Supporting Information

Yuan et al. Arabidopsis cryptochrome 1 functions in nitrogen regulation of flowering

Supplemental Materials and Methods

Plant Materials and Growth Conditions. Seeds of *nia1*,*nia2* double mutant were kindly provided by Dr. Wenhao Zhang (Chinese Academy of Sciences, Beijing, China). All other mutants were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, OH) and were all in the Col-0 background. The seed stock numbers for *cry1*, *cry2*, *fnr1*, *fnr2*, *kin10*, *kin11* and *chl1-5* mutants are CS6955 (*hy4-B104*), CS3732, CS832609, SALK_017328C, SALK_052855, SALK_139618C and CS6384 respectively. *kin10*×*kin11* double mutant was acquired by performing genetic crossing and the progenies were analyzed by PCR. The surface-sterilized seeds were sown on 1/2 MS germination plates (1/2 MS media supplied with 1% sucrose). Blue light was supplied by LED light sources, with light intensity of 50 μmol m^{-2} s^{-1}. White light was supplied by cool-white fluorescent lamps, with irradiance fluence rates of 100 μmol m^{-2} s^{-1}. All plant materials were grown under 16/8 h light/dark cycles at 25±1°C for 11 days or then transferred to continuous white light for additional 4 days.

MS media contain 20 mM NH_4NO_3 and 18.8 mM KNO_3 (58.8 mM nitrogen). For 1/20 N growth condition, NH_4NO_3 and KNO_3 were reduced to 1 mM and 0.94 mM respectively, and complemented with 19 mM NaCl and 17.86 mM KCl. For 1/50 N growth condition, NH_4NO_3 and KNO_3 were reduced to 0.4 mM and 0.38 mM respectively, and complemented with 19.6 mM NaCl and 18.42 mM KCl. For high N growth condition, NH_4NO_3 and KNO_3 were enhanced to 40 mM and 37.6 mM respectively. For 1/20 NO_3^- condition, NH_4NO_3 and KNO_3 were replaced by 2.94 mM $NaNO_3$ and 18.8 mM KCl respectively. For high NO_3^- condition, NH_4NO_3 and KNO_3 were replaced by 117.6 mM $NaNO_3$ and 18.8 mM KCl respectively. For 1/20 NH_4^+ condition, NH_4NO_3 and KNO_3 were replaced by 2.94 mM NH_4Cl and 18.8 mM KCl respectively. For high NH_4^+ condition, NH_4NO_3 and KNO_3 were replaced by 117.6 mM NH_4Cl and 18.8 mM KCl respectively. MS media contains 0.1 mM $FeSO_4 \cdot 7H_2O$. For high Fe & S growth condition, $FeSO_4 \cdot 7H_2O$ was enhanced to 0.4 mM.

To study the role of AMPK in nitrogen-regulated flowering, AMPK inhibitor Dorsomorphin (20 μM; 6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine, Sigma-Aldrich, St Louis, MO, USA) and AMPK agonist aminoimidazole carboxamide ribonucleotide (2 mM; AICAR; Sigma-Aldrich, St Louis, MO, USA) were supplemented in the MS media directly.

To observe phenotypes of Arabidopsis plants grown in soil (2.6 mg kg^{-1} NH_4-N, 3.6 mg kg^{-1} NO_3-N, pH 6.3), low N, medium N and high N treatments were applied by irrigation with water, 1/4 N solution (5 mM NH_4NO_3 and 4.7 mM KNO_3) and full N solution (20 mM NH_4NO_3 and 18.8 mM KNO_3) respectively.

Analysis of Flowering Time. For seedlings grown in MS media in petri dishes, because it was difficult to score the number of leaves at the stage of flowering, we scored the number of rosette leaves at the stage of bolting when stems were about 3 mm, as performed previously (1). We also scored days to bolting. 20 rosettes for each sample were counted. The experiments were repeated three times.

