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Regulation of Maize Leaf Nitrate Reductase Activity Involves Both Gene Expression and Protein Phosphorylation[1]

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Nitrate reductase (NR; EC 1.6.6.1) activity increased at the beginning of the photoperiod in mature green maize (*Zea mays* L.) leaves as a result of increased enzyme protein level and protein dephosphorylation. In vitro experiments suggested that phosphorylation of maize leaf NR affected sensitivity to Mg^{2+} inhibition, as shown previously in spinach. When excised leaves were fed ^{32}P-labeled inorganic phosphate, NR was phosphorylated on seryl residues in both the light and dark. Tryptic peptide mapping of NR labeled in vivo indicated three major ^{32}P-phosphopeptide fragments, and labeling of all three was reduced when leaves were illuminated. Maize leaf NR mRNA levels that were low at the end of the dark period peaked within 2 h in the light and decreased thereafter, and NR activity generally remained high. It appears that light signals, rather than an endogenous rhythm, account primarily for diurnal variations in NR mRNA levels. Overall, regulation of NR activity in mature maize leaves in response to light signals appears to involve control of gene expression, enzyme protein synthesis, and reversible protein phosphorylation.

Nitrate assimilation in plants is an energetically expensive process that is generally coordinated with leaf photosynthetic activity. Thus, light stimulates the rate of nitrate reduction and, ultimately, the formation of amino acids. NR (EC 1.6.6.1), which catalyzes the first step in the reduction of nitrate to ammonia, is highly regulated and may constitute a rate-limiting step in this pathway (Beevers and Hageman, 1980). Fluctuations in NR mRNA pools constitute an important level of control for NR activity (Pilgrim et al., 1993), and rapid de novo synthesis and degradation of the NR protein provides coarse control over the level of enzyme available (Somers et al., 1983; Remmler and Campbell, 1986; Shiraishi et al., 1992). The complex regulation of NR gene expression can involve induction of transcription and translation by light and nitrate (Melzer et al., 1989; Crawford and Campbell, 1990; Solomonson and Barber, 1990; Gowri et al., 1992) and/or ammonium- and Gln-induced repression of NR mRNA (Srivastava, 1980; Martino and Smarrelli, 1989; Deng et al., 1990, 1991). The level of NR activity and rates of nitrate assimilation in leaves of some plant species can be stimulated by nitrate feeding in darkness, but the magnitude of the response is generally very low compared with nitrogen-sufficient leaves in the light. In some plant species, NR expression is under phytochrome control, although this effect appears to be limited to etiolated shoots (Rajasekhar et al., 1988; Deng et al., 1991).

In species such as tobacco and tomato, the pool of NR transcript appears to fluctuate throughout a 24-h dark/light cycle in response to regulation by an endogenous clock. NR mRNA declines during the light period of a typical day/night cycle, even though light is involved in the expression of NR mRNA as well as NR protein and activity. The highest levels of NR mRNA are found at the end of the night period, whereas NR protein levels reach a peak 2 to 4 h thereafter (Galangau et al., 1988). NR activity is required for the rhythmic fluctuations in tobacco NR mRNA to occur, as indicated by the continuous high levels of NR mRNA in tobacco leaves expressing a nonfunctional NR gene. These observations suggest that a product of NR activity may exert negative control over NR expression (Deng et al., 1991).

In maize, Bowsher et al. (1991) observed that changes in NR mRNA and activity levels in 6-d-old seedlings generally correlated during the course of a day (peaking 4–8 h into the light period) and in response to continuous light or dark conditions. NR gene expression in young maize plants was suggested to be controlled by a circadian rhythm. In contrast, Lillo (1991) reported that a 4-fold increase in NR mRNA level in 5-d-old maize seedlings occurred within 1 h after the light was turned on; thereafter, the transcript level decreased rapidly to very low levels. However, it was not clear from the experimental approach whether the initial peak in NR mRNA levels was in response to the light signal or an endogenous rhythm, as has been observed for tobacco. Although an endogenous rhythm may be important for regu-

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Abbreviations: NR, NADH:nitrate reductase; TLE, thin-layer electrophoresis.

lation of maize leaf NR, it is difficult to rule out direct regulation by light or one or more products of photosynthesis.

