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Light suppression of nitrate reductase activity in seedling and young plant tissues

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Abstract
Light is often reported to enhance plant nitrate reductase (NR) activity; we have identified a context in which light strongly suppresses NR activity. *In vivo* NR activity measurements of laboratory-grown seedlings showed strong suppression of nitrate-induced NR activity in cotyledon, hypocotyl, and root tissues of *Ipomoea hederacea* (L.) Jacquin; robust NR activity accumulated in nitrate-induced tissues in the dark, but was absent or significantly reduced in tissues exposed to light during the incubation. The suppressive mechanism appears to act at a point after nitrate perception; tissues pre-incubated with nitrate in the light were potentiated and developed NR activity more rapidly than nitrate-induced tissues not so pre-exposed. Suppression was affected by moderate to low light levels under full-spectrum light sources and by single-wavelength red, green, and blue sources. The suppression phenomenon persisted in early (first through fourth) leaves of glasshouse plants grown in soil, and in artificially rejuvenated cotyledons. Collectively these observations suggest a link between light perception and NR regulation that remains to be fully characterized.

Keywords: plant, nitrate, reductase, regulation, suppression, light

Introduction

Living systems employ reductive enzymes for a range of processes; important among these is the acquisition of nutrients from inorganic pools. Nitrate (NO_3^-) -- the major form of inorganic nitrogen available to plants in the environment -- must be reduced to ammonium (Guerrero, Vega and Losada 1981) prior to its assimilation into the amino acid pool via either the glutamine synthetase/glutamate synthase cycle or the action of glutamate dehydrogenase (Lam *et al.* 1995). The initial step of nitrate reduction is mediated by nitrate reductase (NR), which generates nitrite (NO_2^-). Nitrite is subsequently reduced to ammonium by nitrite reductase. While the occurrence of nitrate-reducing activities in plant tissues has been known for more than a century (Irving and Hankison, 1908) their mechanisms and physiological roles (Campbell 1999), genetics (Hirel *et al.* 2001), modes of regulation (Lillo *et al.* 2004, Lillo 2008), and potential for improvement in the context of nitrogen use efficiency (Zhao, Nie and Xiao 2013) have been the foci of ever increasing numbers of investigations. Beyond assimilation, nitrate reduction plays an important role in the synthesis of nitric oxide, a molecule recognized as mediating signal transduction in plants and animal systems (Desikan *et al.* 2002).

Because N assimilation entails both energetic and metabolic costs in the forms of reducing equivalents and carbon skeletons, respectively, it is to be anticipated that associated processes are physiologically regulated and sensitive to the plant's status. Multiple levels and mechanisms of regulation have been reported to impact NR activity in plants. At the transcriptional level, promoter sequences and other functional elements associated with the *Arabidopsis* NR-encoding *NIA1* gene have been demonstrated to contribute qualitatively and quantitatively to nitrate-dependent induction (Lin *et al.* 1994, Wang *et al.* 2010, Konishi and Yanagisawa 2011). Examination of the relative abundance of transcripts from two NR-encoding isogenes of *Brassica napus* revealed distinct nitrate-independent accumulation patterns associated with different developmental stages and tissue types in microspore culture-derived embryos (Fukuoka *et al.* 1996). NR activity is modulated post-translationally through regulatory phosphorylation (Su, Huber and Crawford 1996) permitting the association of a 14-3-3 family protein that alters electron flow through the enzyme's modular structure (Lambeck *et al.* 2012); this feature appears to be widely conserved among flowering plants (Bachman *et al.* 1996) and may have emerged prior to the divergence of Magnoliophyta (Nemie-Feyissa *et al.* 2013). Evidence has also been provided for regulation through degradation of the NR protein (Gupta and Beevers 1984, Somers *et al.* 1983).

Factors to which NR regulatory mechanisms are responsive include developmental state (Fukuoka *et al.* 1996), available nitrate (Hageman and Flesher 1960), metabolic status (Botrel and Kaiser 1997, Vincentz *et al.* 1993), moisture and pathogen stresses (Bardzik, Marsh and Havis 1971, Yamamoto *et al.* 2003), plant growth regulators (Lu, Ertl and Chen 1990, Zhang *et al.* 2011) and light (Duke and Duke 1984, Huber *et al.* 1992b, Lillo 1994). Light is most often reported to have an enhancing effect on NR activity, and this enhancement may be either the direct result of light perception (Rajasekhar, Gowri and Campbell 1988), or through stimulation by the products of photosynthesis (Cheng *et al.* 1992). In addition, light entrains the plant's circadian rhythm, which has been proposed to influence the cyclic accumulation of NR tran-

script in anticipation of daylight, and corresponding decrease as night approaches (Lillo and Ruoff 1989, Deng *et al.* 1990), though whether this modulation is necessarily integrated with the cell's central diurnal timekeeping function, termed the "central oscillator," has been called into question (Lillo, Meyer and Ruoff 2001). In contrast to evidence for an enhancing effect, the potential for photoreceptor-mediated negative impacts of light on NR activity levels has been suggested in limited cases (Rajasekhar, Gowri and Campbell 1988) with far red treatments reversing red light stimulation of NR activity in etiolated squash cotyledons and red light suppression of cotyledon NR in intact seeds of *Cicer arietinum*, though not in excised tissues (Bueno *et al.* 1996).

In the course of examining the carbon and nitrogen metabolic physiology of the twining forb *Ipomoea hederacea* (L.) Jacquin (ivyleaf morning glory), we noted novel patterns of nitrate reductase activity in seedling tissues. Contrarily to the often-reported enhancement of NR activity by light exposure, we found a robust suppression of nitrate-dependent induction, even at low light levels, in both laboratory-grown seedling root and shoot tissues and in young glasshouse-grown plants. Our objective, therefore, was to characterize the nature of this phenomenon with respect to the relative timing of nitrate-mediated NR induction versus light-mediated suppression; with respect to the light quantity and quality; and with respect to the potential impact of plant growth and development and tissue age.

Materials and methods

Plant materials and culture

Seeds of *I. hederacea* were collected annually from a locally occurring population. Seeds were stored at approximately 30°C to promote drying and subsequent germination. Germination was induced by soaking the seeds in distilled water overnight with gentle shaking. The following day, seeds showing emergent radicles were transferred to growth boxes. The bottoms of clear plastic boxes (13cm x 17cm x 7cm) with close-fitting lids were lined with paper towels dampened with distilled water; the boxes were allowed to drain until no more water dripped freely under gravity. Approximately 20 germinating seeds were planted in each box and placed under constant light. New seedlings were started for each experiment, and in the instances when large numbers of seedlings were required, seedlings from multiple boxes were distributed in a representative fashion among the different treatments; no difference in growth or responsiveness was observed between seedlings started from seed collected during different years. Light was provided by a single light bank (Sun System New Wave T5-44 high output fluorescent fixture), with two Starcoat T5 F54W 830 tubes and two 865

fluorescent tubes (General Electric). Light intensity was controlled by shading the boxes with sheets of white paper. Light intensity was measured by placing a photometer in the same location as the box.

