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Circadian and light-regulated expression of nitrate reductase in *Arabidopsis*

Marsha L. Pilgrim [1], Timothy Caspar [2,3], Peter H. Quail [2] and C. Robertson McClung [1,*]
[1] *Department of Biological Sciences, Dartmouth College, 6044 Gilman Laboratory, Hanover, NH 03755-3576, USA (*author for correspondence);* [2] *UC-Berkeley/USDA Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710, USA;* [3] *Present address: Du Pont Central Research and Development, P.O. Box 80402, Wilmington, DE 19880-0402, USA*

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Abstract

The expression of a number of plant genes is regulated by an endogenous circadian clock. We report that the *Arabidopsis NIA2* (nitrate reductase) gene shows robust circadian oscillations in mRNA accumulation which persist for at least 5 days in plants that have been grown in a light-dark (LD) cycle and then transferred to continuous light (LL). We further show that *NIA2* mRNA accumulation oscillates in a circadian fashion in plants that have been grown in LD and then transferred to continuous darkness (DD). Results from nuclear run-on transcriptional analysis suggest that the oscillations in steady-state levels of *NIA2* mRNA abundance are not primarily due to changes in transcription but, instead, reflect post-transcriptional regulation. The circadian oscillations in *NIA2* mRNA abundance are paralleled by circadian oscillations in nitrate reductase enzyme activity (NR activity) in *Arabidopsis* plants that have been grown in LD and then transferred either to DD or to LL. Etiolated *Arabidopsis* seedlings express neither *NIA2* mRNA nor NR activity. However, both *NIA2* mRNA accumulation and NR activity are induced by exposure to white light. The inductive effects of light on *NIA2* mRNA accumulation are due, at least in part, to a very low fluence phytochrome-mediated response. However, the persistence of circadian oscillations in *NIA2* mRNA abundance for at least 5 days in LL demonstrates that the circadian clock is capable of overriding or gating the inductive effects of light on *NIA2* mRNA accumulation in *Arabidopsis* for an extended, continuous period of time.

Introduction

Circadian rhythms are ubiquitous among higher organisms and are defined as rhythms in biological function whose period length is approximately 24 h. These rhythms persist in the absence of environmental time cues and are therefore thought to reflect regulation by an endogenous pacemaker. Circadian rhythms have been documented in a variety of plant species, and the rhythmic activities observed cover a broad spectrum from leaf movement and CO_2 conductance to regulated gene expression and protein phosphorylation [39, 53]. Although well documented, circadian

regulation is poorly understood at the molecular level. The identification of genes controlled by the biological clock and subsequent definition of their clock-responsive regulatory sequences are important first steps in the characterization of rhythmic responses at the gene level. Many of the genes shown to be regulated by a biological clock in plants encode products involved in photosynthesis such as the chlorophyll *a/b*-binding proteins (reviewed in [31]) and the small subunit of Rubisco [1, 25, 45, 46]. In some species, circadian expression has also been demonstrated for genes whose products have non-photosynthetic roles, such as the *Cat3* (catalase) gene in maize [49].

We have chosen to examine the circadian regulation of nitrate reductase in *Arabidopsis thaliana*. Nitrate reductase (NR) is the first committed enzyme in the nitrate assimilation pathway, and probably represents the rate limiting step in this process. The primary regulator of nitrate reductase activity is its substrate, nitrate [9, 16, 28]. Other factors, particularly those related to photosynthesis, such as light, CO_2, and sugar, strongly influence nitrate reductase expression [4, 12, 16, 20, 28, 56]. In addition to these exogenous regulators, an endogenous circadian clock regulates gene expression, protein accumulation, and enzyme activity of nitrate reductase in some plant species, including barley [36], tobacco [20] and *Chenopodium rubrum* [15].

Arabidopsis thaliana has two nitrate reductase genes, *NIA1* and *NIA2* [14, 18]. Both *NIA1* and *NIA2* mRNA abundance show diurnal oscillations, with peak abundance occurring shortly after dawn, in plants growing in a light-dark (LD) photoperiod [13]. These oscillations persist for one circadian cycle in plants transferred to continuous light [13]. In this report, we confirm the circadian oscillation in *NIA2* mRNA abundance in plants transferred from LD to continuous light (LL), and we extend the observations of Cheng *et al.* [13] to show that the oscillation in *NIA2* mRNA abundance persists for at least 5 continuous cycles in LL, demonstrating the robustness of the circadian oscillator. We also show that a circadian oscillation in *NIA2* mRNA abundance occurs in plants that have been grown in LD and then transferred to continuous darkness (DD). Previous studies have described oscillations in steady-state *NIA2* mRNA abundance but have not addressed the relative contributions of transcriptional and post-transcriptional mechanisms of regulation. Here, we show that the oscillations in *NIA2* mRNA do not appear to be a result of transcriptional regulation, as evidenced at the level of nuclear run-on analysis. The circadian oscillations in *NIA2* mRNA abundance are paralleled by circadian oscillations in nitrate reductase enzyme activity in both DD and LL. Finally, we demonstrate that etiolated *Arabidopsis* seedlings do not express detectable nitrate reductase activity, but that NR activity, as indicated by chlorate sensitivity, is induced by exposure to white light. We show that the induction of *NIA2* mRNA abundance in etiolated seedlings in response to light is due at least in part to the effects of a very low fluence phytochrome response.

Materials and methods

Plant materials

The Columbia ecotype of *Arabidopsis thaliana* was used for all circadian experiments. Plants were sown on potting mix (Pro-Mix BX, Experimental Brands, Stamford, CT) and were grown for five weeks under a 14:10 photoperiod. Light intensity during the day phase of the LD cycle was 126 μmol s^{-1} m^{-2}. Throughout the duration of the experiments, plants were irrigated with a nutrient solution containing 5 mM potassium nitrate pH 5.5, 50 mM potassium phosphate, 2 mM $MgSO_4.7H_20$, 2 mM $Ca(NO_3)_2.4H_2O$, 50 μM FeEDTA, plus 1 ml/liter of micronutrient solution consisting of 70 mM H_3BO_3, 14 mM $MnCl_2$, 0.5 mM $CuSO_4$, 1 mM $ZnSO_4$, 0.2 mM $NaMoO_4$, 10 mM NaCl and 0.01 mM $CoCl_2$.

For all light regulation experiments, including effects of chlorate treatment, the Landsberg *erecta* ecotype was used. Seeds were sterilized and sown onto media containing 0.5 × Murashige-Skoog salts (Gibco-BRL, Gaithersburg, MD) pH 5.7, 0.7% agar and germination was induced as pre-

viously described [11]. The light sources used for the irradiation of plants in the light regulation experiments provided 0.55 W/m^2 for the red source, 2.3 W/m^2 for the far-red source and 1.5 W/m^2 for the white light source [37].