In mature spinach leaves, NR activity varies significantly during a day/night cycle (Huber et al., 1992c), with highest activity occurring in the morning and declining as the photoperiod progresses. The changes in spinach NR activity are largely the result of covalent modification (protein phosphorylation/dephosphorylation) of the enzyme superimposed on relatively small changes in NR protein (de novo synthesis and degradation) (Huber et al., 1992a. In vivo phosphorylation of NR in the dark was correlated with greater sensitivity of the enzyme to inhibition by millimolar levels of Mg^{2+}, and dephosphorylation of NR protein in the light was correlated with decreased inhibition by Mg^{2+} (Kaiser and Brendle-Behnisch, 1991; Kaiser and Spill, 1991; Huber et al., 1992b; Riens and Heldt, 1992; Kaiser et al., 1992). Such in vivo evidence for regulation of NR by protein phosphorylation has only been obtained for spinach, and it is not known whether similar regulation might occur in leaves of other species such as maize.

In the present study, we examined NR activity in mature maize leaves for evidence of enzyme regulation via light signals and covalent modification. We show that (a) the sensitivity of maize NR activity to inhibition by Mg^{2+} is subject to a rapid, reversible light modulation that correlates with phosphorylation of specific seryl residues of the enzyme; (b) NR mRNA, enzyme protein, and activity (assayed $-Mg^{2+}$) levels change primarily in response to light signals and not as a result of an endogenous diurnal rhythm; and (c) Man treatment of darkened excised leaves activates NR (presumably via dephosphorylation) and stimulates NR enzyme protein synthesis but without substantially affecting NR mRNA level. The Man effect suggests that alteration in cellular metabolites can affect NR mRNA translation and/or NR protein stability, and thus, the effect of light may be mediated indirectly by shifts in metabolites.

MATERIALS AND METHODS

Materials

Biochemicals for NR extraction and activity assay were obtained from Sigma.[2] Immunoprecipitin and most of the reagents for RNA isolation were obtained from Gibco-BRL. ^{32}P radionucleotide ([^{32}P]Pi and [α-^{32}P]dCTP) was purchased from New England Nuclear. Trypsin (*N*-tosyl-L-Phe chloromethyl ketone treated) was obtained from either Sigma or Promega (sequencing grade modified enzyme).

Plant Material

Maize (*Zea mays* L. cv Pioneer 3184) was grown in soil in a greenhouse and supplied with standard Hoagland solution containing nitrate (10 mM) as the nitrogen source every 2 d. Generally, the second and third leaves (numbered acropetally) of plants grown for 3 to 4 weeks were used. On the day prior to an experiment, plants were fertilized twice and transferred to a growth chamber at 25°C. As described in the text, leaf tissue was harvested from plants in the dark or light (400 μmol m^{-2} s^{-1} PPFD) and frozen immediately in liquid nitrogen. In other experiments, leaves were excised from plants 30 min into the light period, and the petioles were recut under degassed water before being placed in tubes containing degassed water. The leaves were placed in a darkened chamber for 1 h at 25°C and then allowed to take up any additions by transpiration for 90 min before harvesting into liquid nitrogen. Samples were stored at −80°C until extraction.

Extraction and Enzyme Assays

Frozen leaf tissue was ground rapidly in a chilled mortar with extraction buffer (1 g/2 mL) containing 50 mM Mops-NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 0.1% (w/v) octylphenoxypolyethoxyethanol (Triton X-100). The homogenates were centrifuged at 20,000*g* for 0.5 min in a 1.5-mL microfuge tube. The supernatant fluids were immediately desalted by centrifugal filtration on Sephadex G-25 columns (1 × 5 cm) equilibrated with extraction buffer minus Triton X-100 and with the concentration of DTT decreased to 2.5 mM. NR activity was assayed as the NADH-dependent formation of nitrite from nitrate. The standard 1-mL reaction mixture for the "plus Mg^{2+} assay" contained 50 mM Mops-NaOH (pH 7.5), 5 mM $MgCl_2$, 10 mM KNO_3, and the standard 1-mL reaction mixture for the "$-Mg^{2+}$ assay" contained 50 mM Mops-NaOH (pH 7.5), 1 mM EDTA, and 10 mM KNO_3. The reductant was 0.1 mM NADH. Reactions were initiated by addition of desalted extract (25–100 μL) and terminated after 5 min at 25°C by addition of zinc acetate. Other details of the product detection were as previously described (Huber et al., 1992a).

