Differential Light Induction of Nitrate Reductases in Greening and Photobleached Soybean Seedlings

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ABSTRACT

Soybean (Glycine max [L.] Merr.) seeds were imbibed and germinated with or without NO₃, tungstate, and norflurazon (San 9789). Norflurazon is a herbicide which causes photobleaching of chlorophyll by inhibiting carotenoid synthesis and which impairs normal chloroplast development. After 3 days in the dark, seedlings were placed in white light to induce extractable nitrate reductase activity. The induction of maximal nitrate reductase activity in greening cotyledons did not require NO₃ and was not inhibited by tungstate. Induction of nitrate reductase activity in norflurazon-treated cotyledons had an absolute requirement for NO₃ and was completely inhibited by tungstate. Nitrate was not detected in seeds or seedlings which had not been treated with NO₃. The optimum pH for cotyledon nitrate reductase activity from norflurazon-treated seedlings was at pH 7.5, and near that for root nitrate reductase activity, whereas the optimum pH for nitrate reductase activity from greening cotyledons was pH 6.5. Induction of root nitrate reductase activity was also inhibited by tungstate and was dependent on the presence of NO₃; further indicating that the isoform of nitrate reductase induced in norflurazon-treated cotyledons is the same or similar to that found in roots. Nitrate reductases with and without a NO₃ requirement for light induction appear to be present in developing leaves. In vitro kinetics (light induction and dark decay rates) and in vivo kinetics (Arrhenius energies of activation and NADH:NADPH specificities) of nitrate reductase with and without a NO₃ requirement for induction were quite different. Kₚ values for NO₃ were identical for both nitrate reductases.

The large amplitude of daily rhythms for NR activity extracted from higher plants (8, 25) and the toxic effects of NO₃, the product of NR activity, in higher plants (4) suggest that in many species NR activity is highly regulated and that the close regulation of NR is necessary for healthy plant function. Phytochrome (11, 12, 22), blue light photoreceptors (16, 22), photodynamic pigments (6), NO₃ (30), reduced nucleotides (21), ATP:ADP ratios (21), polysome levels (28), and specific proteinaceous inhibitors and activators of NR (25) have been shown to affect levels of NR activity which may be extracted from higher plants and have been implicated as regulators of NR activity. In soybean plants the regulation of NR activity may be particularly complicated because two isozymes with quite different properties may be present (1, 19). One manifestation of this complexity is the two diurnal peaks in extractable NR activity in soybean leaves (8) as compared to the one diurnal peak in NR activity of maize (8), and wheat (25).

In a previous report, it was demonstrated that the kinetics for light induction and the optimum pH for activity of extractable NR in soybean seedlings greening normally and in those treated with norflurazon (San 9789), a potent inhibitor of carotenoid synthesis and Chl development, were quite different (12). This report indicated that differing forms of soybean NR were induced with the differing treatments. Here we report evidence for the differential light induction of two isoforms of NR with vastly differing properties and requirements for induction in normally greening and photobleached soybean seedlings.

MATERIALS AND METHODS

Plant Tissue. Soybean (Glycine max [L.] Merr. cv Hill) seeds were imbibed in distilled H₂O for 6 h at about 25°C. Seeds were then rolled in germination paper soaked with N-free nutrient solution (10) with added 10 mM KNO₃ or 10 mM KCl. Rolls of seeds were placed in beakers containing nutrient solution and germinated in darkness. After 3 d in darkness, seedlings were removed and wrapped in fresh germination paper soaked in fresh nutrient solution. Seedlings were oriented so that the cotyledons were exposed at the top of the rolled germination paper. Seedlings rolled in paper were then placed in beakers containing fresh nutrient solution and placed in a growth chamber under constant light, with a fluence rate of about 190 μE m⁻² s⁻¹ (180-w Westinghouse cool white fluorescent F72T12/CW/SHO and 40-w Westinghouse incandescent lamps) at plant height, and constant temperature (25°C). Seedlings for NR purification and dark decay studies were grown as before (12).

