

Arabidopsis cryptochrome 1 functions in nitrogen regulation of flowering

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The phenomenon of delayed flowering after the application of nitrogen (N) fertilizer has long been known in agriculture, but the detailed molecular basis for this phenomenon is largely unclear. Here we used a modified method of suppression-subtractive hybridization to identify two key factors involved in N-regulated flowering time control in *Arabidopsis thaliana*, namely ferredoxin-NADP⁺-oxidoreductase and the blue-light receptor cryptochrome 1 (CRY1). The expression of both genes is induced by low N levels, and their loss-of-function mutants are insensitive to altered N concentration. Low-N conditions increase both NADPH/NADP⁺ and ATP/AMP ratios, which in turn affect adenosine monophosphate-activated protein kinase (AMPK) activity. Moreover, our results show that the AMPK activity and nuclear localization are rhythmic and inversely correlated with nuclear CRY1 protein abundance. Low-N conditions increase but high-N conditions decrease the expression of several key components of the central oscillator (e.g., *CCA1*, *LHY*, and *TOC1*) and the flowering output genes (e.g., *GI* and *CO*). Taken together, our results suggest that N signaling functions as a modulator of nuclear CRY1 protein abundance, as well as the input signal for the central circadian clock to interfere with the normal flowering process.

adenosine monophosphate-activated protein kinase | circadian clock | cryptochrome 1 | ferredoxin-NADP⁺-oxidoreductase 1 | nitrogen-regulated flowering

The transition from vegetative to reproductive development is a central event in the plant life cycle, which is coordinately regulated by various endogenous and external cues. In the model dicotyledonous plant species *Arabidopsis thaliana*, five distinct genetic pathways regulating flowering time have been established: the vernalization pathway, photoperiod pathway, gibberellin acid (GA) pathway, autonomous pathway, and endogenous (age) pathway (1). These pathways ultimately converge to regulate a set of floral integrator genes, *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF CONSTANS 1* (*SOC1*), which in turn activate the expression of floral meristem identity genes to trigger the formation of flowers (2–4).

Plants use the circadian clock as the timekeeping mechanism to measure day length and to ensure flowering at the proper season (5, 6). As a facultative long-day (LD) plant, *Arabidopsis* flowers earlier under LD conditions than under short-day (SD) conditions. Forward genetics in *A. thaliana* have identified the *GI-CO-FT* hierarchy as the canonical genetic pathway promoting flowering specifically under LD conditions (5, 7, 8). In this pathway, *GI* (*GIGANTEA*) can be considered the output point of the circadian clock to control flowering by regulating *CONSTANS* (*CO*) expression in the right phase, which activates expression of *FT* and *Tsf* (*TWIN SISTER OF FT*) in the companion cells of the phloem within the vascular tissue (2, 9). *FT* and *Tsf* proteins act as the long-sought florigens that move from leaves to the apical meristem to induce genes required for reproductive development (2–4). Both

GI and *CO* are regulated by the circadian clock and by light signaling simultaneously and at both transcriptional and post-transcriptional levels, to ensure the transcription of *FT* under LD conditions, but not under SD conditions (10).

Nitrogen (N) availability is one of the key factors controlling developmental and growth to ensure plant survival and reproduction (11). *Arabidopsis*, like other plants, flowers earlier under low-nitrate conditions (11). Previous studies have reported that the flowering activators *CO*, *FT*, *LEAFY* (*LFY*), and *APETALA1* (*AP1*) are induced, but the flowering repressor *FLC* (*FLOWERING LOCUS C*) is repressed in low-nitrate conditions (12, 13). There are also reports that nitrate availability controls the GA pathway at different levels (GA biosynthesis, perception, and signaling) to influence the timing of vegetative to reproductive phase change (13, 14). However, Castro Marín et al. (15) showed that low nitrate induced flowering by a pathway downstream of the floral integrators and independent of photoperiod, GA, and autonomous pathways. Thus, the detailed molecular mechanisms, especially the key factors to sense and transmit the N signal to regulate flowering remain elusive.