Quantitation of NR Protein

The approximately 100-kD subunit of NR was immunopurified from the crude extracts using monoclonal antibodies raised against the maize enzyme [II6(69) IgG; Hyde et al., 1989] with Immunoprecipitin as the precipitating agent. In all experiments the efficiency of immunoprecipitation was monitored as removal of NR activity from solution and was found to be greater than 90%. The NR subunit was further purified by SDS-PAGE fractionation of the dissociated immune complexes as described previously for Suc-P synthase (Huber and Huber, 1992). NR protein was quantitated on Coomassie blue-stained gels by scanning band densities on a LKB Ultroscan XL enhanced laser densitomer. This technique allowed us to make relative comparisons within a given experiment of the amount of NR enzyme protein present in leaf extracts.

RNA Isolation and Northern Blot Analysis of NR mRNA

Total RNA was extracted from approximately 1 g of frozen tissue according to a procedure derived from Chirgwin et al. (1979). The purified RNA was dissolved in diethyl pyrocar-

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the North Carolina Agricultural Research Service or the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

bonate-treated water, and the concentration and purity were determined spectrophotometrically. Total RNA was fractionated (10 μg/sample) in formaldehyde-agarose denaturing gels by electrophoresis and transferred to nitrocellulose. Maize NR mRNA levels were probed by hybridization with a ^{32}P-labeled cDNA clone of the maize NR mRNA (Gowri and Campbell, 1989) as previously described (Redinbaugh et al., 1988; Gowri et al., 1992). Briefly, a nonspecifically bound probe was eliminated by washing the membrane in 0.1× SSC (0.15 mM Na-citrate, pH 7.0, 15 mM NaCl, 0.1% [w/v] SDS) at 65°C for at least 1 h. The Zmnr1 cDNA hybridized with an mRNA of about 3.2 kb as previously seen (Gowri and Campbell, 1989). For quantitative analysis, the autoradiographs were scanned with an LKB Ultroscan XL laser densitometer. Similar yields of total RNA were obtained from all samples, and the gels were evenly loaded as determined by visual inspection of gels stained with ethidium bromide and by probing with rDNA (pHA2; Jorgensen et al., 1987). In all cases, lane-to-lane variation in RNA loading was quite low, and normalization of the NR mRNA signal relative to the rRNA signal (data not shown) gave the same result as simply looking at the NR mRNA signal.

Phosphorylation of NR in Vivo

The second and third leaves were excised after 1 h of illumination. The leaf bases were recut under degassed water and placed in 1.5-mL microfuge tubes containing 0.5 mL of a solution made up of 10 mM KNO_3, 10 μM K-phosphate (pH 6.5), and 0.5 to 1.0 mCi [^{32}P]Pi (approximately 3000 Ci/mmol). After the isotope was taken up, the solution was replaced with degassed 10 mM KNO_3. Each microfuge tube contained two leaves (total fresh weight approximately 1.0 g), and tissue from three tubes was pooled for a sample. Excised leaves were allowed to take up the [^{32}P]Pi solution in the light (400 μmol m^{-2} s^{-1} PPFD, 25°C) for 2.5 h, at which time the "light" sample was harvested by freezing the leaves in liquid nitrogen. The lights in the growth chamber were then turned off, and the remaining leaves were harvested as the "dark" sample after exposure to 20 min of darkness. Leaves that served as the light or dark controls (for assay of NR activity) were treated in the same way except that ^{32}P was omitted.

Frozen leaves were ground in a mortar using 5 volumes of extraction buffer. The homogenates were centrifuged at 15,000*g* for 5 min, and NR was quantitatively immunoprecipitated from the crude extracts using a monoclonal antibody (Zm2,69) raised against maize NR (Hyde et al., 1989) and Immunoprecipitin as the precipitating agent. The 100-kD NR subunit was further purified by SDS-PAGE fractionation of the dissociated immune complexes, exactly as described previously for similar studies with spinach NR (Huber et al., 1992a) and Suc-P synthase (Huber and Huber, 1992).

Phosphoamino Acid Analysis

The approximately 100-kD NR subunit was transferred electrophoretically to Immobilon-P. Partial acid hydrolysis was performed on the membrane in 5.7 N HCl for 1 h at 110°C (Kamps and Sefton, 1989). The released phosphoamino acids were resolved by two-dimensional TLE and autoradiography (Manai and Cozzone, 1982). Phosphoamino acid analysis was done in two experiments, and representative results from one experiment are presented.