For the chlorate experiments, sterilized *Arabidopsis* seeds were plated onto media containing 0.8% agar, 2.5 mM potassium phosphate pH 5.7, and either $NaClO_3$ or NaCl at the indicated concentrations. The seeds were stratified at 4 °C for 1 day in the dark, and germination was induced by dim white light treatment (30 min) before being placed into either continuous darkness or a 16:8 photoperiod (white light from fluorescent tubes of 800 $\mu W/cm^2$) at 22 °C. The seedlings were analyzed after 7 days.

RNA preparation and analysis

For all circadian experiments, leaf tissue was harvested in either white light or complete darkness, depending on the light regime, and frozen in liquid nitrogen. Total RNA was prepared from the frozen tissue [46]. For northern analysis, samples (10 μg) of total RNA were separated by electrophoresis in formaldehyde agarose gels and then transferred to nylon membranes (BioTrans; ICN, Irvine, CA) in 20 × SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0). For slot blot analysis, samples (1 μg per well in Fig. 3, 3 μg per well in Fig. 2) of total RNA were applied to membranes of reinforced nitrocellulose (Schleicher and Schuell, Keene, NH) using a Minifold II apparatus (Schleicher and Schuell) according to the manufacturer's instructions. RNA was bound to the membranes either by baking at 80 °C for 2 h or by UV crosslinking (Stratalinker; Stratagene, La Jolla, CA). Plasmid inserts were isolated by agarose gel electrophoresis and labelled with the Klenow fragment of DNA polymerase by the random primer method [23]. An *Arabidopsis NIA2* probe was generated by labelling a 1.6 kb *Bam* HI fragment of pAtc-46 ([18]; a gift from N. Crawford). The soybean actin (*SAC3*) probe was generated by labelling a 3.0 kb *Hin*d III fragment of pSAc3 ([51]; a gift from R. Meagher). An *Arabidopsis* α-tubulin (*TUA3*) probe was generated by labelling a 1.8 kb *Eco* RI fragment of pAtαtub [38]; a gift from P. Snustad). Membranes were prehybridized at 42 °C in 0.01 M PIPES, 0.8 M NaCl, 0.01% sarkosyl, 0.01% Ficoll 400, 0.01% polyvinylpyrrolidone, 0.01% bovine serum albumin, 200 μg/ml salmon sperm DNA, 50% formamide and hybridized at 42 °C in the same solution plus 10% (w/v) dextran sulfate to which DNA probes were added to give 2×10^6 cpm/ml. Membranes were manually rubbed in 2 × SSC plus sodium pyrophosphate (0.2 mg/ml) and sarkosyl (0.5 mg/ml), and then washed at 50 °C in 4 changes of 0.1 × SSC plus sodium pyrophosphate (0.1 mg/ml) and sarkosyl (0.5 mg/ml), for a total of 2 h. Following autoradiography, mRNA abundance was quantified using a MasterScan densitometer (Scanalytics, Billerica, MA). To allow for reprobing, filters were stripped in 0.1 × SSC plus sodium pyrophosphate (0.1 mg/ml) and sarkosyl (0.5 mg/ml) at 90 °C for 30 min.

For all light regulation experiments, seedlings were harvested and RNA was prepared as previously described [11]. Filters were prehybridized and hybridized as described [37] except that the Denhardt's reagent was left out of the prehybridization mix and both the prehybridization and hybridization were incubated at 65 °C. ^{32}P-labelled antisense hybridization probes were synthesized as previously described [11]. *NIA2* DNA used in the antisense reaction was generated by PCR amplification of the *Arabidopsis NIA2* gene using Landsberg *erecta* genomic DNA as the substrate. Ribosomal DNA probes were synthesized by random priming (Amersham, Arlington Heights, IL) using pHA2 and pUCXho3.0 which contain the pea nuclear and chloroplast ribosomal RNA genes [21]. After hybridization, the filters were washed as described [11]. Relative transcript levels were quantified by scanning densitometer as previously described [11]. Filters were stripped to allow reprobing by incubating in 2% SDS at 100 °C for 30 min.

Nuclear run-on analysis

Nuclei were isolated in conditions of either white light or dim green safe-light, depending on the

light regime, at 3 h intervals from leaf tissue of *Arabidopsis* plants as described [22]. Nuclear run-on transcription assays were performed on these nuclei as described [2]. DNA probes were generated by linearizing pAtc-46 (containing the *Arabidopsis NIA2* gene [18]), pMLP-1 (containing the *Arabidopsis CAB1* gene (pMLP-1 was made by ligating a 2.75 kb *Eco* RI-*Pst* I fragment containing the *Arabidopsis CAB1* (AB140) gene [35]; a gift from E. Tobin) into SK^- Stratagene; La Jolla, CA), and pARR16 (containing the *Arabidopsis* rDNA gene [50]; a gift from F. Ausubel). Linearized DNAs were slot-blotted (5 μg per slot) onto nitrocellulose membrane (Schleicher and Schuell) using a Minifold II apparatus (Schleicher and Schuell). Hybridization and washing conditions were as described [2]. Hybridizable counts were quantified in liquid scintillant (National Diagniostics, Somerville, NJ).

Enzyme assays

Leaf tissue was harvested in either white light or total darkness, depending on the light regime, and frozen in liquid nitrogen. Protein extracts were prepared as described [18] and assayed for nitrate reductase enzyme activity as described [17]. Protein concentrations were determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) according to the manufacturer's instructions.

Results

A circadian clock regulates nitrate reductase mRNA abundance

Wild-type *Arabidopsis thaliana* was grown for five weeks in a 14 h light/10 h dark (LD) photoperiod. Tissue was harvested at timed intervals from plants that were maintained in the LD cycle and from plant that had been grown in LD and then transferred to either continuous darkness (DD) or continuous light (LL). Total RNA was extracted and analyzed by northern and slot blot analysis using a coding sequence probe for nitrate reductase (*NIA2*) and, as controls, coding sequence probes for actin (*SAC3*) and α-tubilin (*TUA3*). To facilitate comparison of experiments conducted under different LD regimes, all times are expressed Zeitgeber time (ZT). Zeitgeber, literally time giver, refers to environmental signals, such as the dark-to-light transition, that reset the circadian clock. ZT, then, represents the hours since the onset of light [59]. Circadian oscillations in *NIA2* mRNA abundance were observed with northern blot analysis (Fig. 1). In plants that were grown and maintained in LD (Fig. 1A), a strong peak in *NIA2* mRNA abundance appears at ZT1. However, note that increased *NIA2* mRNA abundance is evident at ZT23, prior to the onset of light. The accumulation of *NIA2* mRNA in anticipation of dawn (lights on) clearly demonstrates that the increase in *NIA2* mRNA does not simply represent an inductive response to light. Rather, this pre-dawn accumulation,