In this study, we used a modified method of suppression-subtractive hybridization (SSH) to identify two key factors involved in N-regulated flowering: ferredoxin-NADP⁺-oxidoreductase (*FNR1*) (16) and the blue-light photoreceptor cryptochrome 1 (*CRY1*) (17). Their loss-of-function mutants are

Significance

Overapplication of nitrogen (N) fertilizer causes delayed flowering and negatively impacts the function and composition of natural ecosystems and climate. In this study, we demonstrate that flowering time variations regulated by altered nitrogen levels are mediated by two key factors: ferredoxin-NADP⁺-oxidoreductase (*FNR1*) and the blue-light receptor cryptochrome 1 (*CRY1*). Nitrogen regulates *FNR1* expression, thereby contributing to changes in NADPH/NADP⁺ and ATP/AMP ratios, which in turn activates adenosine monophosphate-activated protein kinase to modulate nuclear *CRY1* abundance, which further acts in the N signal input pathway to affect central clock function and flowering time. A better understanding of N-regulated floral transition will offer biotechnological solutions to improve sustainable agriculture.

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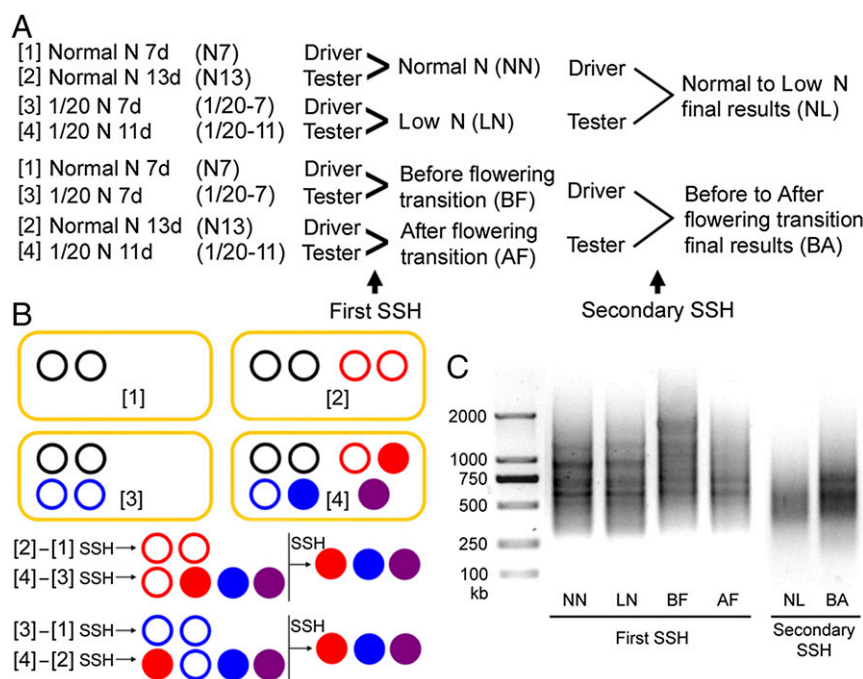


Fig. 1. Procedures, schematic diagram of the SSH screens, and results. (A) Experimental procedure for SSH. (B) Schematic diagram of two-round SSH. Red circles indicate floral transition-induced genes, which can be further induced by 1/20 LN conditions (red disk); blue circles indicate LN-induced genes, which can be further induced after floral transition (blue disk). Purple disks indicate genes expressed only at 1/20 LN conditions and after floral transition (two-condition synergistic genes). (C) PCR verification of first-round and second-round SSH.

insensitive to altered N levels. We show that N regulates *FNRI* at the transcription level, thus affecting ratios of NADPH/NADP⁺ and ATP/AMP, which in turn affect adenosine monophosphate-activated protein kinase (AMPK) activity and nuclear CRY1 protein abundance. Our data imply that the nuclear level of CRY1 functions as an input cue to regulate the amplitude of circadian clock transcripts, thereby controlling flowering time.

Results

Identification of *FNRI* and *CRY1* as N-Responsive Genes. Earlier microarray studies have shown that thousands of *Arabidopsis* genes (~7% of the *Arabidopsis* transcriptome) are N-responsive (18). To search for key factors involved in N-regulated flowering, we performed a modified SSH screen with *Arabidopsis* seedlings grown on media containing different levels of N. We first determined the appropriate N treatment levels and *Arabidopsis* floral transition times. When grown at reduced N levels (1/20 N, NH₄NO₃, and KNO₃ equally reduced), the flowering time was shortened from 21 d from 25 d when grown on normal-N (NN) medium (1/2 MS medium containing 10 mM NH₄NO₃ and 9.4 mM KNO₃). When grown on high-N (HN; 2×N) MS medium (40 mM NH₄NO₃ and 37.6 mM KNO₃), the flowering time was delayed to 32 d. A further reduction of N levels to 1/50 N resulted in a severely stressed phenotype with increased anthocyanin accumulation (*SI Appendix*, Fig. S1).