Two-Round Suppression Subtractive Hybridization (SSH). Low N conditions should trigger some early events (enhancing some gene expression), which would induce an earlier floral transition, while HN would repress these genes. Therefore, we paid attention to floral-transition-related genes induced by the low-nitrogen conditions. Four total RNA samples were collected: [1] Normal N before floral transition at

the 7th day; [2] Normal N after floral transition at the 13th day; [3] 1/20 N before floral transition at the 7th day; [4] 1/20 N after floral transition at the 11th day. Total RNA was isolated using TRIzol Reagent® (Molecular Research Center, Cincinnati, OH, USA). First strand cDNA was synthesized with 1 μg total RNA. The reaction conditions were as follows: 10 pmol anchor oligo dT (**CDS-PT**, containing a *Rsa* I enzyme cutting site) was annealed to 1 μg total RNA in a volume of 10 μl RNase-free water, by heating the mixture at 70°C for 10 min, followed by cooling on ice for 5 min. Transcription was then initiated by mixing the annealed RNA with 200 U PowerScript™ Reverse Transcriptase (Clontech, Mountain View, CA, USA) in a final volume of 20 μl containing 1× first-strand buffer, 10 mM dithiothreitol (DTT), 10 pmol T-S primer (**AD-G**, containing a *Rsa* I site) (2), and 1 mM each of dATP, dGTP, dCTP, and dTTP. The reaction was incubated at 42°C for 90 min.

Subtraction hybridization usually requires 2 μg of driver cDNA. SMARTer™ PCR cDNA Synthesis Kit (Clontech) with the template-switching RT-PCR method produces over 2 μg double-stranded cDNA originated from 1-1000 ng of total RNA (3). Aliquots (1-2 μl) of first strand cDNA were subjected to a 2×EasyTaq PCR SuperMix system (TransGen Biotech. Co. Ltd., Beijing, China) in 50 μl with the T-S PCR primer (**AD-PCR** for single-primer amplification). 95°C 1 min, and then 28-36 cycles: 95°C 30 sec, 58°C 30 sec, 72°C 2.5 min. The double-stranded cDNA products were purified with E.Z.N.A®. Cycle-Pure Kit (Omega Biotech Corporation, Victoria, BC, Canada) and quantified.

Then standard SSH procedures were followed (3). Briefly: [1] Rsa I Digestion; [2] Adaptor Ligation to Tester cDNA; [3] First Hybridization; [4] Second Hybridization; [5] Suppression Nested PCR Amplification (3). For convenience, PCR-Select™ cDNA Subtraction Kit (Clontech) was used. Then 2 μg purified PCR products of the first SSH were used as the driver cDNA and the tester cDNA respectively as indicated in Fig. S3. Then standard SSH procedures were followed (3). All primers for SSH are shown in Table S5.

Results from Sample [2] subtracted from Sample [1] would obtain floral-transition-related genes in NN conditions (Result "NN"); results from Sample [4] subtracted from Sample [3] would obtain floral-transition-related genes at 1/20 N conditions (Result "LN"). Then Result "LN" subtracted from Result "NN" would lead to the final Result "NL". Alternatively, results from Sample [3] subtracted from Sample [1] would reveal low-N-induced genes before floral transition (Result "BF") and results from Sample [4] subtracted Sample [2] would reveal low-N-induced genes after floral transition (Result "AF"). Then Result "AF" subtracted from Result "BF" would lead to the final Result "BA" (Fig. S3).

After secondary SSH, PCR products were ligated into pEASY-T1 Simple vector (TransGen Biotech. Co. Ltd., Beijing, China) and positive clones were screened by blue or white plaques. 50 random positive clones for each PCR product were sequenced.

Quantitative Real-Time PCR. *LFY* (*LEAFY*), *FT* (*FLOWERING LOCUS T*) and *ACT7* (*ACTIN7*) expression levels were detected by common reverse transcription (RT)-PCR. The other transcripts were detected by quantitative real-time PCR analysis. The cDNA was amplified by using 2×EasyTaq PCR SuperMix system (TransGen Biotech. Co. Ltd.). The *Ct* (threshold cycle), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence was first detected, was used as a measure for the starting copy numbers of the target gene (4). Three technical replicates were performed for each experiment. *ACTIN7* gene was used as an internal control. The specific gene expression levels are represented as the percentages relatively to *ACTIN7* expression levels. All primers are shown in Table S6.

For analysis of circadian transcripts, plants were grown side-by-side in petri dishes for 11 days (16/8 h light/dark cycles; when the circadian clock was stable). Some of seedlings were then transferred to

continuous white light for 3 days. Every 4 hours, shoots from five plants were pooled for RNA extraction. Then the transcript levels were detected by quantitative real-time PCR analysis. Excerpt that *FNR1* is not a circadian transcript. Therefore, samples from only two time-points (2:00 and 10:00) were collected for *FNR1* expression detection.