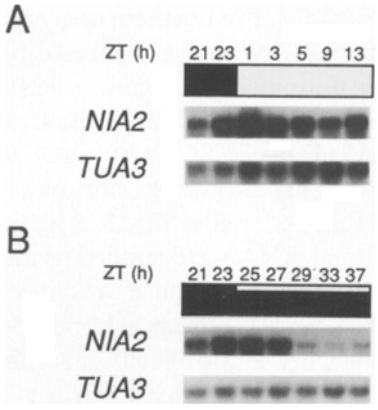

Fig. 1. Northern blots showing circadian regulation of *NIA2* mRNA accumulation. Northern blots containing 10 μg per lane of total RNA from five week old *Arabidopsis* plants were probed with the *NIA2* gene, then stripped and reprobed with an α-tubulin gene (*TUA3*). A. Control light condition showing RNA prepared from plants that were grown in a 14 h light/10 h dark (14:10) photoperiod. Bar above blots indicates the light regime, where the filled area indicates dark, and the open area indicates light. Zeitgeber time (hours after the onset of light) is indicated above the bar. B. RNA prepared from plants that were grown in a 14:10 photoperiod, and then transferred to continuous darkness. Bar above blots indicates light regime, where the filled area indicates dark. Small open inset within the filled bar indicates the subjective day period.

termed dawn anticipation, of mRNA is consistent with regulation by the circadian clock. To confirm that the circadian clock regulates *NIA2* mRNA accumulation, we transferred LD-grown plants to DD (Fig. 1B). At the time when the lights would have come on had the LD regime been maintained (subjective day), *NIA2* mRNA accumulates to maximal levels. Thus, the oscillation in *NIA2* mRNA accumulation results from endogenous control by a circadian clock, rather than in response to exogenous light cues. The accumulation of *NIA2* mRNA at ZT 23 is the DD equivalent of dawn anticipation and the peak observed at ZT 25, during the subjective day, correlates with the ZT 1 peak in LD. No apparent peaks or evidence of dawn anticipation is seen when these northern panels were probed with a control probe, *TUA3*, although minimal fluctuations are observed and probably reflect slight differences in RNA loading. Thus, there appears to be circadian regulation of *NIA2* in LD and DD conditions.

For better quantification, the RNA preparations used in Fig. 1 were analyzed with slot blots and the resulting autoradiograms scanned using a densitometer. Graphic representation of the results [*NIA2* values were normalized to actin *SAC3*-hybridizing values] are shown in Fig. 2. Circadian oscillations in *NIA2* mRNA abundance occurs in plants that have been grown in LD (Fig. 2A) and in plants that have been grown in LD and then transferred to DD (Fig. 2B, where the last day in LD is shown followed by the first day of DD), confirming the results shown in Fig. 1. Quantitatively, the amplitude of the oscillation in *NIA2* mRNA abundance ranges from 1.7-fold to 2.2-fold in LD conditions (Fig. 2A). In plants that have been grown in LD and then transferred to DD, the amplitude of the *NIA2* mRNA abundance oscillation is 1.9-fold (Fig. 2B, second cycle). The results for plants that were grown in LD and then transferred to LL are shown in Fig. 2C, where the last day in LD is shown followed by the first day in LL. In plants

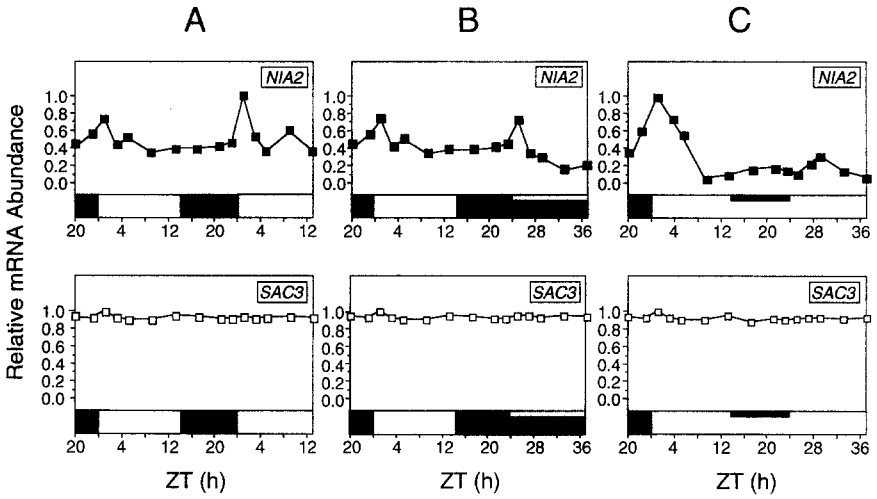

Fig. 2. Quantification of slot blot results showing circadian regulation of *NIA2* mRNA accumulation. Slot blots containing total RNA from five week old *Arabidopsis* plants were probed with the *NIA2* gene, then stripped and reprobed with an actin gene (*SAC3*). Quantification of the resulting autoradiographs was by scanning densitometer. Results are shown for plants that were grown and maintained in a 14:10 photoperiod (A), grown in a 14:10 photoperiod and then transferred to continuous darkness (B), or grown in a 14:10 photoperiod and then transferred to continuous light (C). The *NIA2* values represent the amount of *NIA2* mRNA relative to the amount of actin (*SAC3*-hybridizing) RNA present at each time point. The highest value obtained for actin mRNA accumulation was defined as 1.0 unit of mRNA accumulation. *NIA2* results are represented by filled square symbols, and *SAC3* results are represented by open square symbols. Light regimes are indicated by the bars within the graphs. Filled bars indicate dark, open bars indicate light, and small inset bars indicate subjective day (open) and subjective night (filled). Zeitgeber time is indicated below the bars.

transferred from LD to LL, a low level of *NIA2* mRNA is detectable throughout the subjective night, even though the lights remained on. During the first day in LL, the peak in *NIA2* mRNA accumulation appears to be at ZT 29. The amplitude of the oscillation in LL is 3-fold. In all three light conditions, actin mRNA accumulation shows minimal fluctuations with no apparent circadian regulation (Figs. 2A, B, and C).