Consistent with the observed alteration in flowering time, quantitative RT-PCR analysis revealed that expression of *LFY* and *FT* began to increase at day 11 for plants grown in low-N (LN; 1/20 N) medium, day 13 for plants grown in NN medium, and day 15 for plants grown in HN medium (*SI Appendix*, Fig. S2). This finding validates the suitability of our experimental conditions for screening N-responsive genes.

To identify the key players involved in N regulation of flowering, we performed a modified SSH screen with RNA samples collected before and after the floral transition. Four total RNA samples were collected: in NN before floral transition at the

seventh day, in NN after floral transition at the 13th day, in LN before floral transition at the seventh day, and in LN after floral transition at the 11th day. Pairwise comparison of these samples through two rounds of SSH screens (Fig. 1) revealed that genes encoding *FNRI* and *CRY1* are prominently enriched in both final results (*SI Appendix*, Tables S1 and S2). Induction of *FNRI* and *CRY1* gene expression by LN (increased by 5.2-fold for *FNRI* and by 4.8-fold for *CRY1*) was further confirmed by quantitative RT-PCR (*SI Appendix*, Fig. S3). Statistical analysis revealed that the abundance of *FNRI* and *CRY1* transcript is negatively correlated with flowering time ($P < 0.05$), implying that *FNRI* and *CRY1* act as two positive regulators of N-regulated flowering.

Furthermore, the *FNRI* expression can be induced by high-Fe and S conditions (0.4 mM FeSO₄·7H₂O in the MS medium) and *CRY1* expression can be induced by blue-light treatment (*SI Appendix*, Fig. S3), in disagreement with previous reports (17, 19) but consistent with reported transcriptome analyses (20, 21).

***Arabidopsis fnr1* and *cry1* Mutants Are Insensitive to N Changes.** To test whether *FNRI* and *CRY1* play roles in N-regulated flowering, we examined the responsiveness of *Arabidopsis fnr1* and *cry1* mutants to different levels of N under LD conditions. Both medium-grown and soil-grown *fnr1* mutants exhibited a late-flowering phenotype (29 d in NN medium), that could be altered by changing the N levels in the growth medium ($P > 0.05$). Although the *cry1* mutant exhibited a normal flowering phenotype under regular N supply (25 d in NN medium), the flowering time of the mutant also was not altered by changing N levels ($P > 0.05$; Fig. 2 and *SI Appendix*, Fig. S4). Thus, both the *Arabidopsis fnr1* and *cry1* mutants displayed insensitivity to N level changes in term of flowering time. In addition, blue-light treatment (presumably to induce *CRY1* expression; Fig. 1) led wild type (WT), *fnr1*, and *cry1* plants to flower earlier than under NN conditions ($P < 0.05$); whereas high-Fe and S growth conditions (presumably to induce *FNRI* expression; Fig. 1) did not promote early flowering in the *cry1* mutant ($P > 0.05$; Fig. 2B). These results suggest that *CRY1* may work downstream