Determination of NADPH / $NADP^+$ and ATP / AMP. 11-day-old seedlings were harvested. Plastidial $NADP^+$ and NADPH were quantified by using $NADP^+$/NADPH Quantification Kit (BioVision, Milpitas, CA, USA). The ATP and AMP contents were measured by high-performance liquid chromatography (HPLC, Dionex Corporation, Sunnyvale, CA, USA) as previously described (5).

Preparation of Protein Extracts and Immunoblotting. 11-day-old seedlings were harvested. The plant tissues were ground and homogenized in extraction buffer containing 50 mM HEPES-KOH (pH 7.4), 50 mM NaCl, 10 mM EDTA, 5 mM NaF, 1 mM Na_3VO4, 0.1% Triton-X 100, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1× protease inhibitor cocktail (Sigma) (6). Arabidopsis nuclear extracts were prepared by following the chromatin immunoprecipitation method (7). Briefly, nuclei were purified from total protein extracts with Percoll extraction buffer (95% V/V Percoll, 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 5 mM NaF, 1× protease inhibitor cocktail) and washed twice with Nuclei resuspension buffer (10% Glycerol, 50 mM Tris-HCl, pH 8.0 5 mM $MgCl_2$, 10 mM β- mercaptoethanol, 5 mM NaF, 1× protease inhibitor cocktail) by 10 min of centrifugation at 12,000 *g*. Then nuclei were lysised in Nuclei lysis buffer [50 mM Tris-HCl, pH 8.0 10 mM EDTA, 1% Sodium dodecyl sulfonate (SDS), 5 mM NaF, 1× protease inhibitor cocktail].

SDS - polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis of the extracts were processed according to the method as described previously (6). Total protein extracts or nuclear extracts containing 20 μg proteins were loaded in each lane. For Western Blotting, the proteins were electron-transferred onto nitrocellulose films. Antibodies used were anti-human AMPK alpha 1 [Abcam (Hong Kong) Ltd., New Territories, HK], anti-Arabidopsis CRY1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Arabidopsis HISTONE3 and anti-Arabidopsis ACTIN1 IgG (AgriSera Comp., Umea, Sweden). Alkaline phosphatase-conjugated antibodies were used as the secondary antibodies.

For nuclear CRY1 phosphorylation assay, CRY1 protein from the nuclear protein extract (200 μg) was immunoprecipitated with the CRY1 antibody, and then was subjected to Western blotting with anti-phosphoserine antibody (Abcam). CRY1 protein-half-life assay with cycloheximide treatment was performed according to the method of Ahmad *et al*. (1998) (8). Validity and specificity of CRY1 antibody and anti-phosphoserine antibody were confirmed by enzyme kinetics studies (with the Blue-light treatment and then alkaline phosphatase treatment) or by using the mutants (*cry1* and *cry2*; Fig. S6C).

Immunoprecipitation Nuclear Adenosine Monophosphate-Activated Protein Kinase (AMPK) Assay. For immunoprecipitation kinase assays, nuclear protein extract (200 μg) was incubated with AMPK-specific antibody, for 1 hr at 4°C, and 20 μl protein A-agarose (GE) was subsequently added and incubated for other 3 hr at 4°C (6). The kinase on the beads was collected by centrifugation and washed three times with extraction buffer and one time with 10×reaction buffer (400 mmol/L HEPES, pH 7.4, 50 mmol/L $MgCl_2$, 800 mmol/L NaCl, and 1 mmol/L DTT) (9). AMPK activity was measured using the HMRSAMSGLHLVKRR (SAMS) peptide as previously described (9). Kinase assays on the immunoprecipitated AMPK were performed at 37°C for 30 min in 50 μL of a reaction mixture containing 5 μL of 10×reaction buffer, 10 μL of ATP working stock (0.1 μL of 100 mmol/L ATP, 1 μL of [^{32}P]ATP, and

8.9 μL of H_2O), 10 μL of SAMS peptide (1 g/L), and 25 μL of H_2O (9). Reactions were ended by the addition SDS-PAGE loading buffer. Phosphorylation of SAMS was analyzed by autoradiography after 15% SDS-PAGE (6).