Persistence of a rhythm throughout multiple cycles in the absence of environmental time cues is one of the hallmarks of circadian regulation. We decided to examine the persistence of the *NIA2* mRNA accumulation rhythm during a period of extended LL; Plants were grown as described above and tissue was harvested at 4 h intervals for 6 days in LD and LL conditions. Graphic representation of slot blot results are shown in Fig. 3 (*NIA2* values were normalized to actin values). In LD conditions (Fig. 3A), daily peaks in *NIA2* mRNA accumulation are observed. The peaks on days one and six correspond to ZT 20 while the remaining peaks correspond to ZT 0 (tissue was not harvested at ZT 1 in this experiment), and in each case clear evidence of dawn anticipation can be seen. Daily peaks in *NIA2* mRNA accumulation can also be seen in plants that have been transferred to LL (Fig. 3B). On day 1 of the experiment (which represents the last day in LD), the peak in *NIA2* mRNA accumulation occurs at ZT 0. *NIA2* mRNA abundance reaches a peak on day 2 at ZT 28 (corresponding to ZT 4 in LD conditions). Daily peaks in *NIA2* mRNA accumulation are observed throughout the remainder of the experiment, although the timing and amplitude of peak levels is somewhat variable. In plants that were grown in LD, the amplitude of the oscillation in *NIA2* mRNA abundance ranges from 4-fold (day 4) to 19-fold (day 3). In plants that were grown in LD and then transferred to LL, the amplitude of the oscillation ranges from 3.5-fold (day 4 in LL) to 14-fold (day 2 in LL). Such day-to-day fluctuations in peak values of mRNA accumulation, and even small fluctuations in the exact timing of the peaks are often observed in circadian experiments [53]. As these fluctuations are readily apparent in LD (Fig. 3A), where one would expect the fluctuations in amplitude and timing to be minimal, the degree of variation seen in Figs. 2 and 4 (below) probably reflects the normal range of fluctuation for *NIA2* mRNA abundance. Actin mRNA accumulation showed minimal fluctuations with no apparent circadian regulation (data not shown).

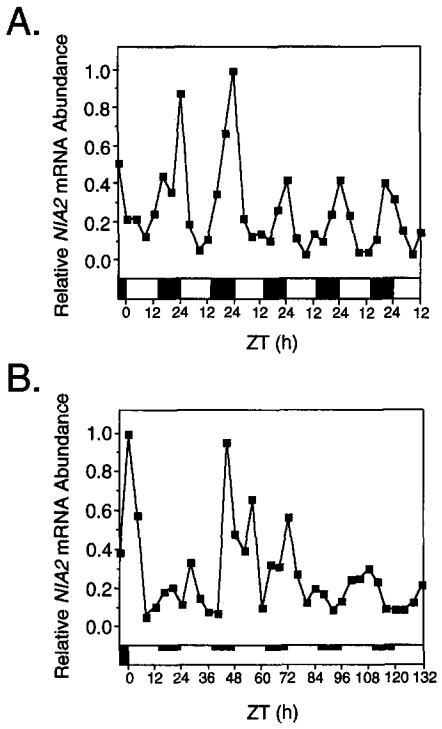

Fig. 3. Circadian regulation of *NIA2* mRNA accumulation persists for 5 days in white light. Slot blots containing total RNA from five-week old *Arabidopsis* plants were probed with *NIA2*, then stripped and reprobed with *SAC3*. Quantification of the resulting autoradiographs was by scanning densitometer. The *NIA2* values represent the amount of *NIA2* mRNA relative to the amount of actin mRNA (*SAC3*-hybridizing) present at each time point. Details of data presentation are described in the legend to Fig. 2. Results are shown for plants that were grown and maintained in a 14:10 photoperiod (A), and for plants that were grown in a 14:10 photoperiod and then transferred to continuous white light (B). Light regimes are indicated by the bars within the graphs. Filled bars indicate dark, open bars indicate light, and small filled inset bars indicate subjective night. Zeitgeber time is indicated below the bars.

Transcriptional regulation is not the primary mediator of clock regulation of nitrate reductase mRNA abundance

Circadian oscillations in steady-state *NIA2* mRNA abundance could be due to changes in rates of transcription, changes in mRNA stability, or a combination thereof. Results from nuclear run-on transcription assays show that in plants grown in an LD cycle, *NIA2* mRNA synthesis appears to be relatively constant (Fig. 4A). *NIA2* transcription is also relatively constant, with minimal fluctuations and no apparent circadian regulation, in plants that were grown in LD and then transferred to DD (Fig. 4B), and in plants that were grown in LD and then transferred to LL (Fig. 4C). Thus, although circadian oscillations in *NIA2* mRNA abundance are observed in LD, DD and LL (Figs. 1, 2 and 3), these oscillations do not appear to be a result of changes in transcription. In all three light conditions, rRNA synthesis is constant with no apparent circadian regulation. As a positive control, *CAB* transcription was examined. *CAB* mRNA abundance is regulated in a circadian fashion in *Arabidopsis* [31, 39, 41]. As shown in Fig. 4, and by Millar and Kay [41], these changes in *CAB* mRNA abundance are due, at least in part, to changes in *CAB* transcription [41]. The rate of *CAB* transcription increases dramatically during the day phase of the cycle for plants that were grown in LD (Fig. 4A), grown in LD and then transferred to DD (Fig. 4B), and grown in LD and then transferred to LL (Fig. 4C), indicating that *CAB* transcription is clearly regulated in a circadian fashion.

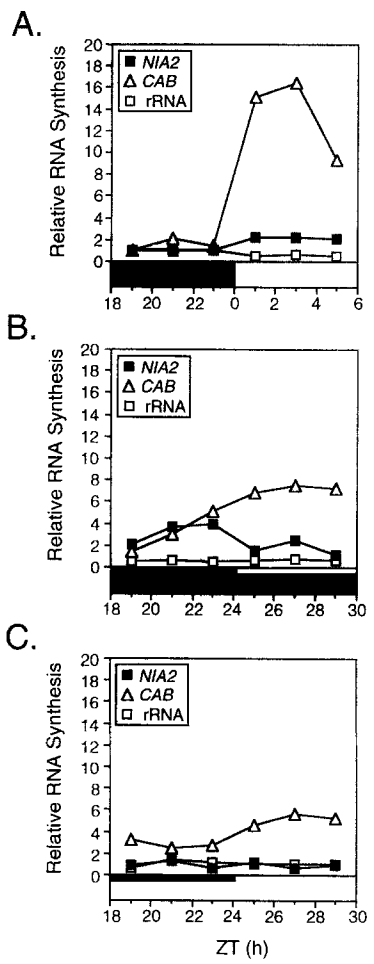

Fig. 4. Nuclear run-on transcription analysis of *NIA2*. Data shown represent the mean for three independent sets of assays. The individual means for *NIA2* and *CAB* have been normalized to rRNA counts. The value obtained at the first time point in LD has been arbitrarily defined as 1.0 unit of RNA synthesis. Results are shown for plants that were grown and maintained in a 14:10 photoperiod (A), grown in a 14:10 photoperiod and then transferred to continuous darkness (B), or grown in a 14:10 photoperiod and then transferred to continuous light (C). Light regimes are indicated by the bars within the graphs. Filled bars indicate dark, open bars indicate light, and small inset bars indicate subjective day (open) and subjective night (filled). Zeitgeber time is indicated below the bars.