Phosphopeptide Mapping

The NR protein, ^{32}P labeled in vivo, was immunopurified, electrophoresed, and transferred to Immobilon-P as described above. The membrane strip containing the NR subunit was excised, and the protein was digested with *N*-tosyl-L-Phe chloromethyl ketone-treated trypsin (1:10 [w/w] with NR protein). After digestion overnight at 30°C, the solution was taken to dryness under vacuum, and the peptide were resolved by two-dimensional TLE/TLC (King et al., 1983). After identification of the ^{32}P-phosphopeptides by autoradiography, the spots were cut from the plates, and radioactivity was determined by liquid scintillation counting. ^{32}P labeling of maize leaf NR following [^{32}P]Pi feeding to excised leaves was observed in four independent experiments. Phosphopeptide-mapping analysis was done in three experiments, and representative results obtained from one experiment are presented.

RESULTS AND DISCUSSION

Kinetics of Light/Dark NR Activity Changes

An initial experiment was performed to characterize the in vivo activation/inactivation kinetics of maize leaf NR activity during light/dark transitions. NR activity, assayed either in the presence or absence of Mg^{2+}, increased rapidly in leaves harvested early in the light period (Fig. 1A). When the plants were subsequently darkened after 1 h of light, the NR activities rapidly decreased to the original dark levels (Fig. 1A).

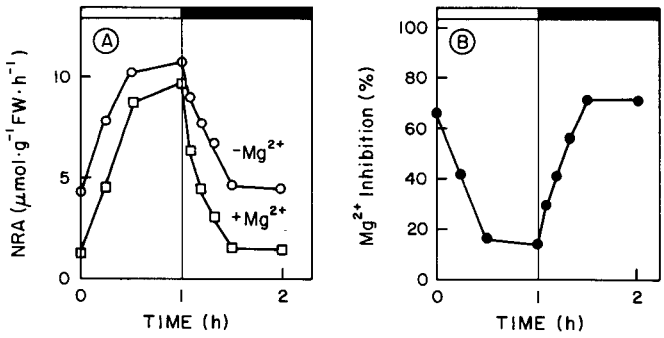

Figure 1. Kinetics of light activation and dark deactivation of NR in attached maize leaves. NR activity (NRA) was assayed in the presence of 5 mM Mg^{2+} and in the absence of Mg^{2+} (with 1 mM EDTA) as indicated (A), and the percentage of inhibition by Mg^{2+} was calculated (B). Plants were grown for 2 weeks in the greenhouse and were transferred to a darkened growth chamber (25°C) 12 h before the start of the experiment. The plants were then exposed to 1 h of light (400 μmol m^{-2} s^{-1} PPFD) at 28°C, followed by 1 h of darkness. As indicated, leaves 2 and 3 (numbered from the base; both fully elongated) were harvested by freezing in liquid nitrogen. NR activities were essentially the same in the two leaf positions, and mean values are plotted. FW, Fresh weight.

Although maize leaf NR activity ($-Mg^{2+}$) fluctuated substantially during a light/dark transition, maize NR clearly showed altered sensitivity to inhibition by Mg^{2+} (Fig. 1B). In the light, NR was largely insensitive to Mg^{2+} inhibition within 30 min but was converted back to an Mg^{2+}-sensitive form in darkness. These results are consistent with the original observations of spinach leaves that NR activity is subject to rapid, reversible modulation by an activation/inactivation mechanism affecting sensitivity to Mg^{2+} inhibition.

A major difference, however, between maize and spinach concerns changes that occur in NR activity, assayed without Mg^{2+}, during dark/light transitions. With spinach, ($-Mg^{2+}$) NR activity (which is thought to reflect primarily the level of NR protein) often remained relatively constant, at least after the initial dark-to-light transition and typically decreased less than 30% after darkening of plants (Huber et al., 1992a; Huber et al., 1992b). In contrast, with maize, ($-Mg^{2+}$) NR activity always changed substantially with dark/light transitions even after the initial cycle. In the experiment presented in Figure 1A, the ($-Mg^{2+}$) NR activity decreased about 60% in the dark. Similar decreases in maize leaf ($-Mg^{2+}$) NR activity have been observed earlier and could not be entirely accounted for by changes in NR enzyme protein (Remmler and Campbell, 1986; Campbell, 1987). Thus, the observed changes in ($-Mg^{2+}$) NR activity in maize leaves could reflect a larger contribution of changes in NR protein relative to spinach and/or maize leaf NR activity could be inhibited independently of divalent cations.