Prokaryotic Protein Expression. The Arabidopsis CDSs (At3g01090, At3g29160, At5g39440 and At2g26980) were used as templates to amplify *KIN10*, *KIN11*, *SnRK1.3* and *CIPK3* respectively, and finally cloned into the EcoR I and Hind III sites of pGEX-KG (Amersham Bioscience, Uppsala, Sweden) Vector. The whole constructs were transformed into *Escherichia coli* BL21(DE3) pLyss Chemically Competent Cell (TransGen Biotech. Co. Ltd.) after DNA sequencing confirming, and expression of the fusion protein was induced by 0, 0.5, 1 or 1.5 mM IPTG (isopropyl thiogalactoside) for 2h at 37°C. The induced *E. coli* were harvested and lysed in PBS buffer with 1mM PMSF (Phenylmethanesulfonyl fluoride) and proteinase inhibitor cocktail (Sigma-Aldrich). GST-AMPKα1 fusion proteins were purified with the Glutathione-Sepharose 4B column (Amersham Bioscience). Then SDS-PAGE and Western blotting with the AMPKα1 antibody were processed according to the method as described previously (6).

Statistics Analysis. Most experiments were repeated three times, and typical results are presented and the standard deviations were shown. SSH was repeated once and the results were merged. Student's *t* test was used for comparison between different treatments. A difference was considered to be statistically significant when $p < 0.05$.

Supplemental References

1. Yanovsky MJ, Kay SA (2002) Molecular basis of seasonal time measurement in Arabidopsis. *Nature* 419:308–312.
2. Huang JC, Chen F (2006) Simultaneous amplification of 5’ and 3’ cDNA ends based on template-switching effect and inverse PCR. *BioTechniques* 40:187–189.
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4. Zhang ZW, et al. (2011) Mg-protoporphyrin, haem and sugar signals double cellular total RNAs against herbicide and high-light-derived oxidative stress. *Plant Cell Environ* 34:1031–1042.
5. Hahn-Windgassen A, et al. (2005) Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. *J Biol Chem*280:32081–32089.
6. Gao M, et al. (2009) Regulation of cell death and innate immunity by two receptor-like kinases in Arabidopsis. *Cell Host Microbe* 6:34–44.
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8. Ahmad M, Jarillo JA, Cashmore AR (1998) Chimeric proteins between cry1 and cry2 Arabidopsis blue light photoreceptors indicate overlapping functions and varying protein stability. *Plant Cell* 10:197–207.
9. Zou MH, et al. (2003) Activation of 5′-AMP-activated kinase is mediated through c-Src and phosphoinositide 3-kinase activity during hypoxia-reoxygenation of bovine aortic endothelial cells. Role of peroxynitrite. *J Biol Chem* 278:34003–34010.