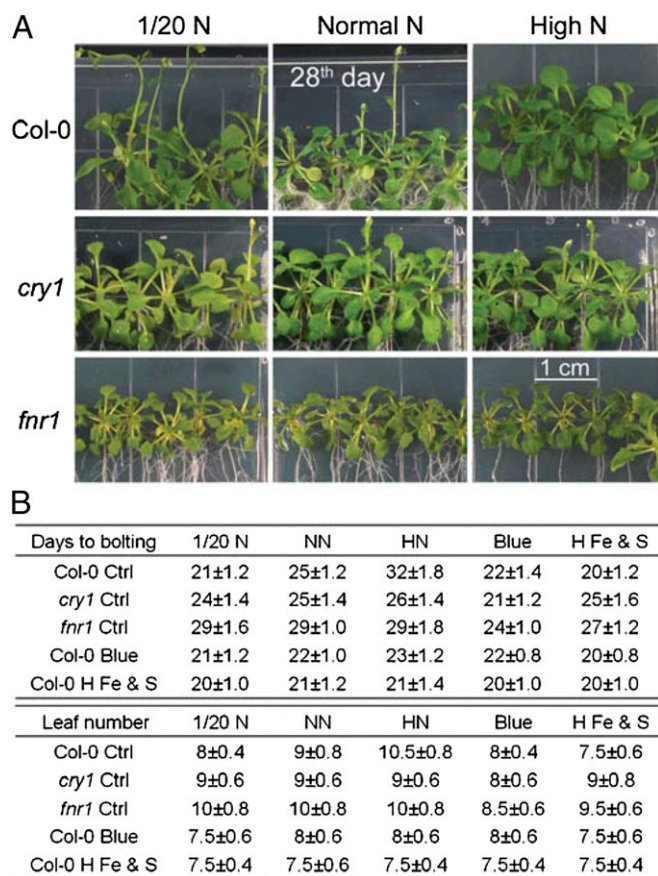


Fig. 2. *fnr1* and *cry1* mutants are insensitive to N levels. (A) 28-d-old WT Columbia (Col-0) plants and the *fnr1* and *cry1* mutant derivatives grown in MS medium under different N conditions. (B) Flowering times of WT, *fnr1*, and *cry1* plants grown in MS medium. Days to flowering and rosette leaf number were scored (mean ± SD; $n \geq 25$ plants). blue, blue-light treatment; H Fe & S, high-Fe and S (0.4 mM FeSO_4) treatment.

of *FNR1* in the N-signaling pathway. Although previous studies reported that both the *fnr2* mutant (16, 22) and the *cry2* mutant (19, 23) had a late-flowering phenotype, here these mutants exhibited normal responses to N changes ($P < 0.05$; *SI Appendix, Table S3*), suggesting that neither *FNR2* nor *CRY2* plays an essential role in N-regulated flowering time.

We next checked the responsiveness of WT, *cry1*, and *fnr1* plants to two different N sources to assess for a preference for ammonium or nitrate. WT plants flowered earlier when grown in medium supplemented with 1/20 ammonium (2.94 mM NH_4Cl ; at 21 d, as in the 1/20 LN condition), but flowered later when grown in medium supplemented with high levels of NH_4^+ nitrogen (117.6 mM NH_4Cl ; at 32 d, as in the HN condition). However, no difference in flowering time was observed for either the *cry1* or *fnr1* mutant when grown on 1/20 ammonium or high- NH_4^+ conditions. WT plants showed severely stressed phenotypes in either the 1/20 NO_3^- (2.94 mM NaNO_3) or high- NO_3^- (117.6 mM NaNO_3) condition, implying a preference for ammonium as the N source; however, the *cry1* and *fnr1* mutants showed less-stressed phenotypes in both the 1/20 NO_3^- and high- NO_3^- conditions (*SI Appendix, Fig. S5*), indicating that the *cry1* and *fnr1* mutants are less sensitive to nitrate changes.

We next questioned whether the LN-promoted flowering is *FNR1*-specific. A previous study reported that the *chl-5* (nitrate transporter *AtNRT1.1*) mutant exhibits a delayed flowering phenotype (24), whereas the double mutant of nitrate reductase, *nia1/nia2*, shows an early flowering phenotype (25). Interestingly, the

flowering time in these mutants exhibited normal responses to altered N levels (*SI Appendix, Fig. S6*), demonstrating that the altered flowering phenotype of the *cry1* or *fnr1* mutant is not a general phenotype of N deficiency and is presumably related to the cooperation between N signaling and the flowering pathway.

LN Conditions Increase NADPH/NADP⁺ and ATP/AMP Ratios. FNR is a ubiquitous flavoenzyme that oxidizes the final reduced product of the photosynthetic electron transport chain, ferredoxin (Fd), to reduce NADP⁺, resulting in ATP production. Therefore, FNR levels (activity) regulate cellular NADPH/NADP⁺ and ATP/AMP ratios (16, 22). Consistent with LN induction of *FNR1* expression, we detected higher NADPH/NADP⁺ and lower AMP/ATP ratios in WT plants in LN conditions than in NN conditions ($P < 0.05$). Conversely, lower NADPH/NADP⁺ and higher AMP/ATP ratios were detected in HN conditions ($P < 0.05$; Fig. 3A). As expected, the *fnr1* mutant always had a lower NADPH/NADP⁺ ratio and a higher AMP/ATP ratio independent of N level compared with the WT plants (Fig. 3B).