Nitrate reductase activity is also regulated by a circadian clock

Are the circadian oscillations in *NIA2* mRNA abundance translated into oscillations in NR enzyme activity? As shown in Fig. 5, circadian oscillations in NR activity are apparent in LD, DD, and LL conditions. In plants that were grown in LD, the peak in NR activity occurs at ZT 8 on both days of the experiment. In plants that were grown in LD and transferred to DD, a peak in NR activity also occurs at ZT 8. In plants that were grown in LD and transferred to LL, the peak in NR activity shows a phase advance and occurs at ZT 24, which corresponds to a time of

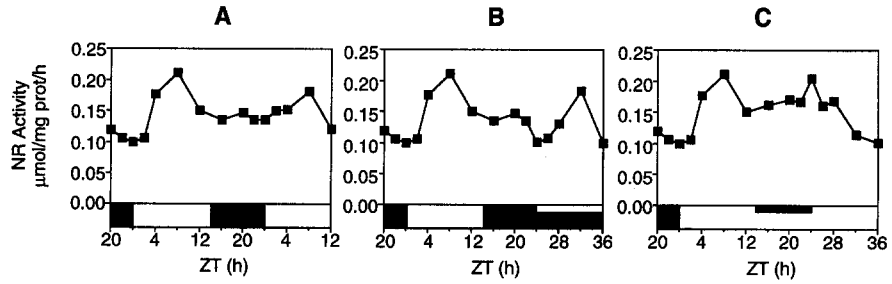

Fig. 5. Circadian regulation of NR activity. Tissue from five week old *Arabidopsis* plants was assayed for NR activity. Shown are the averaged results from eight independent sets of assays. Results are shown for plants that were grown and maintained in a 14:10 photoperiod (A), plants that were grown in a 14:10 photoperiod and then transferred to continuous darkness (B), and plants that were grown in a 14:10 photoperiod and then transferred to continuous light (C). Light regimes are indicated by the bars within the graphs. Filled bars indicate dark, open bars indicate light, and small inset bars indicate subjective day (open) and subjective night (filled). Zeitgeber time is indicated below the bars.

ZT 0 in LD. The amplitude of the oscillation ranges from 1.3- to 2.1-fold in LD. The amplitude of the oscillation is 1.6-fold in DD, and 1.2-fold in LL. Although peak values of NR activity are similar in LD, DD and LL, basal activity is higher in LL, resulting in lower amplitude of the oscillation. The higher trough activity values in LL do not correspond to higher trough *NIA2* mRNA values in LL (Fig. 2C), perhaps indicating additional regulation of NR activity in response to light.

Light induces nitrate reductase mRNA in etiolated seedlings

NIA1 and *NIA2* mRNA accumulation is induced by exposure to white light in mature, dark-adapted *Arabidopsis* plants [13]. We wished to determine whether similar light responsiveness could be observed in *NIA2* mRNA accumulation in etiolated seedlings and, further, to determine the photoreceptor(s) required for light responsiveness. We prepared total RNA from 5- to 6-day old seedlings exposed to various light treatments and assayed the levels of *NIA2* mRNA by northern blot analysis. Northern blots were quantified using scanning densitometry. Measurement of rRNA levels (data not shown) indicated that equal amounts of RNA are present in each lane. As shown in Fig. 6A, the *NIA2* mRNA level in 5-day old etiolated seedlings is induced upon transfer to continuous white light. Although it is difficult to discern at the scale of Fig. 6A, etiolated seedlings that have been transferred to and maintained in continuous white light for 1 h show a 10-fold increase in *NIA2* mRNA compared to the *NIA2* levels in untreated etiolated seedlings. By the time the seedlings have been in white light for 12 h, the *NIA2* mRNA level is 96-fold greater than the level in untreated etiolated seedlings. For comparison, the abundance of *NIA2* mRNA in seedlings grown in white light for 120 h (5 days) is 300-fold higher than that seen in untreated etiolated seedlings.

Induction of *NIA2* mRNA in etiolated seedlings by short pulses (5 min) of red light appears to follow different kinetics from that of white light induction (compare Fig. 6A with Fig. 6B). Etiolated seedlings that have been exposed to a pulse of red light and then incubated in darkness for either 1 h or 3 h show 3.7-fold and 6.8-fold increases, respectively, in *NIA2* mRNA accumulation compared to the level present in untreated etiolated seedlings. This rapid induction of *NIA2* mRNA accumulation in response to a red light flash is very similar to the rapid induction seen when etiolated seedlings are exposed to white light (Fig. 6A), although the amplitude of the response is lower in red light than in white light. However, after a peak *NIA2* mRNA accumulation 3 h after a red light pulse, *NIA2* mRNA levels eventually drop to a point that is roughly equal to that present in untreated etiolated seedlings. In contrast, increasing time in white light results in increasing