Induction and light treatments

Live tissue samples were harvested from seedlings or more developed plants for light and nitrate treatments. Cotyledons were separated from each other, and the seedling root was separated from the hypocotyl, which was typically cut into approximately 1 cm sections. Nitrate reductase activity was induced by the infiltration of nitrate-containing buffer. Both control (non-induced) and treatment (induced) tissues were placed in the wells of 12-well microtiter plates, with a single sample (cotyledon pair, hypocotyl, or root) per well. Tissues were immersed in 2 mL volumes of potassium phosphate buffer (50 mM, pH 6.5) with or without potassium nitrate amendments. Tissues were vacuum infiltrated by placing the sample plate in a vacuum chamber and drawing a vacuum until air bubbles were seen to emerge from the tissues. Cotyledons (abaxial side up) were held submerged by small glass weights, which were removed subsequent to infiltration. Infiltrated tissues (a minimum of eight repetitions per treatment) were incubated under full spectrum (as previously described) or single wavelength red, green, or blue (Thor Labs) LED light sources, typically for periods of 18-22h. Specific exposure times and repetition numbers are reported in the corresponding figure legends. Exposure to the high light treatment had the effect of increasing the temperature of the medium 2-3C relative to the dark (foil shielded) treatment. To test whether this higher temperature contributed to the suppression of NR activity, shielded tissues were incubated in nitrate-containing medium at room temperature (25C) and in a darkened incubator at 37C. Tissues incubated at 37C had a higher measureable NR activity, and as such it was determined that the slightly increased temperature under full illumination was not the cause of NR activity suppression in the light (data not shown).

In vivo detection of nitrate reductase activity

In vivo detection was performed similarly to the method described by Klepper, Flesher and Hageman (1971). At the time of measurement, treatment solutions were removed by aspiration and replaced with 2 mL nitrate reductase assay buffer (1 mM KPO_4, pH 6.5, 0.1 M KNO_3, 0.07% Triton X-100). Tissues were briefly vacuum infiltrated and then incubated in the dark. After 1 hr, 200 µL of the assay solution from each sample was transferred to a tube with 1 mL of color reagent (0.5% sulfanilamide m/v and 0.05% N-1-naphthylethylenediamine hydrochloride m/v in 1.5 N HCl). The nitrite content of each sample was determined spectrophotometrically at 540 nm, and the mass of nitrate

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generated per hour normalized by the fresh mass of the tissue in the assay. In the event of especially high levels of nitrite generated, all samples and standards were diluted proportionally with water to keep measurements within the linear range for the assay (A_{540} < 0.5).

Qualification of the in vivo assay

Tissues treated with nitrate in the dark showed a stimulated capacity to generate nitrite. Tests optimizing the *in vivo* assay showed that a fraction of nitrite produced in the tissues prior to the assay was released during the assay, and this was most pronounced in tissues with the highest NR activity; a similar nitrite release was observed in tissue homogenates used in determining the suitability of the *in vitro* NR assay, leading us to choose the technically simpler *in vivo* assay. The nitrite released from the tissue during the *in vivo* assay is taken to reflect the net of nitrite generated during the assay plus nitrite already present, and less the amount consumed by nitrite reductase (NiR) activity. We did not attempt to quantify NiR activity in this study, though we did observe that tissues, particularly roots, were capable of affecting moderate depletion of applied nitrite from treatment media, suggesting active nitrite uptake and possibly reduction. Consequently, the reported activity is not an absolute measure of activity but rather reflects the relative levels of nitrite-generating activities in the tissues.

Statistical methods

All experiments were analyzed using general linear models (Proc GLM in SAS/STAT® software (Version 9.3 of the SAS System for Windows. Copyright © [2002-2010] SAS Institute Inc.: Cary, NC, USA). The statistical models for the experiments depicted in figures 1B, 3, 4, and 5A-D included nitrate treatment and tissue type as main factors. The statistical models for the experiments illustrated in figures 1C and 2 contained in addition light treatment as third main factor. The models for the experiments shown in figure 6 included as main factors nitrate and light treatments and, with the exception of the experiments shown in figures 6e and f, age was also part of the model. Interactions between all main factors were included in all statistical models. As significance level we used α ≤ 0.05. Prior to analyses, all data were tested for normality and homogeneity of variances and transformed if needed (Sokal and Rohlf 2012). Post-hoc Scheffe's tests were carried out to compare group means of significant main effects or interactions between main effects (Sokal and Rohlf 2012).

Results

Ipomoea hederacea seed germination and growth

Ipomoea species have been employed in eco-physiological, developmental, and genetic studies (Gianoli and González-Teuber 2005, Simms 1993, and other works by these laboratories). In this study *Ipomoea hederacea* (ivyleaf morning glory) was used as a study organism because its large seeds germinate uniformly giving rise to rapidly growing seedlings that are responsive to both light and nitrate, and that provide sufficient tissue mass for multiple samplings from individual seedlings. Following thorough drying at moderately warm temperatures (sustained open storage at approximately 30°C) stratified seeds imbibe rapidly, with the radicle emerging within the first 24h. Figure 1*A* shows germination and development through day 6. Primary growth at the root tips is evident by day 2, and secondary roots are present by day 3. Hypocotyl elongation begins by days 2-3. Under moderate light (approximately 2500 lx) hypocotyls elongate at a rate of >1 cm day^{-1} up to days 6-7, with the most rapid elongation occurring during days 3-5. Seed coat shedding occurs on days 3-4 under the high humidity growth conditions employed herein, followed by unfolding of the cotyledons. Initiation of primary growth from the shoot apical meristem could be observed within the first week, but was less pronounced in laboratory grown plants compared with seedling that were transplanted to the glasshouse within the first week.