Interestingly, the light-induced activation of NR observed with intact plants (Fig. 1) was evident in excised leaves, provided that the leaves were excised from plants that had been exposed to light. Under these conditions, the kinetics of NR modulation in excised leaves with light/dark transitions were essentially the same as those of the intact leaves, with the exception that the steady-state NR activity achieved in the light was slightly higher in excised leaves compared to attached leaves (not shown). The ability to use excised leaves allowed us to test the effect of various compounds (provided via the transpiration stream) on the light modulation of maize leaf NR.

Mg·ATP-Dependent Inactivation of Maize NR

The Mg^{2+}-insensitive NR activity in desalted extracts of maize leaf tissue harvested in the light was converted to the Mg^{2+}-sensitive form as a result of preincubation with Mg·ATP in vitro (Fig. 2A). The time- and ATP-dependent inactivation of NR was observed only when the enzyme activity was subsequently assayed in the presence of Mg^{2+}. The slow decrease in NR activity measured in the absence of Mg^{2+} was independent of ATP because a similar decrease was observed in extracts preincubated without ATP (data not shown). In the absence of ATP, sensitivity of NR to Mg^{2+} inhibition remained low and constant (Fig. 2B). These results suggest that maize NR, like the spinach enzyme, is subject to regulation by an ATP-dependent inactivation reaction most likely involving protein phosphorylation. This was also suggested by the recent demonstration that the rapid, light-induced increase in Mg^{2+}-insensitive NR activity in maize leaves could be prevented by feeding okadaic acid or microcystin-LR to

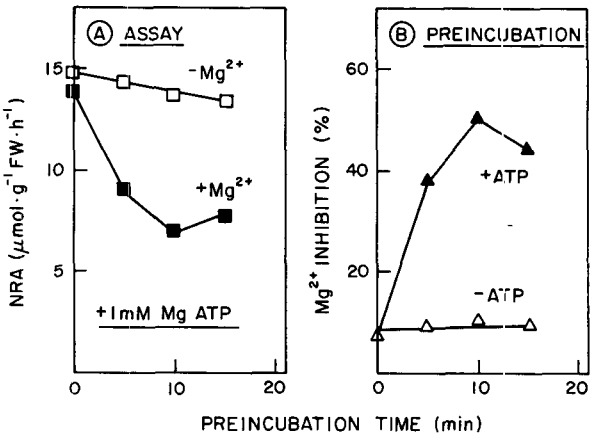

Figure 2. Time- and Mg·ATP-dependent inactivation of maize leaf NRA. Leaves were harvested as described above, about 3 h into the light period, from plants in the greenhouse and plunged into liquid nitrogen. A, Desalted extracts (see "Materials and Methods") were preincubated at 25°C in the presence of 1 mM Mg·ATP and subsequently assayed in either the presence (■) or absence (□) of 5 mM Mg^{2+}. B, Desalted extracts were preincubated in either the presence (▲) or absence (△) of 1 mM Mg·ATP, prior to assay in the presence and absence of 5 mM Mg^{2+}; the percentage of inhibition of NR activity by 5 mM Mg^{2+} is plotted. FW, Fresh weight.

excised leaves prior to their transfer to the light (data not shown). These two toxins are potent inhibitors of type 1 and 2A protein phosphatases (Bialojan and Takai, 1988). Sensitivity of the NR activation mechanism to these inhibitors in vivo and the in vitro inactivation of NR by Mg·ATP support a role for protein phosphorylation in the light/dark control of maize NR activity and suggest that maize NR is activated in the light as a result of dephosphorylation. Collectively, these results prompted us to examine the phosphorylation of the maize leaf enzyme in vivo.

Phosphorylation of Maize Leaf NR in Vivo

Excised maize leaves were fed [^{32}P]Pi via the transpiration stream in the light for 2.5 h and subsequently darkened for an additional 20 min. In a preliminary experiment, it was determined that the approximately 100-kD NR subunit was labeled with ^{32}P when extracted from leaves harvested in the light or after a light-to-dark transition (data not shown). When samples were subjected to phosphoamino acid analysis, label was exclusively associated with P-Ser in both the light and dark samples (data not shown). Similar results were reported for in vivo labeling of NR in spinach (Huber et al., 1992a) and *Arabidopsis* (LaBrie and Crawford, 1994) leaves.