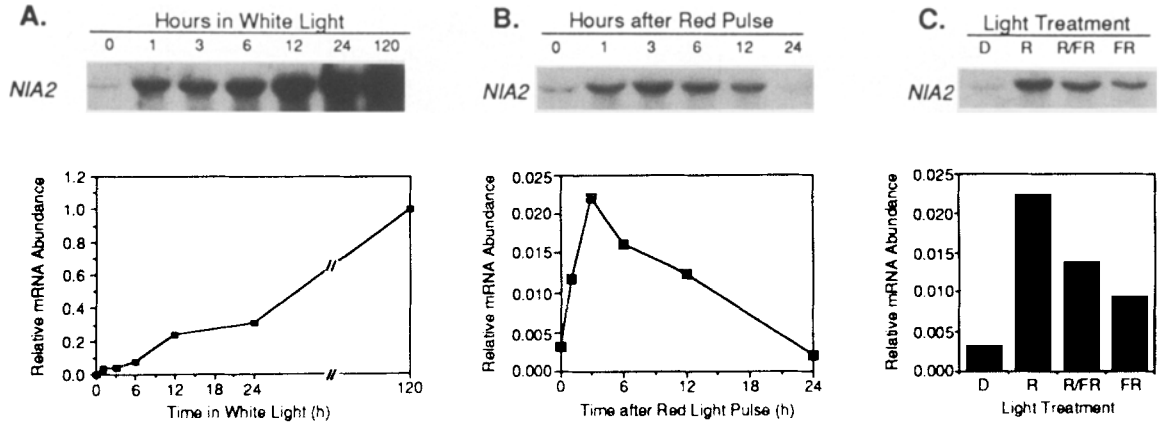

Fig. 6. Light-regulated expression of *NIA2*. Northern blots containing 18 μg of total RNA from 5- to 6-day old *Arabidopsis* seedlings were probed with the *NIA2* gene. Quantification was by scanning densitometer and values represent the amount of mRNA for *NIA2* relative to that present in the 5-day old white light grown seedlings. A. White light induction of *NIA2*. RNA was prepared from 5-day old etiolated seedlings which were transferred to continuous white light for the indicated times or from 5-day old white light grown seedlings. B. Red light induction of *NIA2*. RNA was prepared from 5-day old etiolated seedlings which were irradiated with red light for 5 min then incubated in darkness for the indicated times before harvest. C. Red and far-red regulation of *NIA2*. RNA was prepared from 5-day old etiolated seedlings harvested without irradiation [D] or irradiated (5 min of red light [R], 5 min of red light followed immediately by 5 min of far-red light [R/FR], or 5 min of far-red light [FR]) and then incubated in darkness for 3 h before harvest.

amounts of *NIA2* mRNA. As shown in Fig. 6C, far-red light alone substantially induces *NIA2* mRNA accumulation (2.9-fold increase over dark grown control). A far-red light pulse, given after a red light pulse, only partially reverses the effect of red light on *NIA2* mRNA accumulation (ca. 62% reduction). These responses to far-red light are characteristic of the very low fluence response mediated by phytochrome.

Light regulates nitrate reductase activity

Our results show that light is required for induction of *NIA2* mRNA abundance. We have also measured the induction of NR activity *in vivo* in etiolated seedlings by taking advantage of the ability of NR to catalyze the conversion of the nitrate analog chlorate into the nitrite analog chlorite. Because chlorite is toxic to plants whereas chlorate is relatively non-toxic, the light induction of NR activity can be determined by measuring the effect of light on seedling sensitivity to chlorate. Two parameters of growth were measured: total seedling length (Fig. 7) and fresh weight of seedlings (Fig. 8). Wild type *Arabidopsis* seedlings were grown for 7 days in either white light (16:8 photoperiod) or in constant darkness on medium containing either $NaClO_3$ or NaCl, as a control for Na^+ toxicity and non-specific osmotic effects. When grown in white light, growth of *Arabidopsis* seedlings, as measured by seedling length (Fig. 7A) and seedling weight (Fig. 8A), is inhibited by 0.09 mM chlorate. Only at concentrations 1000-fold higher are the non-specific effects of NaCl toxicity observed. In contrast, dark grown seedlings show only a slightly greater toxicity due to $NaClO_3$ (at 28–94 mM) than to NaCl (at 94 mM). Overall, light-grown seedlings are at least 300–1000 times more sensitive to chlorate than are dark-grown seedlings. These results indicate that, in seedlings, NR activity *in vivo* is very strongly induced by white light.

Discussion

We are interested in the mechanisms by which light and the biological clock interact to regulate

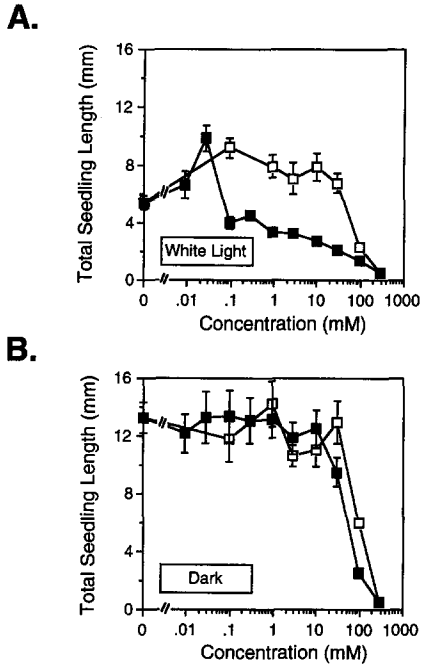

Fig. 7. Effect of light on chlorate sensitivity as measured by *Arabidopsis* seedling length. *Arabidopsis* seedlings were grown for 7 days either in a 16:8 photoperiod (A) or in total darkness (B) in the presence of various concentrations of either $NaClO_3$ (filled square symbols),or NaCl (open square symbols). The maximum lengths of the seedlings are lower for seedlings that were grown in the presence of white light (7–10 mm) than for seedlings that were grown in complete darkness (13–14 mm) due to the inhibitory effect of light on hypocotyl elongation. Each symbol represents the mean (± standard error) seedling length (hypocotyl and root lengths combined) of 10 seedlings.

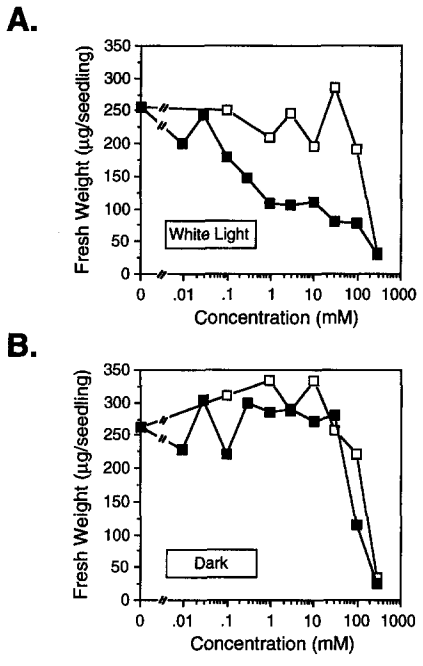

Fig. 8. Effect of light on chlorate sensitivity as measured by *Arabidopsis* seedling weight. *Arabidopsis* seedlings were grown for 7 days either in a 16:8 photoperiod (A) or in total darkness (B) in the presence of various concentrations of either $NaClO_3$ (filled square symbols) or NaCl (open square symbols). Each symbol represents the mean seedling weight of approximately 20 seedlings.

plant gene expression, particularly the expression of nitrate reductase. We have chosen to investigate circadian regulation of nitrate reductase in *Arabidopsis*, a model plant system that has many advantages for molecular genetic analyses and that is amenable to genetic manipulation. A previously report by Cheng *et al.* [13] indicated that the steady state levels of *NIA1* and *NIA2* mRNA oscillate in a circadian fashion in *Arabidopsis* plants that were grown in a light-dark cycle and then transferred to continuous light. Here, we present data confirming this report and extend those results to show that the circadian oscillation in *NIA2* mRNA abundance is also present in plants that have been grown in a light-dark cycle and then transferred to continuous darkness. The oscillations in mRNA abundance in LD, DD and LL conditions are easily detectable with Northern blot and slot blot analysis of total *Arabidopsis* RNA. Nitrate reductase mRNA accumulation has been shown to be regulated by the biological clock in other plant systems such as tobacco [20], and maize [6]. Results from the circadian regulation experiments (in which plants are transferred from an LD cycle to DD and/or LL) done in maize [6] and tobacco [20], and diurnal regulation experiments (in which plants are maintained in an LD cycle) done in tomato [4, 24] and tobacco [24] are consistent with our findings that nitrate reductase mRNA accumulation peaks early in the day period. Similarly, daytime peaks have also been observed for photosynthetic genes whose mRNA accumulation is under circadian regulation [1, 25, 41, 42, 45, 46].