Supplemental Figure 1

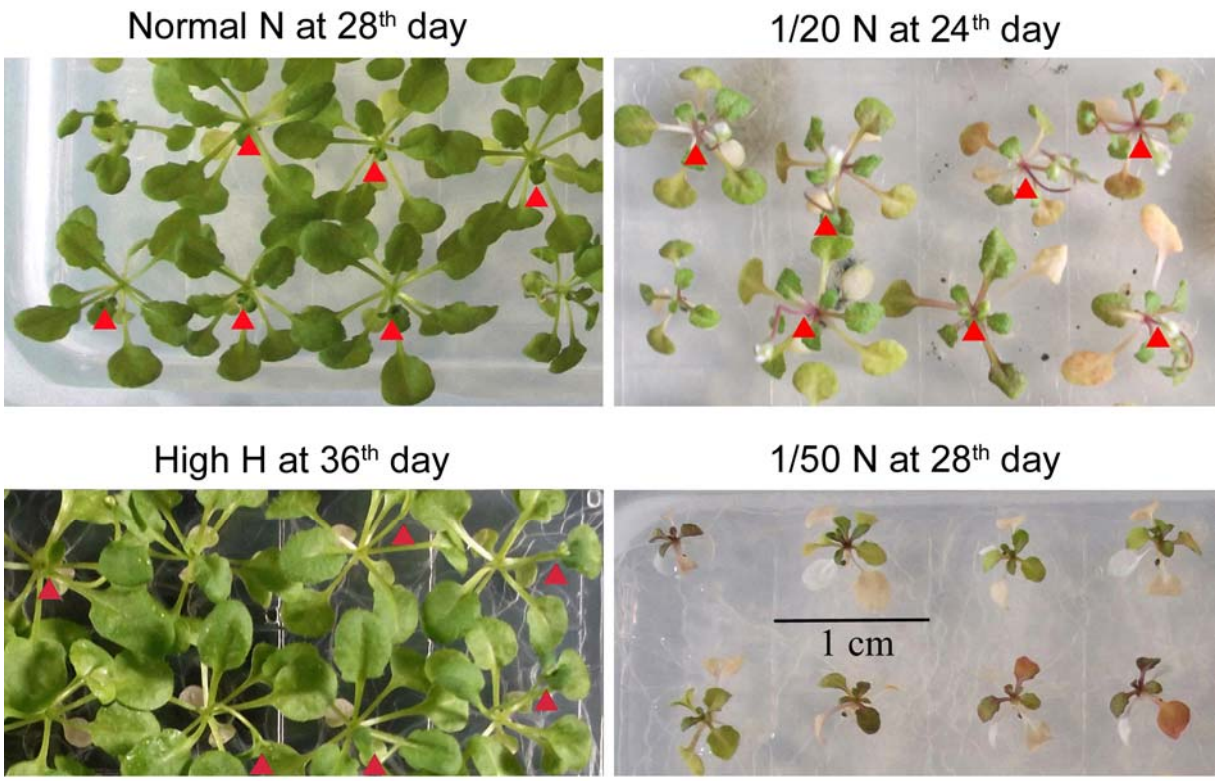


Fig. S1. Phenotypes of wild-type plants grown in MS media supplied with different levels of nitrogen. Red triangles indicate bolting stems.

Supplemental Figure 2

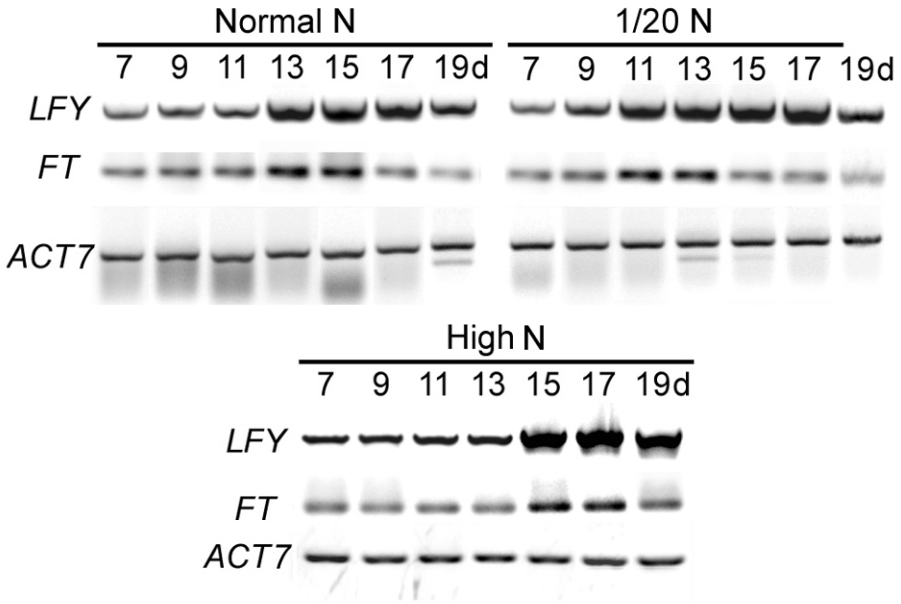


Fig. S2. *LFY* and *FT* expression patterns show the floral transition times. *ACTIN7* (*ACT7*) was used as an internal control. d, days after germination.

