased slightly during the initial part of the light period and then reached a peak after the lights were on for 12 h. These variations in mRNA did not appear to have a noticeable effect on the level of NR activity. When the plants were grown in continuous dark, NR activity decreased slowly. However, unlike the results found for shoot tissue, the activity decreased more slowly and was still present at the end of the experimental period.

Effect of Light Regimes on NiR Activity and mRNA in Shoots

There was no obvious cycling of the shoot NiR enzyme activity under any of the experimental conditions used. For

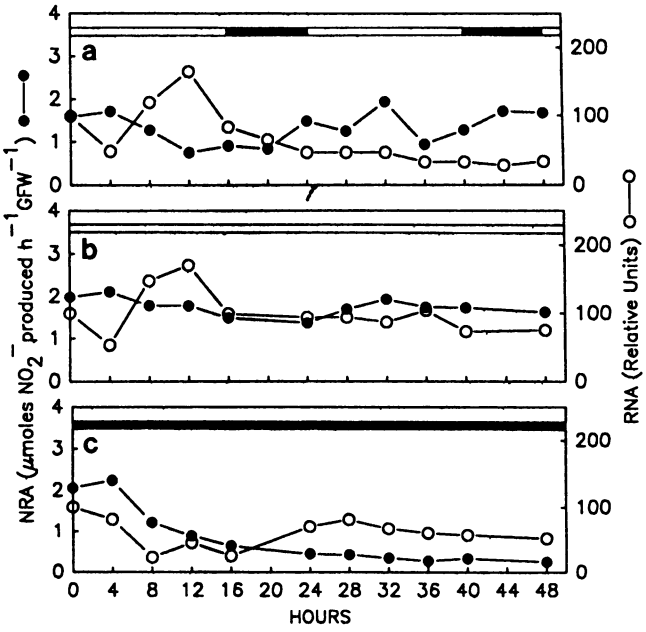

Figure 3. Variations in the amount of NR activity (●) and mRNA levels (○) in the roots of 6-d-old maize plants either (a) maintained in a 16 h light/8 h dark regime, (b) transferred at time 0 h to continuous light, or (c) transferred at time 0 h to continuous darkness. The light conditions are indicated as for Figure 2. Experiments were repeated twice and representative data are shown.

example, when plants were grown under a 16-h light/8-h dark regime, the activity slowly decreased during the experimental period, with only minor fluctuations detected (Fig. 4a). However, the level of NiR mRNA did show considerable diurnal cycling. The pattern of mRNA expression was almost exactly the same as that found for the shoot NR mRNA (Fig. 4a), although the level of NiR mRNA started to increase during the dark period to a considerably greater extent than did the NR mRNA. The NiR mRNA in root tissue showed a similar pattern of expression to that found in the shoots (data not shown).

When plants were grown in continuous darkness, the level of NiR activity actually did not decrease any more rapidly than in the light (Fig. 4b). In contrast, the level of NiR mRNA was eventually affected, decreasing to an undetectable level at 36 h (Fig. 4b). This decrease was considerably slower than that found for the NR mRNA. There was a slight increase in the level of the mRNA at the same time as was seen in the plants grown with a 16-h light/8-h dark cycle. However, it was of too small an amplitude to conclude that the NiR mRNA levels follow a diurnal rhythm even in the absence of light.

DISCUSSION

A variety of environmental and developmental stimuli regulate nitrate assimilatory genes. The primary signal is the presence of nitrate, which stimulates the transcription of both NR (5–7, 10) and NiR (2, 14) genes and the subsequent production of the proteins. In the presence of nitrate, the expression of these genes is also modulated by light conditions, time of day, nutrient conditions, and seedling age (4, 28). However, the mechanisms by which these factors modulate expression and their physiological role in determining the appropriate level of enzyme activity remain unknown.