LN Conditions Decrease Nuclear AMPK Activity and Nuclear CRY1 Phosphorylation. In mammalian cells, the AMP/ATP ratio affects AMPK activity, which in turn affects nuclear cryptochrome phosphorylation and peripheral clock phase (26). The *Arabidopsis* SnRK superfamily is homologous to mammalian AMPK and is composed of three distinct subfamilies, SnRK1, SnRK2, and SnRK3 (27). Members of the *Arabidopsis* SnRK family have been reported to play roles in diverse stress and metabolic signaling (27). To test whether *Arabidopsis* AMPK is regulated by N levels and involved in the regulation of nuclear cryptochrome phosphorylation, we examined the activity of *Arabidopsis* AMPK α 1 (the catalytic

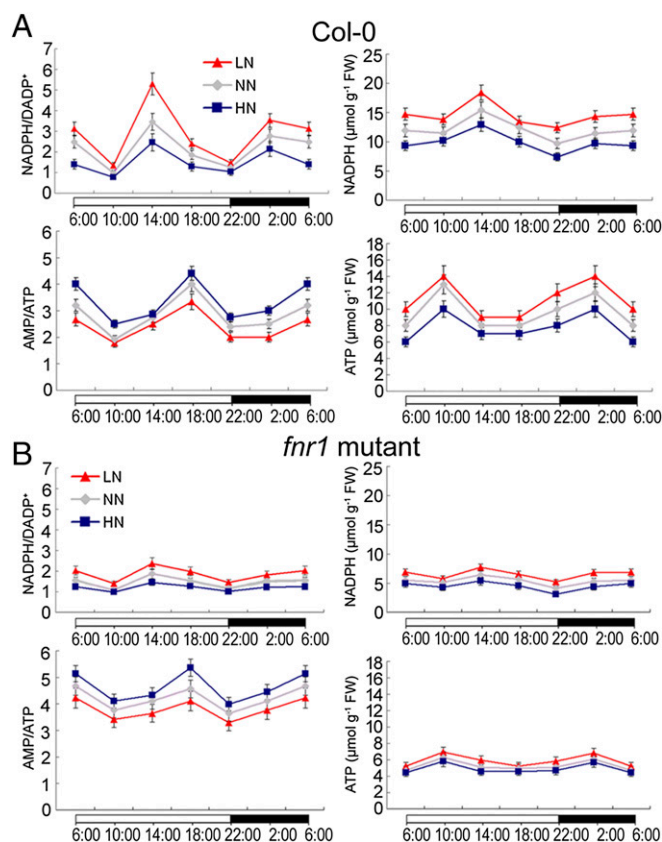


Fig. 3. NADPH levels, ATP levels, and NADPH/NADP⁺ and AMP/ATP ratios in WT (A) and *fnr1* mutant (B) plants over a 24-h period. Error bars show SD ($n = 5$).

subunit of AMPK) under different N levels. AMPK α 1 is highly conserved in eukaryotes (27). *Arabidopsis* AMPK α 1 homologs KIN10 (AT3G01090) and KIN11 (AT3G29160) proteins share 79.3% similarity with the human AMPK α 1 (*SI Appendix, Fig. S7*). Western blot analysis showed that the anti-human AMPK α 1 antibody raised against conserved peptides of the catalytic subunit can also recognize the *Arabidopsis* AMPK α 1 homologs KIN10 and KIN11 specifically (*SI Appendix, Figs. S7 and S8*). An immunoprecipitation kinase assay showed that *Arabidopsis* AMPK α 1 is activated by high levels of AMP under HN conditions (Fig. 4A). Similar to the reported robust circadian rhythm of nuclear localization for the mouse AMPK α 1 subunit (26), nuclear AMPK α 1 protein levels in *Arabidopsis* also exhibited a robust circadian rhythm, although the total cellular AMPK α 1 content remained relative stable. In addition, nuclear AMPK α 1 activity was much higher during the day than at night (Fig. 4A). Strikingly, we found that HN conditions increased the nuclear AMPK α 1 protein level and its oscillation amplitude, whereas LN conditions decreased them (the phase was not changed; Fig. 4A). These observations suggest that N levels affect nuclear AMPK α 1 level (activity) via the circadian clock, which in turn regulates flowering time in *Arabidopsis*. Consistent with this hypothesis, simultaneous loss of KIN10 and KIN11 function, which encode two homologs closely related to the human AMPK α 1, caused reduced sensitivity to N level alteration, despite the normal phenotype of the *kin10* or *kin11* single mutant (*SI Appendix, Fig. S9*).