Seedling responsiveness to nitrate

Efforts to measure NR activity in untreated four day old (4 d) tissues of seedling grown without nutrient amendment showed no detectable activity (twelve observations on each tissue); this was also the case for tissues seedlings grown in soil under a 16h/8h light/dark regime (data not shown). The potential responsiveness of seedling NR levels to applied nitrate was established using 4 d seedlings separated into root, hypocotyl, and cotyledon explant fractions. Each tissue was provided potassium phosphate buffer (10 mM, pH 6.5) supplemented with potassium nitrate (0, 0.1, 1.0, 10, 50, and 100 mM final concentration). Infiltration of treatment solutions into apoplastic spaces was promoted by placing the samples under a vacuum until complete infiltration of cotyledons was apparent. Tissue NR activity was measured following 22 h incubation in the dark at room temperature. The amount of nitrate applied had a significant effect on NR activity ($F_{5, 126}$ = 98.94, P < 0.0001; Fig. 1*B*) and all tissues showed a similar response (effect of tissue type: $F_{2, 126}$ = 2.61, P = 0.002; interaction between nitrate concentration and tissue type: $F_{10, 126}$ = 1.72, P = 0.08). Tissues incubated with 0, 0.1, and 1mM nitrate showed no activity or minimally distinguishable activity, while unambiguous induction of NR activity was observed in tissues receiving 10 mM and higher levels of nitrate, with the greatest proportional changes occurring typically between 1.0, 10, and 50 mM treatments. Subsequent experiments used 10 mM nitrate so that it would be possible to discern factors either increasing or decreasing the degree of NR activity induction.
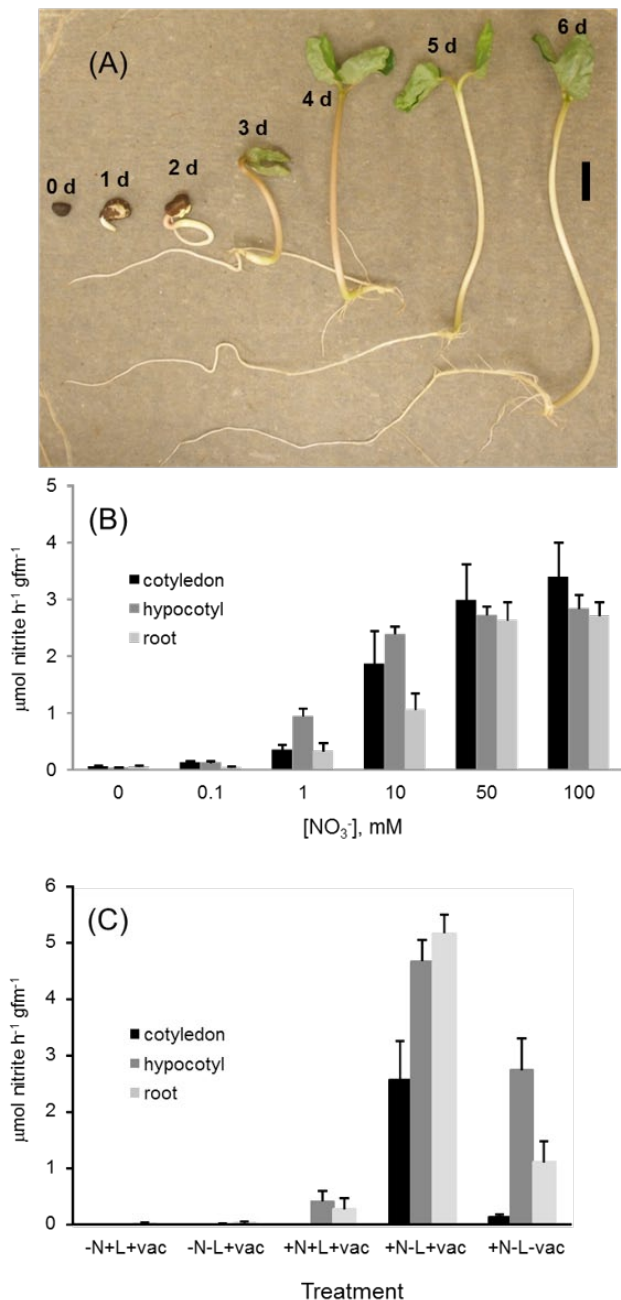
Figure 1. Growth of *Ipomoea hederacea* seedlings and responsiveness of NR activity to nitrate and light. (A) Representative seedlings grown under laboratory conditions at days 0-6 (left to right) post imbibition. Scale bar: 1 cm. (B) NR activity in 4 d *I. hederacea* seedling tissues following 22 h incubation in the dark with indicated concentrations of nitrate. Column height reports the mean of eight measurements; error bar is standard error. Results are representative of three experiments. (C) Impact of light on nitrate-induced NR activity in seedling tissues. NR activity in 4 d seedlings following 22 h incubation with (+N) or without (-N) 10 mM nitrate in dark (-L) or light (+L). All samples vacuum infiltrated (+vac) except "-vac." Column height reports the mean of eight measurements; error bar is standard error. Results are representative of three experiments.

Light suppression of nitrate reductase activity induction

Preliminary studies examining NR activity induction in *I. hederacea* tissues suggested a suppressive effect by light. These observations led us to further examine the phenomenon of NR activity suppression by light in *I. hederacea* seedlings. Seedling (4 days post-imbibition) cotyledons, hypocotyls, and roots were incubated in phosphate buffer with or without 10 mM potassium nitrate amendment in darkness or under continuous light (approx. 12,000 lx). Both the nitrate ($F_{1, 85}$ = 187.93, $P < 0.0001$) and the light treatment ($F_{1, 85}$ = 116.20, $P < 0.0001$) had a significant effect on NR activity, but tissues exposed to light responded differently to the nitrate treatment than tissue exposed to dark (nitrate-by-light treatment interaction: $F_{1, 85}$ = 114.06, $P < 0.0001$; Fig. 1*C*). Consistent with Fig. 1*B*, tissues incubated in the dark without nitrate showed little or no measureable NR activity, while those incubated in the dark with nitrate showed a strong induction. Light-treated tissues without nitrate did not show NR activity induction, and seedlings incubated with nitrate in the light showed a marked reduction in activity relative to those provided nitrate in the dark. NR activity did not differ between tissues in the absence of nitrate; in the presence of nitrate, however, hypocotyls and roots had substantially higher NR activity than cotyledons (tissue type: $F_{2,85}$ = 6.02, $P = 0.004$; nitrate treatment-by-tissue type interaction: $F_{2, 85}$ = 5.38, $P = 0.006$; Fig. 1*C*).

To determine whether vacuum infiltration of tissues was necessary for consistent induction of tissues, nitrate-treated samples were incubated in the dark without initial vacuum infiltration. All tissues showed a reduced level of NR activity relative to the dark-treated vacuum infiltrated tissues (vacuum infiltration: $F_{1, 41}$ = 45.92, $P < 0.0001$; vacuum infiltration-by-tissue type interaction: $F_{2, 41}$ = 1.79, $P = 0.18$; Fig. 1*C*), with cotyledon NR activity being significantly lower compared to hypocotyl and root NR activity (tissue type: $F_{2, 41}$ = 17.80, $P < 0.0001$, Fig. 1*C*). As a consequence, all subsequent experiments employed vacuum infiltration to ensure thorough NR induction. Additional experiments using intact seedlings instead of explant tissues showed a similar trend with respect to light inhibition of NR activity induction; these experiments had greater variability in the tissue responses however, and we speculate that it may have been the result of incomplete infiltration of the intact plant tissues. Further, observations using tissues of 4 d seedlings grown in soil showed a similar trend, except that the variability between samples, particularly in roots, was substantially higher (data not shown). The potential interaction of root tissue exposure to light and soil nutrients will be the topic of subsequent investigations; for simplicity, and to establish a base-line of response, seedlings grown without soil or nutrient amendment were used for the laboratory investigations reported here.