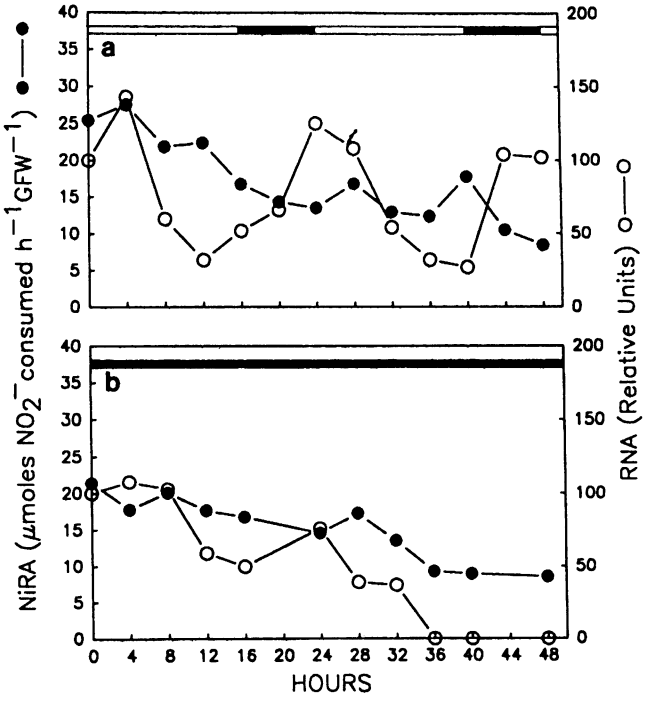

Figure 4. Variations in the amount of NiR activity (●) and mRNA levels (○) in the shoots of 6-d-old maize plants either (a) maintained in a 16 h light/8 h dark regime or (b) transferred at time 0 h to continuous darkness. The light conditions are indicated as previously described in Figure 2. Experiments were repeated twice and representative data are shown.

There are obviously great differences in the expression of these genes in maize depending on seedling age. Between d 4 and 5, the levels of NR and NiR differ substantially. The seedling is going through considerable physiological change, because it is beginning to rely less on catabolized endosperm protein and more on exogenous sources of nitrogen (20). Srivastava *et al.* (29) observed that, in maize, NR activity is low in young leaves when there is a maximum import of nutrients, reaches a maximum just as they approach maximum size, and then declines in older leaves. As the leaves mature and export rather than import nutrients, the control of NR production is altered (29). Although the present work does not study this phenomenon in individual leaves in detail, the age variability in the overall shoot activity levels is apparently due to differences in the level of mRNA. For example, the NR mRNA level on d 7 is considerably lower than on d 6 (Fig. 2a) which corresponds to the decrease in enzyme activity. However, the mechanism of suppression of the expression of these genes early in seedling development and after the peak activity levels are reached is unknown at this time.

The level of maize shoot NRA varies considerably over the course of a day. This oscillation has been reported for a variety of species (28 and references therein). The level of maize NR mRNA in the shoot was found to increase and decline concurrent with activity. However, there are some disparities between the two. For example, the amount of NR mRNA remains fairly constant at around one-third maximal level during the dark period, while NR activity remains very low until the light period commences (Fig. 1a). Furthermore, in constant light there appears to be a superinduction of the NR mRNA with peaks at twice the level of that found for the first day, while the activity maximum for the 2 d is the same. Therefore, while the amount of NR activity is in general correlated with the amount of mRNA, there may be other control mechanisms.

The shoot NR mRNA and NRA decreases rapidly in continuous darkness. This is in agreement with previous work on NR (24) and implies that light is required for NR gene transcription or mRNA stability. Deng *et al.* (8) observed substantial decreases in NR protein and NRA in tobacco leaves under similar conditions, although the protein and NRA did not vary in parallel. However, in contrast to our results, levels of tobacco NR mRNA continued to display rhythmic oscillations in the dark and did not completely disappear in leaves until subjected to darkness for 56 h. There is very little variability of NR activity and mRNA levels during a light/dark or continuous light regime in root tissue. This is possibly due to the fact that nonphotosynthetic tissue may obtain reducing power for these enzymes from either photosynthate translocated to the roots or from reserves maintained in the root tissue itself. Furthermore, between 6 and 8 d of age, ample seed reserves of carbohydrates are probably still

available to sustain root growth and supply reductant. The slow decline in root NR activity and mRNA caused by continuous darkness might reflect the depletion of these sources of reducing power.

NiR is a more stable protein than NR (3), which may explain the lack of oscillations seen in the activity of this enzyme during any of the treatments. Interestingly, the rhythmic oscillation of the shoot NiR mRNA continued even in continuous darkness until it finally disappeared after 36 h. Therefore, there was little correlation between the NiR level of mRNA present and enzyme activity. Posttranscriptional events must be important for maintaining a sufficient level of this enzyme. In root tissue, NiR mRNA levels varied considerably more than NR mRNA, showing a similar oscillation to that seen in shoot tissue. NR and NiR are found in the cytosol and plastids, respectively (4). Posttranslational processing of NiR may account for the differences observed.

A variety of plant activities are controlled by the cyclic alternation of day and night. Expression of a series of nuclear genes coding for chloroplast proteins has been shown to be stimulated by light and to undergo diurnal oscillations (11, 21, 22). For some of the genes, this diurnal rhythm is maintained in the absence of light. In studies of both tobacco and tomato (10), it was found that NR also maintained this circadian rhythm in the dark. In maize, although NR mRNA levels clearly are not maintained in the dark, NiR mRNA levels are more stable and there is some maintenance of the circadian rhythm in the dark.

Although it is clear that the light regime can have a considerable effect on the expression of NR and NiR, the biochemical mechanism of regulation is unknown. However, given that the diurnal fluctuations do not necessarily correspond to the light/dark cycle, it seems unlikely that light itself directly regulates mRNA levels during the diurnal cycle. Either mRNA synthesis or translation might also be influenced by any one of a variety of factors, including the level of reductant present or the amount of reduced carbon available.

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