It was previously shown that in mammalian cells, AMPK-mediated phosphorylation of CRY1 promotes ubiquitin-dependent CRY1 degradation (28), and thus the peak time of AMPK α 1 nuclear localization coincides with minimal nuclear CRY1 (26). Similarly, we found that nuclear AMPK α 1 peaked synchronously with nuclear CRY1 phosphorylation in *Arabidopsis*, and that the peak time of nuclear AMPK α 1 level and its activity coincided with maximal nuclear CRY1 phosphorylation and minimal nuclear CRY1 protein level (Fig. 4B). This result suggests that *Arabidopsis* AMPK α 1 also mediates nuclear CRY1 phosphorylation and its subsequent degradation. In support of this notion, we found that the half-life of nuclear CRY1 protein

levels decreased in response to increased N levels (*SI Appendix, Fig. S10*). Moreover, the AMPK agonist aminoimidazole carboxamide ribonucleotide (AICAR) (26) mimicked the effect of HN conditions by causing late flowering, whereas the effect of AMPK inhibitor dorsomorphin (29) resembled the effect of LN conditions by causing early flowering (*SI Appendix, Figs. S11 and S12 and Table S3*). Furthermore, nuclear AMPK α 1 level (activity) and nuclear CRY1 phosphorylation were always higher and nuclear CRY1 protein level was always lower in the *fir1* mutant than in WT plants (*SI Appendix, Figs. S11 and S12*). Taken together, these results support the hypothesis that AMPK α 1 plays a key role in nuclear CRY1 phosphorylation and degradation, and that this process is regulated by FNR1 activity.

N Regulates the Circadian Clock Through the CRY1 Input Pathway. In *Arabidopsis*, cryptochromes act as part of the input pathways to the circadian clock, which subsequently affects the expression of key components of the central oscillator, such as *LHY* (*LATE ELONGATED HYPOCOTYL*), *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), and *TOC1* (*TIMING OF CAB EXPRESSION 1*) (30–32). As shown in Fig. 5A, the oscillation phase and abundance of *CCA1* and *LHY* transcripts were inversely correlated with the expression of *TOC1*. Transcripts of two output genes, *CO* and *GI*, also fluctuated inversely with each other (Fig. 5A). To further test the role of N signaling in regulating the circadian clock, we compared the effects of altered N levels on the phase and amplitudes of *CCA1*, *LHY*, and *TOC1*. We found that LN conditions increased the amplitudes of all circadian transcripts throughout the circadian cycle, whereas HN conditions decreased the amplitudes of these genes in WT plants ($P < 0.05$); however, no phase shift was observed for these circadian clock genes (Fig. 5A and B). Furthermore, the AMPK agonist AICAR affected the expression pattern of these genes similarly to HN conditions, whereas the AMPK inhibitor dorsomorphin affected the expression pattern of these circadian transcripts in a manner similar to LN conditions (*SI Appendix, Fig. S13*). These results support the notion that altered N levels serve as a signal to regulate circadian clock function, and that AMPK plays an essential role in this regulation.

Interestingly, we found that the expression levels and amplitudes of these circadian transcripts were insensitive to N changes in the *cry1* mutant, but were always lower in the *fir1* mutant, independent of N level ($P > 0.05$; *SI Appendix, Fig. S14*). These results suggest that FNR1 and CRY1 may play key roles in sensing and transmitting the N signal into the central clock to regulate the clock function and thus flowering.

Discussion

In this study, we have identified FNR1 and CRY1 as two critical players in N-regulated flowering time in *Arabidopsis*. We found that AMPK activity and nuclear localization are rhythmic and inversely correlated with nuclear CRY1 protein abundance. Correlation coefficients (R^2) between parameters of NADPH/NADP $^+$, AMP/ATP, nuclear AMPK activity, nuclear CRY1 phosphorylation level, and nuclear CRY1 protein level range from 0.83 to 0.99 (*SI Appendix, Table S4*). Our collective results lead us to propose a working model in which the nuclear level of CRY1 acts as a modulator in the N-signaling pathway to regulate the amplitudes of the circadian clock, thereby controlling flowering time (Fig. 5C). In *Arabidopsis* sugar signaling, photosynthesis also has a profound effect on the entrainment and maintenance of robust circadian rhythms, and thus regulates floral transition (33).