Treatments. Norflurazon [4-chloro-S-((methylamino)-2-(a,a,a-trifluoro-m-tolyl)-3-{2H-pyridazinone})] treatments were during imbibition as before (12), except that the norflurazon was applied directly to the dry seeds in all studies except purification and dark decay studies. Actinomycin D treatments were during imbibition (25 μg/ml) and for 10 h at the beginning of the light treatment (10 μg/ml). Aluminum foil was wrapped around rolls of seedlings exposed to light to prevent photodegradation of actinomycin D. After 2 d in light, some seedlings were placed under a 100% N₂ atmosphere for 24 h. Seeds treated with...
FIG. 1. Light induction of extractable NR activity in soybean seedling cotyledons grown with (■, □) and without (●, ○) norflurazon and with (■, ●) and without (□, ○) 10 mM NO$_3$; Seedlings were germinated and grown in darkness for 72 h prior to being exposed to light. Each data point represents the mean of three tissue samples.

**RESULTS AND DISCUSSION**

**Induction and Characteristics of Cotyledon Nitrate Reductases.** Light induction of extractable NR activity in cotyledons of normally greening seedlings was rapid and of equal magnitude in seedlings grown with and without NO$_3$ (Fig. 1). In contrast, the light induction of NR activity in photobleached cotyledons of seedlings grown with norflurazon (cf. 12) did not occur until after 24 h of light and there was an absolute requirement for NO$_3$. The requirement for light in the induction of activity of either enzyme is absolute (12) and neither type of soybean NR activity should be referred to as "endogenous," as has been proposed (1). We prefer to designate the NR without a NO$_3$ requirement for induction as NR - S (–S to indicate that there is no substrate requirement for light induction) and NR with a substrate requirement as NR + S (+S to indicate that substrate

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Form of NR Induced</th>
<th>pH Optimum</th>
<th>Arhenius Energy of Activation</th>
<th>NADH:NADPH Activity Ratio</th>
<th>$K_m$ (NO$_3^-$)</th>
<th>Tungstate Inhibition</th>
<th>In Vivo Dark Decay After 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotyledons</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-NO$_3$</td>
<td>NR - S</td>
<td>6.5</td>
<td>11.5</td>
<td>ND</td>
<td>145°</td>
<td>90</td>
<td>ND</td>
</tr>
<tr>
<td>+NO$_3$</td>
<td>NR - S</td>
<td>6.5 (6.5)$^b$</td>
<td>ND</td>
<td>1.3</td>
<td>ND</td>
<td>115</td>
<td>121</td>
</tr>
<tr>
<td>+NO$_3$ and norflurazon</td>
<td>NR + S</td>
<td>8.5 (7.5)$^b$</td>
<td>14.1</td>
<td>2.5</td>
<td>145°</td>
<td>4</td>
<td>8.5</td>
</tr>
<tr>
<td>-NO$_3$ and norflurazon</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+NO$_3$</td>
<td>NR + S</td>
<td>8.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>46.8</td>
</tr>
<tr>
<td>-NO$_3$</td>
<td>None</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>+NO$_3$ and norflurazon</td>
<td>NR + S</td>
<td>8.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0</td>
<td>34.5</td>
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<tr>
<td>-NO$_3$ and norflurazon</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

$^a$ ND, not determined.

$^b$ NR purified by affinity chromatography.
is necessary for induction). Maximum light induction of NR - S and NR + S was at 72-h illumination after which both forms of NR rapidly decreased in activity. The decreases in activity after 72 h were not observed in our previous study (12). These differences in results are due to differing experimental conditions.

Cotyledonary NR - S activity had an optimum activity at pH 6.5 (crude and partially purified) and tungstate had little or no effect on its induction (Table I). In contrast, the pH optimum for crude NR + S activity was at 7.5 to 8.5 (a broad peak was found from 7.5 to 8.5 for the crude enzyme) and tungstate inhibited the induction of its activity. Aslam (1) and Harper and Nicholas (14) also demonstrated the presence of a form of NR in soybeans which is resistant to tungstate inhibition. The Arrenius energy of activation for NR + S was 24% greater than for NR - S (Fig. 2, Table I). In vivo dark decay of NR + S activity was almost complete after 48 h whereas there was a slight increase in NR - S activity over the same period of darkness. Specificities for NADH and NADPH are also quite different for NR + S and NR - S (Table I). The only kinetic parameter found to be the same for cotyledonary NR + S and NR - S was the K_m for NO_3. These data indicate that NR + S and NR - S are two differing forms of NR. Whether or not these two forms of NR are differing isozymes, differing conformers of one isozyme, or differing post-translational modifications of a single NR apo-protein is unknown; hence, we will refer to them as isozymes at this time.