To determine whether there was differential phosphorylation of specific sites in the light versus dark, tryptic digests were analyzed by two-dimensional TLE/TLC. Shown in Figure 3 are the phosphopeptide maps for maize leaf NR labeled in vivo in the light (Fig. 3B) and dark (Fig. 3A). In both the light and dark samples, four phosphopeptide fragments were evident in the autoradiograms (designated Pp1 to Pp4). One of the apparent sites (Pp2) was minor and accounted for less than 5% of the ^{32}P incorporated. In the dark NR, phospho-

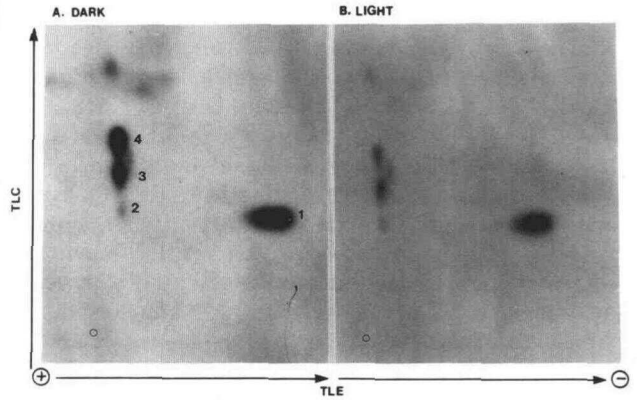

Figure 3. Tryptic peptide maps of maize leaf NR phosphorylated in vivo in the dark (A) and light (B). The origins are marked with a small open circle in the lower left corner of each panel. The four major phosphopeptides (Pp) resolved are designated Pp1 to Pp4 as indicated. Each sample corresponds to the NR extracted and immunoprecipitated from 3 g of maize leaf tissue. FW, Fresh weight.

peptides 1 and 4 were most heavily labeled (together accounting for 80% of the total ^{32}P incorporated). In the light NR, total labeling was reduced about 60% relative to the dark NR. In particular, labeling of Pp4 and Pp3 was reduced about 93 and 67%, respectively, relative to the corresponding fragments in the dark sample, whereas labeling of Pp1 was reduced in the light by only about 50%. NR activity in the light leaves was less sensitive to Mg^{2+} inhibition than was NR in the dark leaf extracts (15 versus 55% inhibition, respectively). Thus, one or more of the three major ^{32}P-phosphopeptides (Pp1, Pp3, and Pp4) could be involved in modulation of sensitivity to Mg^{2+} inhibition. The results obtained with maize NR are very similar to those reported earlier with spinach leaf NR (Huber et al., 1992a). In both cases, four distinct ^{32}P-phosphopeptides can be resolved from the Mg^{2+}-sensitive phosphoenzyme form, and the peptide maps are generally similar, with the exception that one of the maize phosphopeptides (Pp1) has greater mobility during electrophoresis than the spinach homolog; this may indicate a slightly higher charge/mass ratio for the maize phosphopeptide.

Light and Man Modulation of NR Protein Level

The changes in ($-Mg^{2+}$) NR activity during a dark-to-light-to-dark transition (Fig. 1A) were reflected somewhat in the steady-state level of NR protein present in the leaf extracts (Fig. 4). Among several experiments, a positive correlation between ($-Mg^{2+}$) NR activity and enzyme protein levels was evident ($r = 0.81$). The changes in NR protein levels in response to light/dark signals suggest that NR gene expression and/or NR turnover are regulated by light signals, consistent with previous results from other laboratories (Remmler and Campbell, 1986; Galangau et al., 1988; Melzer et al., 1989; Vincentz and Caboche, 1991).

Feeding Man, a phosphate-sequestering agent (Herold and Lewis, 1977), to maize leaves through the transpiration stream in the dark induced increases in ($-Mg^{2+}$) NR activity and NR protein levels similar to those measured in response to a dark-to-light transition (Fig. 4). The basis for these Man-induced increases in NR activity and protein in the dark is not clear but could involve effects on NR protein stability. However, it is clear that its influence is not a simple "hexose" effect, since Glc was less effective and 3-*O*-methyl-Glc at 50 mM had no effect on the subsequent activity of NR (data not shown). Man feeding clearly does not completely mimic the light response since the apparent stimulation of NR protein synthesis did not involve elevated NR transcript levels (Fig. 5).

Effect of Extended Darkness on NR Activity and NR mRNA Levels

NR activity ($-Mg^{2+}$ assay) in maize increased rapidly within the 1st h of the light period and was sustained at a high level throughout the day (Fig. 6), consistent with similar diurnal studies of spinach NR activity (Huber et al., 1992c). In contrast, the sensitivity of maize NR activity to Mg^{2+} inhibition remained low throughout the day (Fig. 6), whereas previous results indicated that spinach NR was partially converted to the Mg^{2+}-sensitive form as the light period progressed (Huber et al., 1992c).