Our model is analogous to the earlier finding that AMPK α 1 plays an important role in peripheral circadian clock entrainment in mammals in response to nutritional status through regulation of nuclear CRY1 phosphorylation and degradation (26, 34–36). Thus, the nutritional status-AMPK-CRY1 pathway may represent a conserved mechanism in higher eukaryotes. Our model is also consistent with a

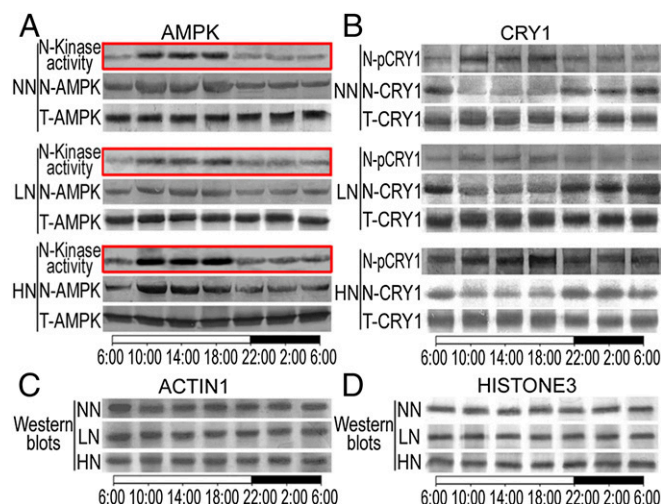


Fig. 4. N enhances nuclear AMPK activity and phosphorylates and destabilizes nuclear CRY1. (A) Nuclear AMPK activity (N-kinase activity), nuclear AMPK protein level (N-AMPK), and total cellular AMPK protein level (T-AMPK) of WT plants grown under 1/20 LN, NN, or HN conditions over a 24-h period. Autoradiographs are marked with red boxes. (B) Nuclear phosphorylated CRY1 (N-pCRY1), nuclear CRY1 protein level (N-CRY1), and total cellular CRY1 protein level (T-CRY1) of WT plants grown under LN, NN, and HN conditions over a 24-h period. (C) ACTIN1 served as a loading control for total cellular proteins. (D) HISTONE3 served as a loading control for nuclear proteins.

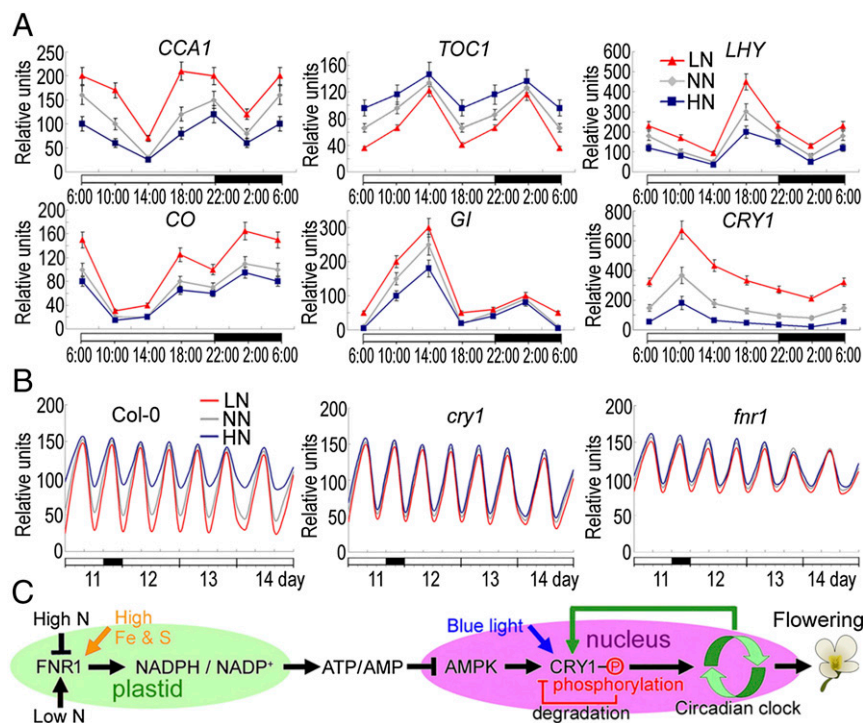


Fig. 5. N suppresses the photoperiod pathway by inhibiting circadian transcript oscillation. (A) mRNA abundances of *CCA1*, *TOC1*, *LHY*, *CO*, *GI*, and *CRY1* in WT plants grown in LN, NN, and HN conditions over a 24-h period. Specific gene expression levels are represented as the percentage relative to *ACTIN7* expression level. Error bars show SDs ($n = 5$). (B) Circadian accumulation of *TOC1* mRNA in WT (*Col-0*), *fnr1*, and *cry1* plants under 3-d continuous light. Samples were collected from seedlings grown in 16-h light/8-h dark for 11 d, and from seedlings that were then transferred to continuous white light for 3 d. (C) Diagram of the putative N-regulated flowering pathway. Phosphorylation of CRY1 triggers its degradation. The CRY1 transcription is feedback-regulated by the circadian clock.