The induction of NR - S in soybean cotyledons appears to be dependent on the presence of normally developing plastids, as evidenced by its absence in norflurazon-treated cotyledons, whereas the induction of NR + S is inhibited by the presence of normally developing plastids. However, the induction of NR - S does not seem to be dependent on the greening of tissues. Both NR - S and NR + S may be induced by far-red light, which will not cause greening (12). Hence, the effects of norflurazon on NR induction in soybean cotyledons are not due to effects on development.

For the cycloheximide treatment, seedlings were germinated as in Table I. Cycloheximide-treated seedlings died between 12 and 24 h of treatment and NR activity was assayed after 12 h of light and cycloheximide treatment.

Table II. Effects of Inhibitors of Protein and RNA Synthesis on the Induction of Soybean Cotyledon Nitrate Reductases and Alcohol Dehydrogenase

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Form of NR Induced</th>
<th>Actinomycin</th>
<th>Cycloheximide</th>
<th>Chloramphenicol % control</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NO_3^-</td>
<td>NR - S</td>
<td>99</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>+NO_3^-</td>
<td>NR - S</td>
<td>101</td>
<td>199</td>
<td>0</td>
</tr>
<tr>
<td>+NO_3^- and</td>
<td>NR + S</td>
<td>224</td>
<td>162</td>
<td>0</td>
</tr>
<tr>
<td>norflurazon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*ND, not determined.

+ADH experiments were under anaerobic conditions. ADH could not be detected under aerobic conditions.

The nitrogen assimilation and photosynthesis.
Cotyledonary NR - S appears to be the same or similar to the NR reported by Orihuel-Iranzo and Campbell (19) as a soybean cotyledon NDH-dependent NR. The pH optimum is the same (6.5) and the NADH:NDPH activity ratio is similar (about 1.2-1.3). We did not detect a form of soybean cotyledon NR with properties similar to the NADPH-dependent NR reported by Orihuel-Iranzo and Campbell, nor did they detect a form of NR with properties similar to the NR + S reported here. NR + S has a much higher optimum pH for activity and NADH:NDPH activity ratio than either NR reported by Orihuel-Iranzo and Campbell. Differences between our studies may be due to the differing cultivars used or experimental treatments.

Nitrate Concentrations in Seeds and Seedlings. Nitrate was
Differential Induction of Soybean Nitrate Reductases

These findings and the observation that NR + S activity fails to develop in normally greening tissues with high levels of NO$_3^-$, we conclude that there is no simple relationship between NR + S induction and tissue NO$_3^-$ concentrations. Our previous studies indicate a low negative correlation between tissue NO$_3^-$ concentrations and NR + S induction (12). Other studies demonstrating a positive correlation between soybean NR activity and NO$_3^-$ uptake have utilized the in vivo NR assay (3, 5). In utilizing the in vitro assay, such correlations may be primarily due to NO$_3^-$ pool availability and may not be a function of potential NR activity.

Effects of Actinomycin D, Cycloheximide, and Chloramphenicol on Induction of NR – S and NR + S. The induction of NR – S in greening cotyledons in the absence of NO$_3^-$ could be explained by the presence of preformed NR mRNA in soybean seeds. Preformed mRNAs may be expressed after germination and exposure to light. Ihle and Dure (15) have demonstrated that a large amount of protein synthesis in germinating cotton seed is a result of preformed mRNAs. Studies with soybean seedlings (27) have suggested that preformed mRNA exists for certain enzymes. Also, actinomycin D has been shown to have little effect on the induction of NR activity in soybean (3), barley (13), and cotton (20) seedlings, suggesting that NR mRNA exists in the seeds of several species; therefore, we tested the effect of the mRNA inhibitor actinomycin D on the induction of NR + S and NR – S (Table II). We found that actinomycin D has no effect on the induction of NR – S and actually increases the induction of NR + S. Although these data suggest that preformed mRNA or unactivated NR protein exists for both forms of NR in soybean seedlings, we questioned these data.

As a check on the effectiveness of actinomycin D in inhibiting nuclear mRNA synthesis we assayed for ADH activity under aerobic and anaerobic conditions with and without actinomycin D (Table II). The induction of ADH activity is known to require synthesis of mRNA in anoxia-sensitive plant species (18). Anaerobic conditions were necessary for ADH induction and actinomycin D caused more than a 2-fold increase in activity over controls (Table II). Considering these data, we question the efficacy of actinomycin D in preventing mRNA synthesis under our experimental conditions and those of others. In the past we observed a positive effect of actinomycin D on the induction of ADH and NR + S, it is apparent that this compound is affecting some aspect of protein synthesis and/or enzyme activation or protein processing.