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Timing of NR activity induction

In order to characterize the dynamics of NR activity induction and to further determine the nature of light-mediated NR activity suppression, we examined the timing of NR activity induction. Figure 2 reports the timing and degree of NR activity in seedling tissues (4-5d over the course of the experiment) infiltrated with nitrate-containing solution incubated under continuous dark or light. Length of induction period strongly affected NR activity ($F_{5, 228}$ = 123.72, P < 0.001). The interaction between induction period and tissue type was not significant, meaning that all tissues responded in the same pattern to changes in the induction period ($F_{10, 228}$ = 1.69, P =0.08). Cotyledons, hypocotyls, and roots incubated with light showed a different pattern than those incubated in the dark (light treatment: $F_{1, 228}$ = 297.76, P < 0.001; induction period-by-light treatment interaction: $F_{5, 228}$ = 108.46, P < 0.001). Tissues assayed immediately after infiltration, or at 1, 2, or 4 h in either light or dark, did not show measureable NR activity (not shown). Dark-incubated cotyledons, hypocotyls, and roots showed an increase in NR activity following 12 h incubation, with activity increasing up to the 26 h incubation, with the most rapid increase occurring between 16 and 20h; longer incubation periods were not tested. In contrast to the dark-incubated tissues, light-incubated tissues showed a strikingly reduced induction, distinct from dark-incubated tissues by the 20 h and longer incubation periods. Activities in hypocotyls and roots were overall higher than in cotyledons (effect of tissue: $F_{2, 228}$ = 3.80, P = 0.02) and were low but measureable when light-incubated, while activity in cotyledons was essentially undetectable.

We hypothesized that exposure to light may generate a suppressive factor whose effect might persist after the end of light exposure, and that would alter the rate at which the tissue responded to nitrate. To test this hypothesis, explant tissues and whole plants were exposed to high light (12,000 lx) or darkness prior to nitrate infiltration and incubation in the dark. Multiple experiments, typically employing light pre-treatments in the range of several hours, failed to demonstrate a consistent difference in the induction patterns resulting from pre-incubation light or dark exposure (data not shown).

Interrupted light exposure and potentiation of NR induction during light exposure

It remained to be established whether the suppressive effect of light required continuous light exposure, or whether interruption of the dark period would suffice to affect suppression. Tissues exposed to nitrate and incubated in darkness for 26 hours, punctuated by 2 minute exposures to full (12,000 lx) light at 5, 10, 15, and 21 hours ("interrupted dark") had NR activity levels comparable or higher than nitrated-exposed tissues in continuous 26 hour darkness (treatment: $F_{4,81}$=46.29, P < 0.0001, Fig. 3).
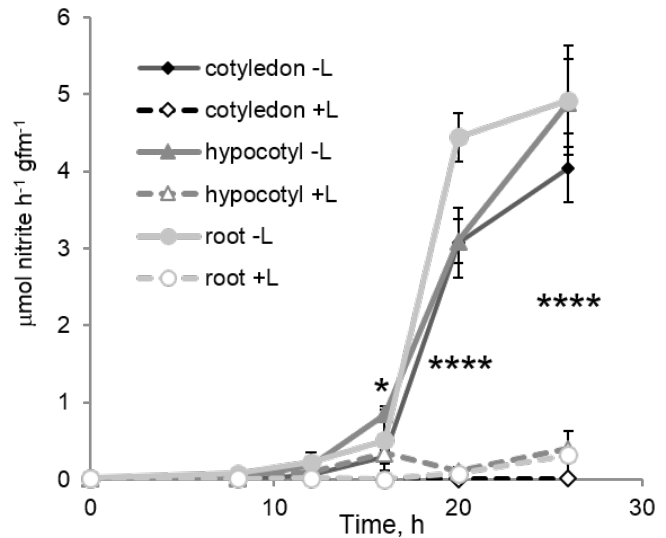
Figure 2. Timing of NR activity in *I. hederacea* 4 d seedling tissues following vacuum infiltration of 10 mM nitrate and incubation in the dark or light. Each point reports the mean of eight measurements; error bar is standard error. Results are representative of three experiments. *P* for difference between light and dark incubated tissues at a given time point: *: P < 0.05, **: P < 0.01, ***: P < 0.001; ****: P < 0.000.
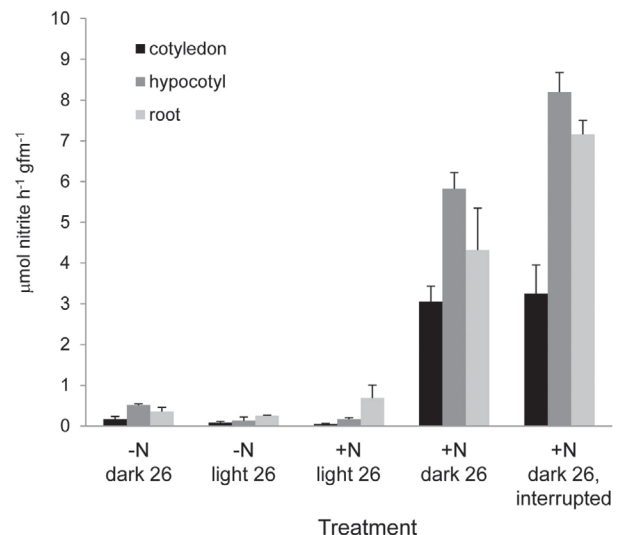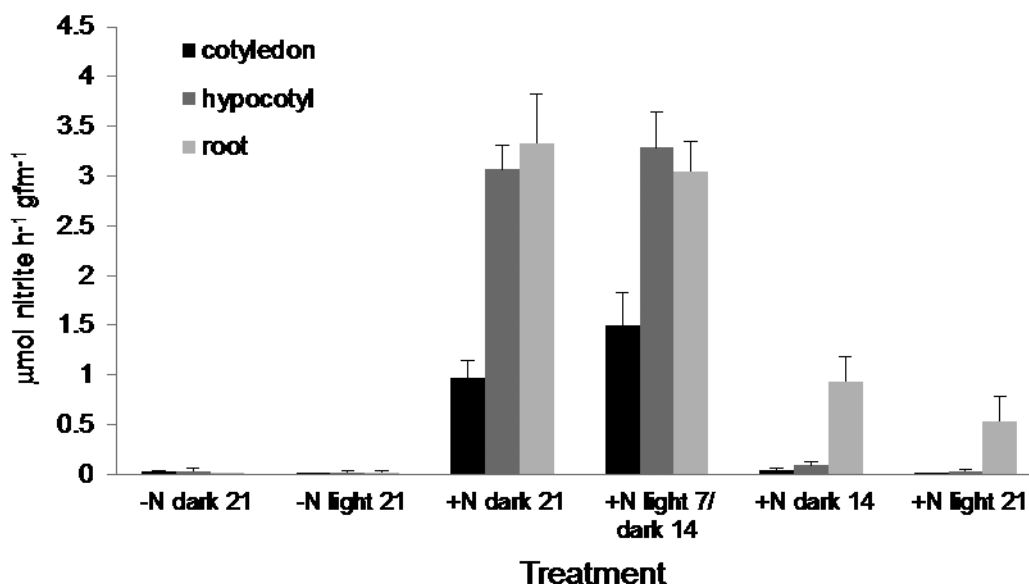

Figure 3. Post-induction effect of light on NR activity. NR activity of 4 d seedling tissues was measured following infiltration with (+N) or without (-N) 10 mM nitrate solution and incubation for a total of 26 h. Column labels indicate the hours of dark or light in the regime; "interrupted" indicates that the tissues were incubated in the dark, and exposed to full light (12,000 lx) for two minute intervals at 5, 10, 15, and 21 hour points. Column height reports the mean of eight measurements with the exceptions of the -N dark and -N light controls, which comprised four each; error bar is standard error. Letters above columns indicate a significant difference at *P* < 0.05 between treatments.