previous finding that the central clock gene *CCA1* acts as a “master regulator” of the organic N response network (37). Expression of *CCA1* is repressed by organic N, and this in turn affects the expression of *glutamine synthetase 1.3* (*GLN1.3*), *glutamate dehydrogenase 1* (*GDH1*), *bZIP1*, and *asparagine synthetase 1* (*ASN1*) (37). Furthermore, chromatin immunoprecipitation assays have shown that *CCA1* directly binds to the promoter regions of *GLN1.3*, *GDH1*, and *bZIP1*. These results support the hypothesis that *CCA1* is a key regulator of N assimilation, and that in turn N metabolites modulate *CCA1* expression, allowing N assimilation to regulate the *Arabidopsis* circadian clock (37).

It has been shown that the major function of CRY1 is to mediate the blue light-dependent de-etiolation process, whereas CRY2 mediates primarily the photoperiod regulation of floral initiation (19, 23). However, in the present study, the *cry2* mutant exhibited normal responses to N, suggesting that CRY2 does not play an essential role in N-regulated flowering. Previous studies showed that CRY2 may mediate photoperiodic control of floral initiation by mediating light suppression of the COP1-dependent degradation of CONSTANS (10, 38), regulating light entrainment of the circadian clock to affect the expression of *CO* (39), or directly modulating the transcription of *FT* through interaction with CIBs, a group of basic helix-loop-helix transcription factors (40). In the present study, we have shown that in response to HN conditions, AMPK-mediated nuclear CRY1 phosphorylation triggers nuclear CRY1 degradation when total cellular CRY1 protein levels remain relatively stable and then inhibit circadian clock oscillations to interfere with flowering control. Consistently, it has been reported that *hy4/cry1* alleles cause late flowering in SD conditions (41), and that a gain-of-function mutation in CRY1 promotes flowering in *Arabidopsis* (42). It also has been reported that the *cry1*, *cry2* double mutation delays flowering in monochromatic blue light, whereas neither monogenic *cry1* nor *cry2* single mutant exhibits late flowering in blue light, suggesting

that CRY1 and CRY2 play a somewhat redundant role in regulation of flowering (43).

Of note, some previous studies have indicated that *Arabidopsis* CRY1 is a light-stable protein, and that blue light-induced CRY1 phosphorylation is not accompanied by a decrease in its steady-state protein level (44). The discrepancies in CRY1 stability and degradation in response to blue light and N signal might be related to CRY1's differing subcellular localizations under different light conditions (45). It is also interesting to note that the blue light-cryptochrome pathways show epistatic effects on plant flowering control, as N-delayed flowering can be overcome by blue light. Although our data suggest that post-translational regulation of nuclear CRY1 protein abundance represents a major regulatory mechanism, transcriptional regulation of *CRY1* also may play a role in this process. Alternatively, the induction of *CRY1* expression by LN conditions might be due to a regulatory feedback mechanism (green arrow in Fig. 5C).

Like HN conditions, nitric oxide (NO) also represses floral transition by inhibiting circadian output of *CO* and *GI* expression in *Arabidopsis* (46), rather than key components of the central oscillator (*LHY*, *CCA1*, or *TOC1*). Therefore, N and NO may regulate plant flowering through different molecular signaling pathways.

In agricultural practice, N shortage causes early flowering, whereas excessive application of N fertilizers usually results in undesirable late flowering and delayed maturation (11). Other environmental stresses, such as salt, drought, heat, cold, and UV stress, also promote flowering, and this has been interpreted as a strategy to ensure seed production for plants grown under unfavorable conditions (47, 48). Designing strategies to control the timing of flowering is of pivotal importance for crop production. Our results reported here suggest that the N-regulated flowering

pathway is adjusted by treatment with blue light or by regulation of *CRY1* expression in *Arabidopsis*, which may offer a new testable solution to managing flowering time in crops.

Materials and Methods

Plant materials and growth conditions, N level adjustments, flowering time analysis, and details of SSH, quantitative RT-PCR, NADPH/NADP⁺ and ATP/AMP determination, protein extraction and immunoblotting (including the

nuclear CRY1 phosphorylation assay), and the immunoprecipitation nuclear AMPK assay are described in detail in *SI Appendix, Materials and Methods*.

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