Supplemental Figure 3

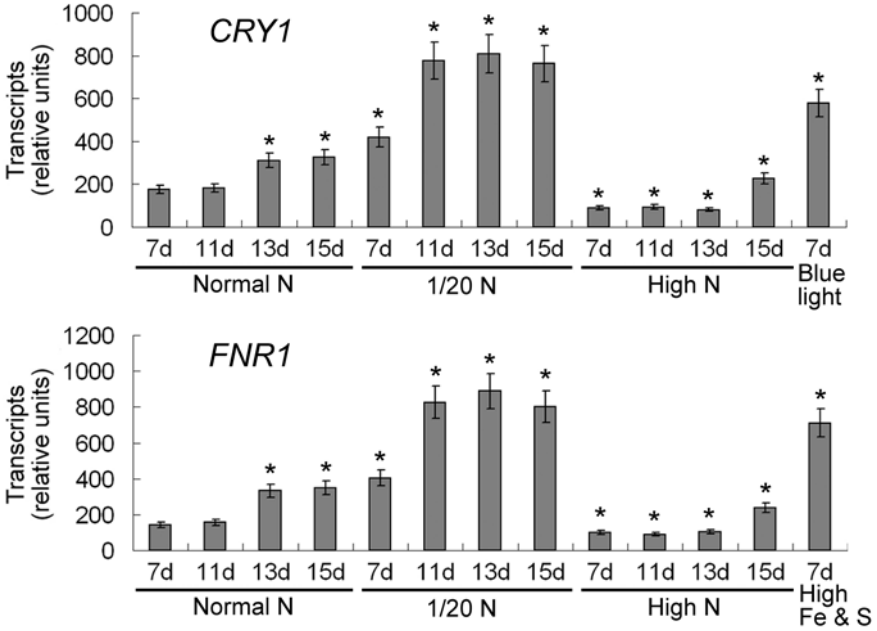


Fig. S3. *CRY1* and *FNR1* expression patterns prove the validity of SSH results. *CRY1* and *FNR1* transcripts were detected by quantitative real-time PCR analysis. The specific gene expression levels are represented as the percentages relatively to *ACTIN7* expression levels. Error bars show standard deviations (n = 5). *Statistically significant different from the sample of Normal N at the 7th day ($p < 0.05$).