Competing hypotheses could be advanced regarding whether light's role in suppression is in the impairment of nitrate perception or, alternatively, in the blocking of signal transduction events following nitrate perception. To determine whether perception could occur even as light suppresses the response, we looked for evidence of "potentiation," defined in this instance as the capacity of light-exposed tissues to perceive nitrate and show a reduced response time after transfer to darkness, relative to response time of tissues incubated with nitrate in darkness without the initial pre-exposure. Because tissues start to show a strong increase in activity between 12 and 16 h (Fig. 2), we chose 14 h as the post-light incubation time, as it should allow discrimination between a potentiated response and a non-potentiated response. Treatment ($F_{5, 102}$ = 74.55, P < 0.0001), tissue type ($F_{2, 102}$ = 17.79, P < 0.0001) and the interaction between treatment and tissue type ($F_{10, 102}$ = 5.32, P < 0.0001), had a significant effect on NR activity, with cotyledons showing lower NR activity than hypocotyls and roots (Fig. 4). NR activity was not detected in non-induced (-N) treatments in either 21 h light or dark, while strong induction was observed in induced (+N) tissues incubated in the dark for 21 h. Tissues pre-incubated for 7 h in the light with nitrate, and subsequently moved to the dark for the remaining 14 h showed activity comparable to the strong induction observed in induced tissues incubated in the dark for the full 21 h. By contrast, tissues incubated with nitrate in the dark for only 14 h had substantially lower NR activity, comparable to the activity of N-treated, light-suppressed tissues. Thus, during the 14 h dark incubation, tissues that had been pre-treated with nitrate in the light for 7 h were able to develop a greater NR activity than those exposed to nitrate during 14 h darkness alone, an observation consistent with the hypothesis that, though light suppresses the NR activity response, it does not fully prevent them from perceiving the nitrate stimulus.

Impact of light quantity and quality

Plants perceive and are capable of responding to both light quantity and light quality. To examine the potential relationship between NR activity suppression and light quantity, seedling tissues incubated with or without nitrate were exposed to full-spectrum light at a range of intensities modified using shading material. NR activity was affected by light intensity treatment ($F_{5, 126}$ = 55.31, P < 0.0001) and tissue type ($F_{2, 126}$ = 14.31, P < 0.0001) as well as the interaction between these effects ($F_{10, 126}$ = 8,03, P < 0.0001; Fig. 5*A*). Full light (12,000 lx) was effective in suppressing NR activity in all nitrate-exposed tissues, relative to the nitrate-exposed tissues incubated in the dark; light did not function to induce NR activity in non-nitrate-ex-

Figure 4. Potentiated NR activity response during exposure to nitrate during light suppression. NR activity of 4 d seedling tissues was measured following infiltration with (+N) or without (-N) 10 mM nitrate solution and incubation in dark or light. Associated numbers indicate the hours incubated in the light and/or dark. Column height reports the mean of eight measurements with the exceptions of the -N dark and -N light controls, which comprised four each; error bar is standard error. Results are representative of three experiments.

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posed tissues. Under reduced full-spectrum light intensities, roots, and to a lesser extent hypocotyls, showed a recovery of NR induction. At both 250 and 50 lx root tissues showed activity levels not greatly reduced relative to the nitrate-treated roots in the dark, while at these same light levels, hypocotyl NR activity levels, while measurable, were reduced relative to the high levels of NR activity occurring in the nitrate-treated hypocotyls in the dark. Nitrate-exposed cotyledons continued to be very sensitive to light, with only moderately measurable activity occurring even at light levels as low as 50 lx.

Plants employ multiple photoreceptors to sense photon flux in different portions of the spectrum. Full-spectrum light comprises the whole range of visible wavelengths, with each individual wavelength occurring only as a minor fraction. We examined NR activity suppression under single-wavelength illumination in hopes of finding evidence for the participation of specific photoreceptors in the suppressive mechanism. Each single-wavelength treatment was conducted as a separate experiment, and each included its own negative, induction, and suppression controls, to which the nitrate- and single-wavelength-exposed samples were compared. Similarly to full-spectrum light, NR activity was reduced as light intensity increased (red: $F_{7, 132} = 87.26$, $P < 0.0001$; green: $F_{6, 123} = 36.90$, $P < 0.0001$; blue: $F_{7, 156} = 153.20$, $P < 0.0001$) and dependent on tissue (red: $F_{2, 132} = 46.53$, $P < 0.0001$; green: $F_{2, 123} = 3.26$, $P = 0.04$; blue: $F_{2, 156} = 35.33$, $P < 0.0001$). Tissue NR activity responded differently to red, green, and blue light intensities (treatment-by-tissue type interaction: red: $F_{14, 132} = 14.86$, $P < 0.0001$; green: $F_{12, 123} = 1.16$, $P = 0.32$; blue: $F_{14, 156} = 14.18$, $P < 0.0001$; Fig. 5B-D). In the absence of nitrate, illumination with red (600 nm), green (525 nm), or blue (470 nm) wavelengths did not induce NR activity in seedling tissues to a level distinguishable from activity in seedlings incubated in the dark without nitrate. All single wavelengths were effective in reducing NR activity induction in the presence of nitrate relative to dark-incubated tissues receiving nitrate. Separately conducted experiments with each wavelength showed what appeared to be inherent variability between and within sample sets and tissue types. As such, representative results are presented, and we are cautiously circumspect about the relative potency of the wavelengths, simply noting that all three (red, green, and blue) were potent in suppression at low flux in some or all tissues.