Maize leaves illuminated after about 8 h of extended darkness retained the capacity to effect an increase in ($-Mg^{2+}$) NR activity concurrently with reduced sensitivity to Mg^{2+} inhibition. Transfer of the plants to light at the end of the extended darkness resulted in increases in activity of similar magnitude but somewhat slower kinetics relative to those observed at the end of a normal dark period (Fig. 6). In contrast, spinach NR activity was only partially activated when illuminated after extended darkness (Huber et al., 1992c), implying the existence of a light-independent mechanism (e.g. circadian rhythm) controlling NR gene expression or protein turnover in spinach. This level of control appears to be less important than the light effect in maize leaves.

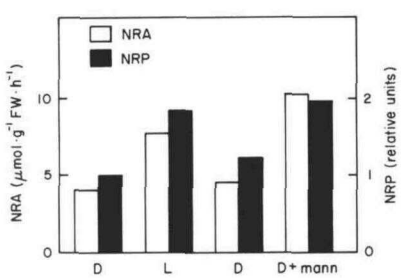

Figure 4. Light induction and Man induction of NRA and NR protein in excised maize leaves. Plants, grown in the greenhouse for 2 weeks, were transferred to a darkened growth chamber (25°C) 12 h before the start of the experiment. Leaves from position number 3 (fully elongated) were sequentially harvested at the end of the night period (first dark [D] sample), after 45 min of light (L; 400 μmol m^{-2} s^{-1} PPFD), after transfer to darkness for 45 min (second dark sample), and finally after transfer of dark-adapted leaves to a solution of 50 mM Man for an additional 1.5 h in the dark. Each leaf sample was harvested in liquid nitrogen, and ($-Mg^{2+}$) NR activity (NRA) and NR enzyme protein (NRP) levels were measured as described in the "Materials and Methods." FW, Fresh weight.

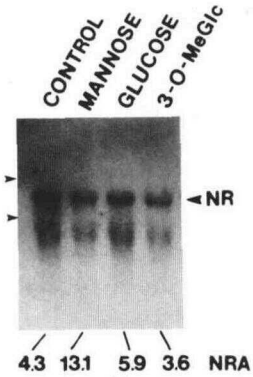

Figure 5. Comparison of the effects of various hexose sugars on the ($-Mg^{2+}$) NR activity and steady-state level of NR mRNA in detached maize leaves in the dark. Leaves were excised from 2-week-old plants in the greenhouse, approximately 2 h into the light period. Excised leaves were placed in water in a darkened growth chamber (25°C) for 2 h. The cut leaves were then placed in water (control) or solutions containing 25 mM Man, Glc, or 3-*O*-methyl-Glc (3-O-MeGlc). After an additional 1.5 h of darkness, leaves were harvested in liquid nitrogen. Total RNA was extracted from the frozen leaf samples and subjected to electrophoresis on an agarose gel (10 μg/lane) and then transferred to a nitrocellulose membrane. The blots were probed with a ^{32}P-labeled probe (Gowri et al., 1992). The position of the approximately 3.2-kb NR transcript (NR) is indicated by the arrowhead on the right, and the positions of the 28S and 18S rRNA bands are indicated by the arrowheads on the left. Equivalent loading of RNA in each lane was verified as described in "Materials and Methods." The ($-Mg^{2+}$) NR activity (NRA) in each sample is indicated at the bottom of the figure.

NR mRNA levels were measured to determine to what extent changes in NR transcript level were correlated with alterations in NR activity and NR protein levels. During a normal diurnal cycle, the steady-state level of NR mRNA increased 2- to 4-fold during the first 1 to 2 h of the light period but then began to decrease and by afternoon approached the levels measured in the dark (Fig. 7, top). A second, small peak of NR mRNA approximately 5 h into the light period was occasionally observed (data not shown) but was usually more pronounced in young (6-d-old) maize plants and was much reduced or absent in mature leaf tissue. The results reported here with mature maize leaves are generally consistent with those reported by Lillo (1991) and Bowsher et al. (1991).