The circadian oscillations in *Arabidopsis NIA2*

mRNA abundance do not appear to be due to changes in *NIA2* transcription. Although minimal fluctuations in *NIA2* transcription are observed they do not appear to vary in a circadian fashion, and the fluctuations in DD and LL do not correlate in a temporal sense with *NIA2* mRNA accumulation. Additionally, the minimal fluctuations in *NIA2* transcription observed do not appear to be sufficient to result in the obvious circadian oscillations observed in *NIA2* mRNA abundance (Figs. 1, 2 and 3). Circadian regulation of transcription of *CAB* genes has been demonstrated in several plant systems, including wheat [42], tomato [25], and *Arabidopsis* (Fig. 3; see also [41]). In tobacco carrying a full-length NR cDNA expressed from the constitutive CaMV 35S promoter the circadian rhythm in NR mRNA abundance is lost, implying a major role for transcription in this circadian rhythm [55]. In contrast, we do not see evidence that circadian oscillations in *Arabidopsis NIA2* mRNA accumulation reflect transcriptional regulation and hypothesize that circadian control of *NIA2* expression is primarily due to post-transcriptional regulation. We are currently investigating *NIA2* sequences required for this circadian response with a gene fusion approach, although recent attempts to characterize the *nia1* and *nia2* promoters of tobacco with a gene fusion approach have been problematic [54].

Many circadian clock-regulated processes oscillate with robust amplitude after the initial transfer to continuous conditions, but dampen rapidly thereafter. Damping is a decrease in the amplitude of an oscillation with increasing time in continuous environmental conditions. In particular, damping is commonly observed in oscillations in plant processes, such as mRNA accumulation, in conditions of continuous darkness (e.g. [25, 41]). Damping in continuous darkness could indicate either that the circadian clock does not continue to run in continuous darkness, or that the circadian clock runs but that some additional signal is required to see an oscillation in mRNA abundance. An obvious candidate for a positive effector of gene expression, which would be missing in continuous darkness, is light. Our results (Fig. 6) and data from other labs (e.g. [13]) indicate that *NIA2* mRNA abundance in *Arabidopsis* is positively regulated by light. Therefore, we conducted an extended circadian experiment in continuous light and observed persistent (over 5 days in LL) circadian oscillations in *NIA2* mRNA abundance. This provides strong evidence that the circadian clock (as evidenced at the level of gene expression) can continue to act over a continuous, extended period of time.

The robust, persistent circadian oscillations in *NIA2* mRNA abundance support the hypothesis that the clock acts as a gate which governs the timing of light-regulated phenomena, such as gene expression [31, 42]. The gating action of circadian clocks on phytochrome-regulated plant gene expression was proposed by Nagy *et al.* [42] on the basis of their results from examining phytochrome and circadian regulation of *Cab-1* expression in wheat. They showed that at times of day (late afternoon) where Pfr (the active form of phytochrome) is presumably present in saturing amounts, *Cab-1* mRNA abundance decreases. They proposed that this decrease is due to negative regulation of *Cab-1* mRNA accumulation by the circadian clock. The gating theory was further supported by results from transgenic experiments that showed that when rice phytochrome is overexpressed in tobacco, again producing amounts of the active form of phytochrome that are presumably saturating, oscillations in *Cab* mRNA accumulation still occur, and that damping of the oscillation in DD is greatly reduced [32]. Our results agree with this interpretation of the interactive effects of light and/or phytochrome, and the circadian clock. Clearly, when, phytochrome as well as other photoreceptors are presumably in a state of continuous stimulation, and when the clock is operating, the circadian clock regulates when *NIA2* mRNA will accumulate in *Arabidopsis*, overriding the inductive effects of light.

Gating of phytochrome and light responses by the circadian clock may be similar to the gating of light responses by a developmental program suggested by Brusslan and Tobin [8] and Kubasek *et al.* [33]. Brusslan and Tobin [8] have shown that a developmental clock regulates the

age at which a red light pulse (1 min) can induce *CAB1* mRNA accumulation in etiolated *Arabidopsis* seedlings. Similarly, a developmental program appears to govern the timing of light-independent induction of the *CAB* and *RBCS-1A* genes in etiolated *Arabidopsis* seedlings [8]. A developmental program also appears to regulate the developmental timing of white light and UVB light inducibility of flavonoid gene expression in *Arabidopsis* seedlings [33]. Additionally, Kubasek *et al.* [33] showed similar regulation of the developmental timing of light-independent expression of *PAL1*.

We observed that the circadian oscillations in *NIA2* mRNA abundance in *Arabidopsis* are paralleled by similar circadian oscillations in NR activity. In tobacco, circadian oscillations in nitrate reductase mRNA are paralleled, with a 3–5 h lag, by oscillations in NR protein and activity [20]. However, in addition to *de novo* NR protein synthesis, other levels of regulation, either translational and/or post-translational, may affect NR activity *in vivo* because the trough level of NR activity is higher in LL than in DD. Vincentz and Caboche noted changes in tobacco NR protein levels associated with shifts to dark and to light that were not accompanied by changes in mRNA, and postulated light effects on the translation of the NR mRNA or on the stability of the NR protein [55]. In spinach, Huber *et al.* [29] showed that the nitrate reductase enzyme can be inactivated reversibly by phosphorylation. Dephosphorylation of inactivated NR restores enzyme activity. However, Shiraishi *et al.* [52] recently argued that changes in NR activity in spinach cells appears to be a result of changes in the amount of NR protein present, rather than changes in activation and inactivation of pre-existing protein. At this time we cannot conclude whether the oscillations in NR activity that we observe in *Arabidopsis* reflect changes in the amount of NR protein present or modulation of NR activity, or both. However, the elevated trough values for NR activity detected in LL, but not in DD, suggest a positive effect of light on NR activity. Clearly, regulation of nitrate reductase is complicated and further studies are required to better understand the regulatory processes involved.