Light suppression persists in primary growth tissues

Glasshouse-grown seedlings were used to determine whether light-mediated suppression of nitrate-induced NR activity could be observed in epicotyl tissues (leaves) or cotyledons at an advanced age, and whether the phenomenon would persist under ambient day-night cycles
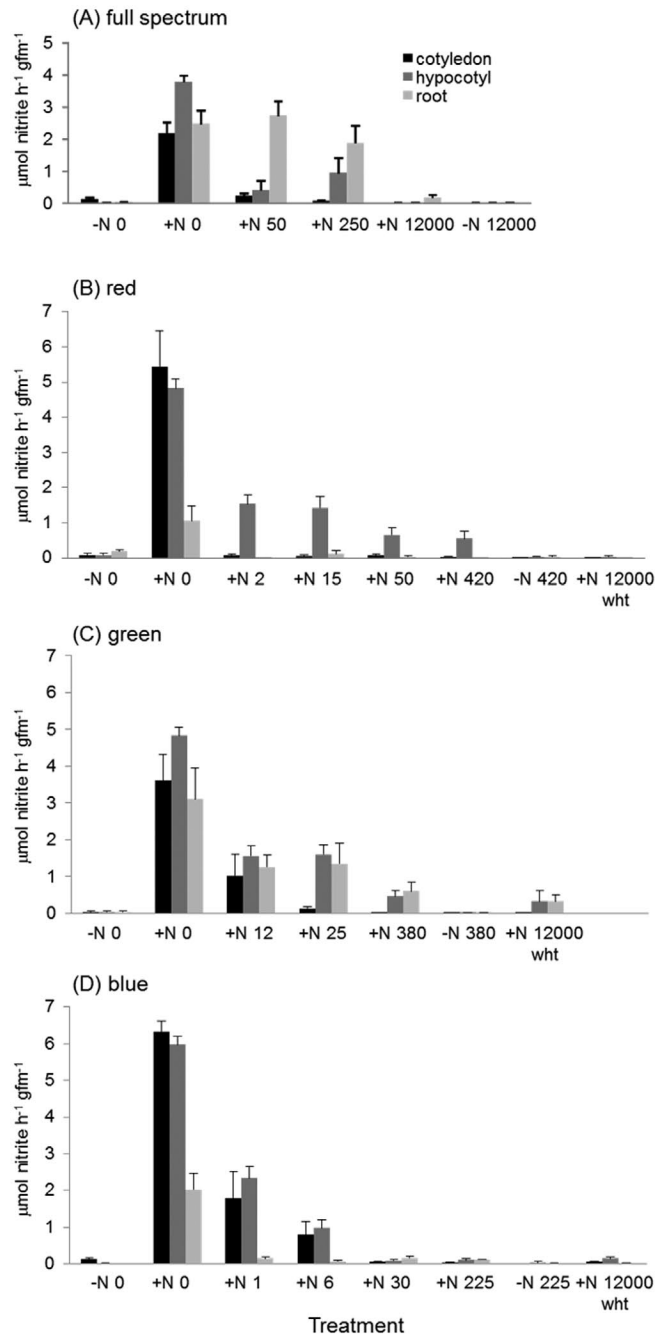
Figure 5. Impact of light quantity and wavelength on NR activity induction by nitrate. NR activity of 4 d seedling tissues was measured 22 h following infiltration with (+N) or without (-N) 10 mM nitrate solution incubated in the dark (0) or to different intensities of full-spectrum (A), red (B), green (C), or blue (D) light. Associated numbers report measured luminous flux (lx) incident on the samples during the experiment; "wht" indicates the full-spectrum suppression control in panels (B)-(D). Column height reports the mean of eight measurements with the exceptions of the -N dark and -N light controls, which comprised four each; error bar is standard error. Results are representative of two experiments for each panel.
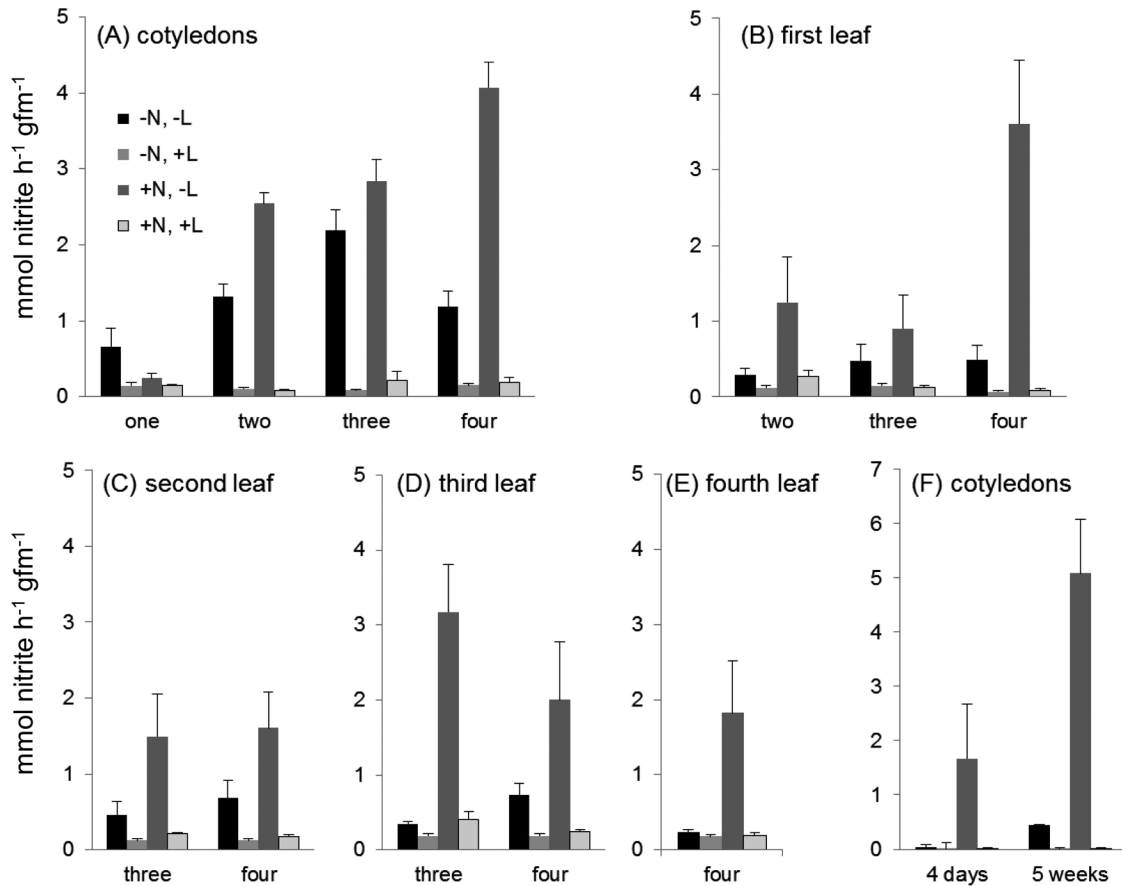
Figure 6. Impact of seedling age on cotyledon and leaf tissue NR activity responsiveness to nitrate and light under glasshouse conditions, and in rejuvenated cotyledons. NR activity of glasshouse-grown seedling cotyledons (A) and first, second, third and fourth leaves (B-E, respectively) was measured 22 h following infiltration with (+N) or without (-N) 10 mM nitrate solution and incubation in dark (-L) or light (+L); individual organs were quartered (or halved, in the case of cotyledon pairs) and resulting quarters exposed to each of the four treatments. Category labels indicate whole plant age in weeks. Column height reports the mean of ten measurements; error bar is standard error. Results are representative of two experiments. Impact of cotyledon rejuvenation resulting from epicotyl removal in laboratory grown seedlings on NR activity responsiveness to nitrate and light was compared to 4 d seedling cotyledons (F). Category labels indicate whole plant age in days or weeks. Column height reports the mean of eight measurements; error bar is standard error. Results are representative of two experiments.