To determine the effect of light signals on NR mRNA levels, plants were retained in darkness for the first 8 h of the normal light period. While in extended darkness, NR mRNA levels remained low, but the transcript accumulated rapidly upon subsequent transfer of the plants to the light (Fig. 7, bottom). Consequently, light itself or metabolites produced by photosynthetic metabolism apparently influence NR mRNA levels. Taken together, the results presented in Figures 6 and 7 suggest that light is an important regulator of both maize leaf NR gene expression and enzymatic activity.

CONCLUSIONS

Overall, the present study demonstrates that maize leaf NR is light activated in part by increased steady-state levels

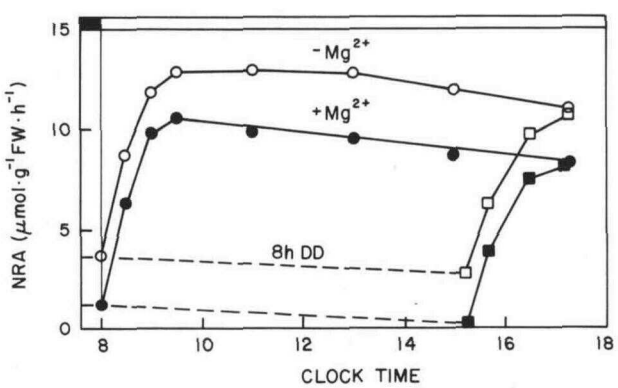

Figure 6. Effect of extended darkness on the light stimulation of NRA in attached maize leaves. Plants were grown in the greenhouse and transferred to a darkened growth chamber (25°C) overnight. Extended darkness (DD) was started at the beginning of the experiment (8 AM [8h]), which was close to the end of the normal dark period. Either at 8 AM or at 3:30 PM (approximately 8h DD) plants were transferred to an illuminated growth chamber (400 μmol m^{-2} s^{-1} PPFD, 28°C). At the times indicated, leaf samples were harvested into liquid nitrogen. Leaves were extracted and NR activity (NRA) was assayed in the presence (closed symbols) and absence (open symbols) of 5 mM Mg^{2+}. At 9 AM and at 4 PM, leaf CO_2 assimilation rates were measured under ambient conditions using an ADC portable photosynthesis system; mean values were, respectively, 17 and 14 μmol m^{-2} s^{-1}. FW, Fresh weight.

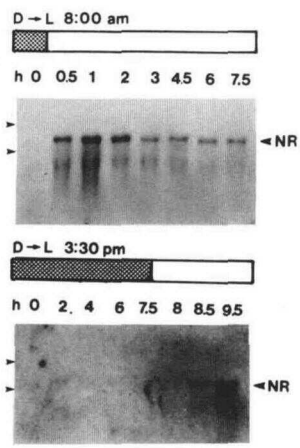

Figure 7. Effect of extended darkness (D) on the light (L) stimulation of NR mRNA accumulation in attached maize leaves. Experimental conditions were as described in the legend of Figure 6, except that total RNA was extracted from leaf samples collected at different times. Total RNA was subjected to electrophoresis on an agarose gel (10 μg/lane) and then transferred to a nitrocellulose membrane. The blots were probed with a ^{32}P-labeled NR probe (Gowri et al., 1992). The position of the 3.2-kb NR transcript (NR) is indicated by the arrowheads on the right, and the positions of the 28S and 18S rRNA bands are indicated by the arrowheads on the left. Equivalent loading of each lane with RNA was verified as described in "Materials and Methods."

of NR protein (Fig. 4) and by covalent modification that is manifested as decreased sensitivity to Mg^{2+} inhibition (Fig. 1B). Similar to the spinach (Huber et al., 1992a) and *Arabidopsis* (LaBrie and Crawford, 1994) leaf enzymes, maize NR has been shown to be phosphorylated on specific seryl residues (Fig. 3), and in general, phosphorylation was associated with increased inhibition by Mg^{2+}. These findings are direct evidence for phosphorylation of NR in a C_4 monocotyledonous species. Thus, this regulation may be a general phenomenon in higher plants. However, unlike spinach and the other dicotyledonous species examined in other studies, NR expression in mature maize leaves was not controlled primarily via an endogenous rhythm but rather appeared to respond primarily to light signals. Thus, light (or a light product) appeared to be directly involved in maize leaf NR expression. Sequestration of intracellular phosphate by Man feeding in the dark partially mimicked some of the effects of light, suggesting that at least part of the light effect was indirectly mediated by changes in cytoplasmic metabolites (e.g. decrease in cytosolic Pi). However, the exact mode of Man action remains to be characterized.

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