In addition to the evidence showing that nitrate reductase mRNA is regulated by an endogenous circadian clock in some plant species, strong evidence exists that shows that nitrate reductase mRNA is also regulated by exogenous factors, such as light [10, 19, 28]. We observed that *NIA2* mRNA accumulation in etiolated *Arabidopsis* seedlings is induced by exposure to white light. Similar results have been observed with etiolated barley seedlings [40], maize seedlings [6, 26], squash cotyledons [47], and tomato cotyledons [4]. Our results also show that the effects of continuous white light are cumulative; maintaining *Arabidopsis* seedlings in white light for up to 5 days results in increased accumulation of *NIA2* mRNA. *NIA2* mRNA accumulation is also induced in etiolated *Arabidopsis* seedlings that have been exposed to a pulse (5 min) of red light; increased *NIA2* mRNA is detectable within 1 h. This increase in *NIA2* mRNA following a red light pulse presumably is due to responses mediated by the active form of phytochrome, Pfr. In tomato, red light induction of NR mRNA in etiolated seedlings is attenuated in the phytochrome-deficient *aurea* mutant, further demonstrating phytochrome control of NR gene expression [4]. In contrast to the results observed in continuous white light, the increase in *Arabidopsis NIA2* mRNA accumulation in response to a pulse of red light is transient. When seedlings are allowed to incubate in the dark for increasing amounts of time (6 h or longer) following the red light pulse, *NIA2* mRNA abundance decreases. This decrease in *NIA2* mRNA abundance with increasing time (> 3 h) in darkness following a red light pulse is not understood, but one possibility is that it results from degradation of PfrA in the dark. Furthermore, white light treatment results in higher overall levels in *NIA2* mRNA accumulation (compare the increasing phase of the graphs in Figs. 6A and 6B). These results suggest the possible involvement of other receptors, in addition to the red light photoreceptor phytochrome, in the induction of *NIA2* mRNA accumulation. Supporting the hypothesis of the involvement of

other photoreceptors is the finding that in etiolated barley seedlings, blue light induces nitrate reductase mRNA accumulation [40]. In addition, light-grown barley seedlings, when dark-adapted, were not phytochrome-inducible (did not exhibit red light induction that was far-red reversible), but instead responded weakly to blue and strongly to white light [40].

The transient nature of the accumulation of *NIA2* mRNA abundance in response to a brief pulse of red light may also reflect the absence of a plastidic factor which comes from mature chloroplasts. The hypothesis of a plastidic factor positively regulating nitrate reductase is based on results from experiments using chloroplast-ribosome deficient mutants in barley and maize [5], and from experiments using inhibitors of intraplastidic protein synthesis and carotenoid synthesis in mustard [43, 48]. These experiments show that, even in the presence of inducing conditions such as nitrate and light, NR apoprotein and NR enzyme activity fail to accumulate to normal levels, if at all, in plants whose chloroplasts fail to develop properly. Perhaps the cumulative effects of white light on *Arabidopsis NIA2* mRNA accumulation are due in part to positive feedback from mature chloroplasts. A 5 min pulse of red light is insufficient to induce mature chloroplasts as chlorophyll accumulation, and therefore, development of mature chloroplasts, requires continuous exposure to light [3]. Thus, the brief red light pulse does not result in the generation of the plastidic factor, and therefore only induces a transient accumulation of *NIA2* mRNA.

Although low fluence phytochrome-mediated responses are induced by red light, the low fluence effects of red light are completely reversible by far-red light [30]. In contrast, very low fluence phytochrome-mediated responses are induced by red light and far-red light, and are only partially reversible by far-red light [30]. The far-red light inducibility of *NIA2* mRNA accumulation and the inability of far-red light to fully reverse the effects of red light on *NIA2* mRNA accumulation clearly indicate that *NIA2* mRNA accumulation is mediated by a very low fluence phytochrome response in *Arabidopsis*. In contrast, nitrate reductase mRNA accumulation is regulated by a low fluence response in etiolated barley seedlings [40]. The effects of red and far-red light treatment on *NIA2* mRNA accumulation are similar to those observed with the *FEDA* and *CAB* genes in *Arabidopsis* [11].

Induction of nitrate reductase by white light is also observable at the level of NR activity *in vivo*, as determined by chlorate sensitivity. *Arabidopsis* plants that were grown in white light (16:8 photoperiod) show increased sensitivity to chlorate compared to plants that were grown in complete darkness. This result suggests that, in *Arabidopsis*, there is insufficient NR activity in etiolated plants to result in chlorate sensitivity and that NR activity is induced upon exposure to light. Light inducibility of NR has also been demonstrated in barley [40], squash [47], and maize seedlings [26].

The circadian oscillation in NR activity suggests that the negative chlorate selection might provide a means to identify clock mutants or light regulatory mutants in which NR activity is high at inappropriate circadian phases, or is high in dark-grown seedlings. *chl3* is an example of an *Arabidopsis* mutant that was isolated based on its resistance to chlorate [7]. Wilkinson and Crawford [58] have shown that *CHL3* corresponds to the gene encoding *NIA2*. Thus, loss of *NIA2* expression in the *chl3* mutant results in reduced NR activity and in increased resistance to chlorate. Presumably, mutations which alter clock or light regulation of *NIA2* expression could also confer chlorate resistance. A complicating factor that needs to be considered when selecting for chlorate resistance is that resistance can arise by multiple mechanisms (altered chlorate uptake, loss of function of one of the many genes required for assembly of the Mo cofactor, etc.) in addition to altered expression of structural genes encoding the NR apoprotein [57]. Additionally, for a circadian selection to be successful, several criteria must be met. First, one must be able to supply the substrate, chlorate, in precisely defined temporal pulses. However, nitrate (and presumably chlorate) is not necessarily reduced immediately following uptake. Instead, nitrate (and chlorate) can

be diverted to stored pools [27], presumably located in the vacuole. Subsequent release and reduction of the stored chlorate would dissociate the temporal application of the selectable substrate from the NR-catalyzed reduction which is the basis of the selection. This problem is similar to that faced in experiments employing pulses of metabolic inhibitors, in which prolonged washout and recovery times greatly complicate the interpretation of the observed responses to the inhibitors (e.g. [44]). A second requirement of a selection for clock mutants is that exposure to a toxin must kill rapidly. Again, experimental results suggest that *Arabidopsis* requires several day's exposure to chlorate to exhibit symptoms of toxicity [34, 57]. Thus, we conclude that chlorate selection against NR activity is not promising for use in the isolation of clock mutants. However, identification of light regulatory mutants using chlorate selection would require neither the precise temporal control of chlorate pulses nor the rapid killing required for the selection of clock mutants. Moreover, the profound differences in sensitivity to chlorate observed in etiolated versus light grown *Arabidopsis* seedlings suggests that chlorate selection may provide a useful tool for the identification of mutants in which responses to light are altered.

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