Our data indicate that de novo synthesis of nuclear and organelle proteins is necessary for NR induction in soybean seedlings (Table II). Cycloheximide completely inhibited the induction of both NR – S and NR + S in seedling cotyledons, whereas chloramphenicol decreased induction of both by about 75%. In contrast, past studies have demonstrated that chloramphenicol enhances NR induction in maize (24) and peas (26). Because NR is known to be a cytosolic enzyme (29), it is doubtful that the results of chloramphenicol were on the synthesis of the NR apoprotein. Effects of both protein synthesis inhibitors could have been on the production of proteinaceous NR activators (25) or other substances which affect activity. Unfortunately, more definitive studies with purified NR and NR antibodies could not be conducted due to the in vitro instability of NR – S and NR + S.

Induction and Characterization of Root and Leaf Nitrate Reductases. As with cotyledonary NR + S, the induction of root NR activity requires NO$_3^-$, has a pH optimum of 7.5 to 8.5, and is inhibited by tungstate (Fig. 4, Table I). Thus, soybean root NR has many properties similar to and may be the same as cotyle- donary NR + S. In our past study, the induction of root NR activity was found to be stimulated by direct exposure of roots to light (12). Here we only exposed the aerial portion of seedlings to light; hence, the light stimulation of root NR activity was indirect (dark control root NR rates of activity were only about...
20% of those from light-treated seedlings after 48 h light; data not shown). Root NR activity peaked at 48 h after the beginning of seedling illumination and declined rapidly thereafter (Fig. 4). Data for root NR induction in photobleached tissues are similar to those in our past study but those for normally greening seedlings are not (12). This may be due to differing light treatments (direct versus indirect exposure of roots to light and differing light intensities).

After 72 h of light, primary leaves of normally greening seedlings were expanding rapidly and had fresh weights of about 80 mg/seedling. In contrast, primary leaves of norflurazon-treated seedlings failed to develop. Primary leaf NR activities from seedlings with and without NO\textsubscript{3} were approximately equal at 72 h (Fig. 5). After 96 h illumination, primary leaves of seedlings treated with NO\textsubscript{3} had substantially higher NR activities than those from seedlings with no NO\textsubscript{3}. Aslam (1) has shown that the increase in NR activity in soybean plants fertilized with NO\textsubscript{3} could be inhibited by tungstate, whereas NR activity in unfertilized plants was unaffected by tungstate. This observation coupled with our observations (Fig. 5) indicate that both NR - S and NR + S activities may occur in soybean leaves fertilized with NO\textsubscript{3}. This is very different from the situation in cotyledons where we were only able to induce NR + S and NR - S separately.

Increases in NR activity in leaves were concomitant with increases in leaf protein between 72 and 96 h after the beginning of light treatments. Soluble leaf protein increased from about 2.2 mg/g fresh weight to 30.7 mg/g fresh weight over this period regardless of whether seeds were treated with NO\textsubscript{3}. Leaf NR activity decreased between 96 and 120 h of light (Fig. 5) as concentrations of soluble protein (data not shown) decreased. These data suggest that NR activity may be in part developmentally regulated in soybean leaves. Although NR + S has a NO\textsubscript{3} requirement for induction, stage of development has a great influence on the magnitude of induction.

CONCLUSIONS

Two very distinct forms of NR can be differentially light-induced in cotyledons of soybean seedlings. One form, NR - S, has no NO\textsubscript{3} requirement for induction (Fig. 1), and its induction is not inhibited by tungstate (Table I). NR - S cannot be induced in roots under the experimental conditions employed here (Fig. 4, Table I). The inhibition of plastid development in cotyledons by norflurazon completely inhibited the induction of NR - S (Fig. 1, Table I); however, primary leaves of light-treated, which does not cause greening, will also cause NR - S induction, Chl and photosynthesis do not appear to be necessary for its induction (12).

A second form of soybean cotyledonary NR, NR + S, has an absolute requirement for NO\textsubscript{3} and is completely inhibited by tungsten (Fig. 1, Table I). NR + S could only be induced in cotyledons when plastid development was disrupted with norflurazon. A form of NR similar or identical to cotyledon NR + S is the only form which can be induced in roots (Fig. 4). Both NR + S and NR - S seem to be present in leaves of seedlings grown with NO\textsubscript{3}; however, NR + S becomes predominant in leaves as they expand (Fig. 5). The biological significance of the presence of very different isoforms of NR in soybean plants is currently unclear. This is the subject of our continuing studies.

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