that might entrain circadian NR regulatory patterns. Further, these observations would serve to show the occurrence of the light suppression phenomenon in soil-grown plants as opposed to seedlings grown on a soil-less medium. Seedlings were established in soil at one week intervals. Induction and suppression was monitored only in organs (cotyledons or leaves) that had expanded sufficiently to permit quartering so that each organ could be tested in each of the four standard treatments. In the glasshouse, cotyledons expanded and persisted through the fourth week and then yellowed and senesced. The first leaf expanded to sufficient size during the second week; the second and third leaves became available during the third week, and the fourth leaf became available

during the fourth week. By the end of the fourth week, the plant had initiated a twining growth habit; subsequent leaves were present, but were not sufficiently expanded and were not tested.

In cotyledons of glasshouse-grown seedlings the application of nitrate generally served to enhance NR activity induction during weeks two, three, and four (nitrate treatment: $F_{1, 144} = 45.68$, $P < 0.0001$; nitrate-by-age interaction: $F_{3, 144} = 17.16$, $P < 0.0001$). NR activity was measurable in cotyledons incubated in the dark, even without nitrate amendment (light treatment: $F_{1, 144} = 436.51$, $P < 0.0001$; Fig. 6A). This level of activity, relative to light-treated tissues increased to higher levels in subsequent weeks (age: $F_{3, 144} = 36.66$, $P < 0.0001$; light-by-age interaction: $F_{3, 144} = 35.55$, P

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< 0.0001) and was comparable to levels observed in nitrate-treated seedling tissues (compare with Fig. 1*C*). By contrast, incubation of cotyledons under light, whether with or without nitrate, had the effect of suppressing NR activity induction (nitrate-by-light interaction: $F_{1, 144}$ = 39.84, P < 0.0001). Light suppression of nitrate-mediated NR induction was evident in the first, second, third, and fourth leaves (Fig. 6 *B-E*). By two weeks, and to an even greater extent at four weeks, the first leaf showed both induction of NR activity by nitrate in the dark, and strong suppression when this incubation occurred in the light (light treatment: $F_{1, 108}$ = 38.02, P < 0.0001; age: $F_{2, 108}$ = 0.93, P = 0.40; light-by-age interaction: $F_{2, 108}$ = 5.32, P = 0.006; nitrate treatment: $F_{1, 108}$ = 11.09, P = 0.001; nitrate-by-age: $F_{2, 108}$ = 1.88, P = 0.16; nitrate-by-light interaction: $F_{1, 108}$ = 5.49, P = 0.02). Though the absolute levels of induction differed, evidence of retained sensitivity to light persisted through the fourth leaf (light treatment: 2nd leaf: $F_{1, 72}$ = 41.48, P < 0.0001; 3rd leaf: $F_{1, 72}$ = 89.92, P < 0.0001; 4th leaf: $F_{1, 36}$ = 14.04, P = 0.0006) suggesting that the mechanism of light-mediated NR activity suppression is not limited to tissues derived immediately from the embryo. Efforts to characterize the effect of light, whether inductive or suppressive, in field-grown plants yielded equivocal results (unpublished data). These observations are addressed in the following discussion.

Persistence of light suppression of NR activity in "rejuvenated" cotyledon tissue

Under glasshouse conditions, cotyledons yellowed and senesced after four weeks and could not be used for subsequent induction or suppression experiments. We were interested in knowing whether cotyledons whose persistence was artificially lengthened through removal of the epicotyl -- a method termed "rejuvenation" (Skadsen and Cherry, 1983) -- would change their responsiveness to either nitrate or light. Seedlings were established in soil and grown in the laboratory for five weeks. During this time, primary growth initiating from the apical meristem was removed using a sharp needle. This process was repeated as necessary, as growth from established axillary buds was initiated. After five weeks the cotyledons remained healthy and green instead of senescent. The responsiveness of these "rejuvenated" cotyledons was compared to the responsiveness of one-week old cotyledons grown under the same conditions. Both one-week old and five-week old rejuvenated cotyledons showed sensitivity to light, with all activity reduced to levels not distinguishable from background when incubated in light ($F_{1, 56}$ = 327.14, P < 0.0001; Fig. 6*F*). When incubated in the dark without nitrate, five-week old rejuvenated cotyledons showed a moderate amount of NR activity, and a strong induction of NR activity when incubated in the dark with nitrate (nitrate treatment: $F_{1, 56}$ = 123.27, P < 0.0001; nitrate-by-light interaction: $F_{1, 56}$ =

110.01, P < 0.0001). Both measurements showed activity levels higher than the same treatments performed with one-week old cotyledons from plants grown under the same conditions (age: $F_{1, 56}$ = 39.81, P < 0.0001; age-by-nitrate interaction: $F_{1, 56}$ = 0, P = 0.95; age-by-nitrate-by-light interaction: $F_{1, 56}$ = 0.12 P =0.74; Fig. 6*F*).

Discussion

The seedlings of *Ipomoea hederacea* (ivyleaf morning glory) provided a facile system for experimentation on the regulation of nitrate reductase (NR) activity in embryonically-derived tissue; the seeds germinated rapidly and uniformly, the seedlings grew quickly (Fig. 1*A*) and demonstrated inducible NR activity *in vivo* in response to a range of nitrate concentrations (Fig. 1*B*). Seedling cotyledons, hypocotyls, and roots infiltrated with phosphate buffer regularly showed little or no measurable NR activity. By contrast, tissues infiltrated with phosphate buffer with nitrate developed distinguishable NR activity in cotyledons, hypocotyls, and roots at concentrations as low as 0.1 mM, and stronger induction at higher concentrations, after an initial delay of 12 or more hours (Figs. 1*B* and 2). Activity increased rapidly after this time, typically ranging from 1 to 3 µmol nitrite h^{-1} gfm^{-1}, and though we measured activity in tissues incubated for up to 26 hours, it is possible that the activity may have continued to increase given additional time. While high levels of NR activity were induced in all tissue types in the dark, incubation of these tissues in the light during the same period reduced or eliminated detectable NR activity (Figs. 1*C* and 2).

Interruption of dark incubation with brief exposures to light did not affect suppression (Fig. 3), suggesting that the mechanism of suppression is not one that "purges" the perception of nitrate, thus requiring the subsequent passage of time for the nitrate stimulus to re-accumulate. Tissues exposed to nitrate in the light, and that were subsequently moved in to the darkness, showed higher levels of NR activity after 14 hours of darkness than tissues that were exposed only to nitrate in darkness for 14 hours (Fig. 4). This difference suggests that, while light suppressed the accumulation of NR activity, it did not prevent nitrate perception. As a consequence, the tissues were able to accumulate NR activity more rapidly following the move to darkness; these tissues appeared to have been potentiated toward this more rapid response in a fashion comparable to the accelerated defensive response in plants that have been systemically sensitized to the presence of a pathogen threat (Conrath, Pieterse and Mauch-Mani 2002). Mechanistically, this suggests that the influence of light in suppressing NR activity induction does not occur at the initial nitrate perception event, but at a subsequent stage in perception and transduction, and that the accelerated response occurs possibly as the result of an accumulation of a signaling intermediate.

The suppressive effect of light was not limited to high light levels; rather, full-spectrum light was capable of a suppressive effect at fluences as low as 50 lx (Fig. 5*A*) in cotyledon tissue. All tissues demonstrated sensitivity to full spectrum "white" light, and to single wavelength red (630 nm), green (525 nm), and blue (470 nm) light sources (Figs. 5*B-D*); cotyledons generally showed the greatest sensitivity as determined by the extent to which NR activity was reduced relative to dark-incubated controls. Rajasekhar, Gowri and Campbell (1988) implicated phytochrome in the regulation of NR activity in etiolated squash cotyledons, noting the photoreversibility of red light induction by subsequent far red treatment. In our study -- which did not use etiolated plants -- the sensitivity to different single-wavelength sources suggests either participation of multiple photoreceptors, or else a single perceptive mechanism that does not discriminate between incident wavelengths.

Cotyledons of glasshouse soil-grown seedlings under ambient day/night light cycles showed higher levels of NR activity in cotyledons infiltrated with phosphate (Fig. 6 *A-E*) than laboratory-grown seedlings. However, these tissues continued to show light-mediated NR activity suppression, up to the fourth leaf, showing that light-mediated NR suppression is not limited to embryonically derived tissues. In addition, cotyledons made to persist for an artificially long time showed light suppression at five weeks (Fig. 6*F*), a time by which cotyledons would typically have undergone senescence. These results suggest that light-mediated NR activity suppression might be a significant phenomenon even as the plant matures. We have attempted comparable determinations in field-grown (non-cultivated) tissues of *I. hederacea* at different times during the growing season: in separate experiments, light had an enhancing effect, a suppressive effect, and no effect on nitrate-induced NR activity levels in leaf tissues. As such, we are not prepared to extend our interpretation beyond laboratory- and glasshouse-grown plants; rather we are currently undertaking studies on *I. hederacea* grown under controlled field conditions to determine whether factors such as plant maturity, tissue age, soil fertility, or seasonal conditions can be demonstrated to impact light-mediated regulation of NR activity. Further, these studies will attempt to determine whether there occurs measureable genetic diversity within *I. hederacea* for the light suppression mechanism, as quantitative trait loci have recently been described as influencing NR activity responsiveness in maize (Morrison, Simmons and Stapleton 2010). The impact of plant maturation on NR activity has been noted; reviewing the state of knowledge of signal transduction cascades mediating light enhancement of nitrate metabolism, Lillo (2008) discerned perceptive mechanisms and signaling pathways at work during early stages of seedling development as distinct from those active during later stages of plant maturation, citing as an example post-translational mechanisms modulating circadian changes in NR activity.

Our demonstration of light suppression of NR activity in seedlings contrasts with reports by other investigators; Beevers *et al.* (1965), for example, showed that nitrate induced radish seedling tissue NR activity in the dark, and that light enhanced the nitrate-dependent NR induction. The authors observed a parallel increase in tissue nitrate content and NR activity after application of nitrate solution to intact seedlings, and nitrate accumulation would therefore have been a function of the rate of nitrate uptake at the roots and movement in the transpiration stream. By contrast, our experiments employed explant tissues, and treatment solutions were delivered directly to the apoplastic spaces through vacuum infiltration; Fig. 1*C* illustrates the reduced degree of NR activity induction observed when tissues were not vacuum infiltrated. Our system is the more artificial of the two, removing both the potential influence of the intact plant system and circumventing the natural rate of nitrate uptake and transport in the plant, and associated regulatory mechanisms. Despite this artificiality, our methods demonstrate a heretofore minimally explored aspect of NR activity regulation: a mechanism by which light can suppress, rather than enhance NR activity.

The physiological significance of light suppression of NR activity is not immediately clear; subsequent experiments will be designed to test whether the suppressive effect can be detected under less artificial conditions. It is conceivable that NR activity suppression by light occurs only under conditions comparable to our current method, and thus reveals a connection between light perception and signaling and nitrogen metabolism that would not typically contribute to plant function. Alternatively, it may be proposed that light's negative impact is physiologically genuine, but that its effect is less pronounced under typical growth conditions, and possibly occurs in conjunction with, or is modified by, other signals. It will be of interest to determine whether a similar suppression can be demonstrated with intact plant systems, which are more commonly used in NR regulation studies; if not, it may suggest that the suppressive mechanism is itself part of, or affected by, a larger integrative scheme through which other signals, such as overall plant nitrogen status, communicated either locally or systemically, serving to prevent NR activity suppression.

Known mechanisms mediating the influence of light on plant NR activity include light perception by phytochrome, acting by way of HY5 and similar proteins, and the sensing of products generated through photosynthesis (Lillo 2008). The potency of red, green, and blue wavelengths in suppressing NR activity prevents us from attributing the light perception event to a single photo-

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receptor; however the observation that strong suppression is observed in non-green tissues argues that suppression is not a function of functional photosystems or their products. Beyond perception, it will be interesting to determine whether the suppressive effect of light is mediated by an entirely distinct mechanism operating through a separate signaling pathway, or through a similar or derived pathway whose effect has either been modified, or is conditional upon plant age, developmental stage, or environmental conditions. The use of the *in vivo* NR activity assay did not permit the clear discrimination of the point at which activity suppression occurred; the goal of subsequent works will be to determine whether the light suppression acts at the level of gene expression, transcript abundance, NR protein abundance, or post-translational modulation of NR activity. Comparison of immunodetectable NR protein levels in induced and suppressed tissues will provide a clue; in addition, as phosphorylation-mediated suppression at the protein level is dependent upon the availability of Mg^{2+} (Huber *et al.* 1992a), the employment of an *in vitro* assay to compare NR activity levels, with or without Mg^{2+} sequestration, may help to determine if light suppression is mediated by post-translational modifications.

In conclusion, we have provided evidence for a connection between plant light perception and a mechanism by which nitrate-induced NR activity is suppressed. While the physiological significance of this connection remains to be established, it is nevertheless important to explore the possible ramifications in order to have a more thorough appreciation of nitrogen nutrition and its regulation in plants. Efforts to improve plant nitrogen use efficiency have focused on different physiological functions, including transport, assimilation, partitioning, and the regulation of each. As such, researchers have been encouraged to adopt a systems biology approach that integrates the best understanding of these processes (Gutiérrez 2012). Further examination of the suppressive effects of light on NR activity might reveal additional unexpected nuances in plant nitrogen metabolic physiology.

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