Supplemental Figure 4

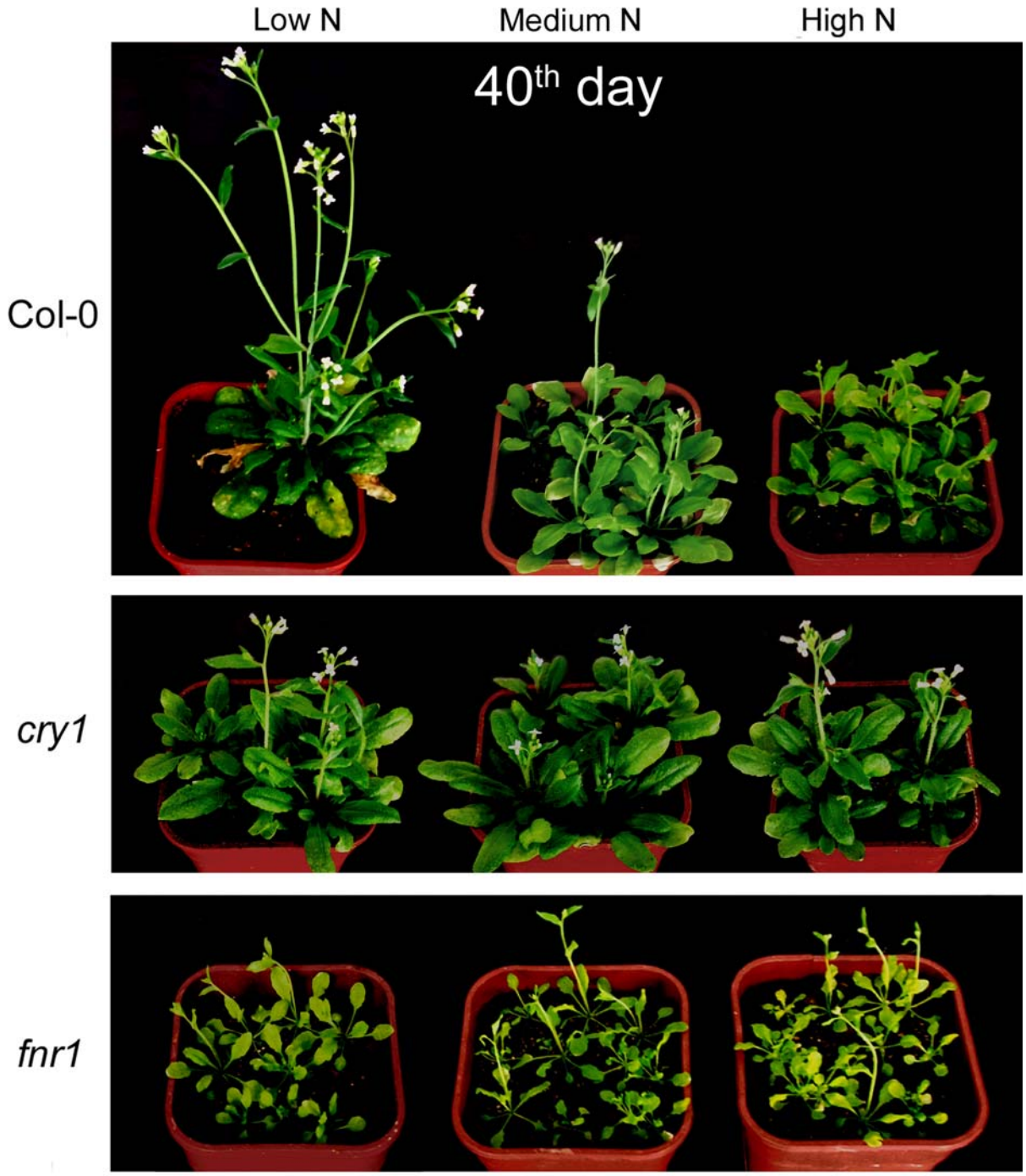


Fig. S4. 40-day-old plants of *fnr1* mutant, *cry1* mutant and wild-type Columbia (Col-0) grown in soil under low-N, medium-N or high-N conditions. See the METHODS on line for details.

Supplemental Figure 5

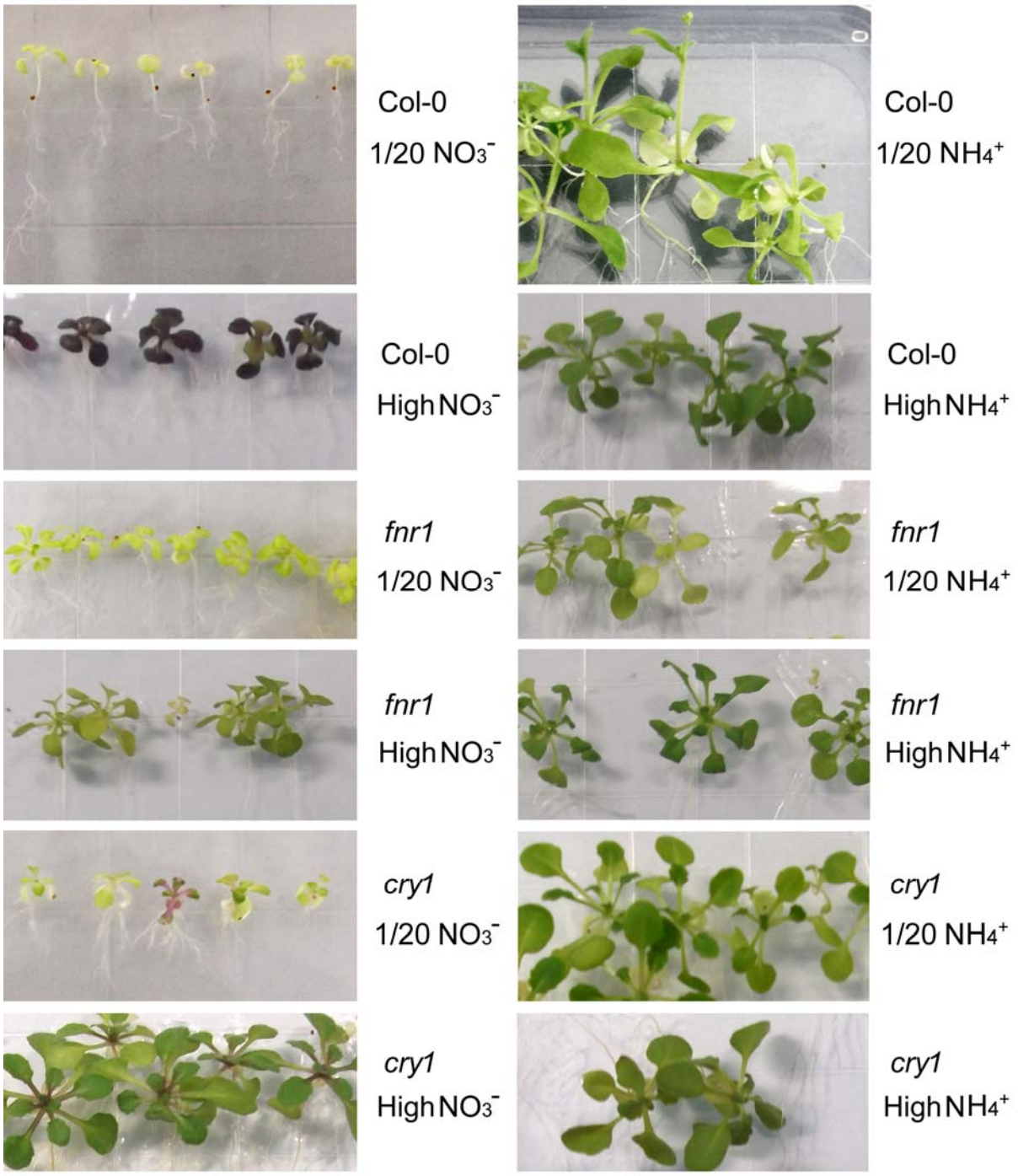
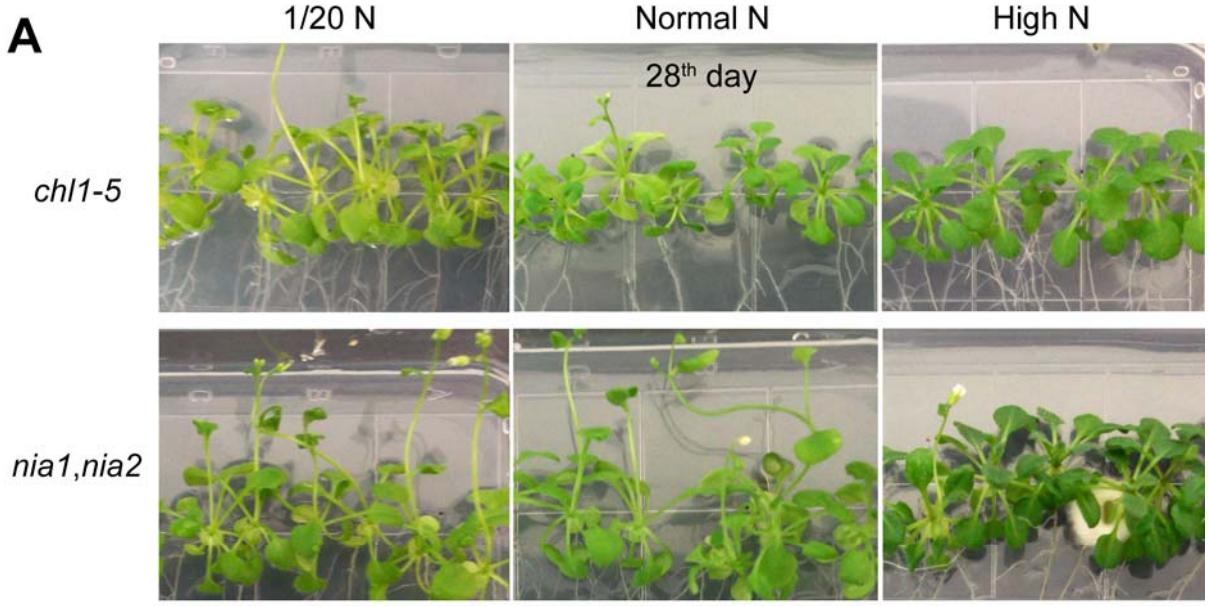


Fig. S5. Phenotypes of *cry1* mutant and wild-type plants (Col-0) grown in MS media supplied with different levels of nitrate nitrogen or ammonium nitrogen. 28-day old seedlings are shown.

Supplemental Figure 6



B

Days to bolting	1/20 N	NN	HN
Col-0	21±1.2	25±1.2	32±1.8
chl1-5	24±1.0	29±1.4	35±1.8
nia1,nia2	20±0.8	21±1.0	28±1.6

Leaf number	1/20 N	NN	HN
Col-0	8±0.4	9±0.8	10.5±0.8
chl1-5	9±0.6	10±0.8	11±0.8
nia1,nia2	7.5±0.4	8±0.6	9.5±0.6

Fig. S6. *chl1-5* mutant and *nia1*,*nia2* double mutant show normal responses to nitrogen. (*A*) 28-day-old *chl1-5* mutant and *nia1*,*nia2* double mutant grown in MS media under different nitrogen conditions. (*B*) Flowering times of *chl1-5* mutant and *nia1*,*nia2* double mutant grown in MS media. Days to flowering and rosette leaf number were scored (mean ± SD; $n \geq 25$ plants). 1/20 low-N condition; NN, normal N condition; HN, high N condition.

Supplemental Figure 7

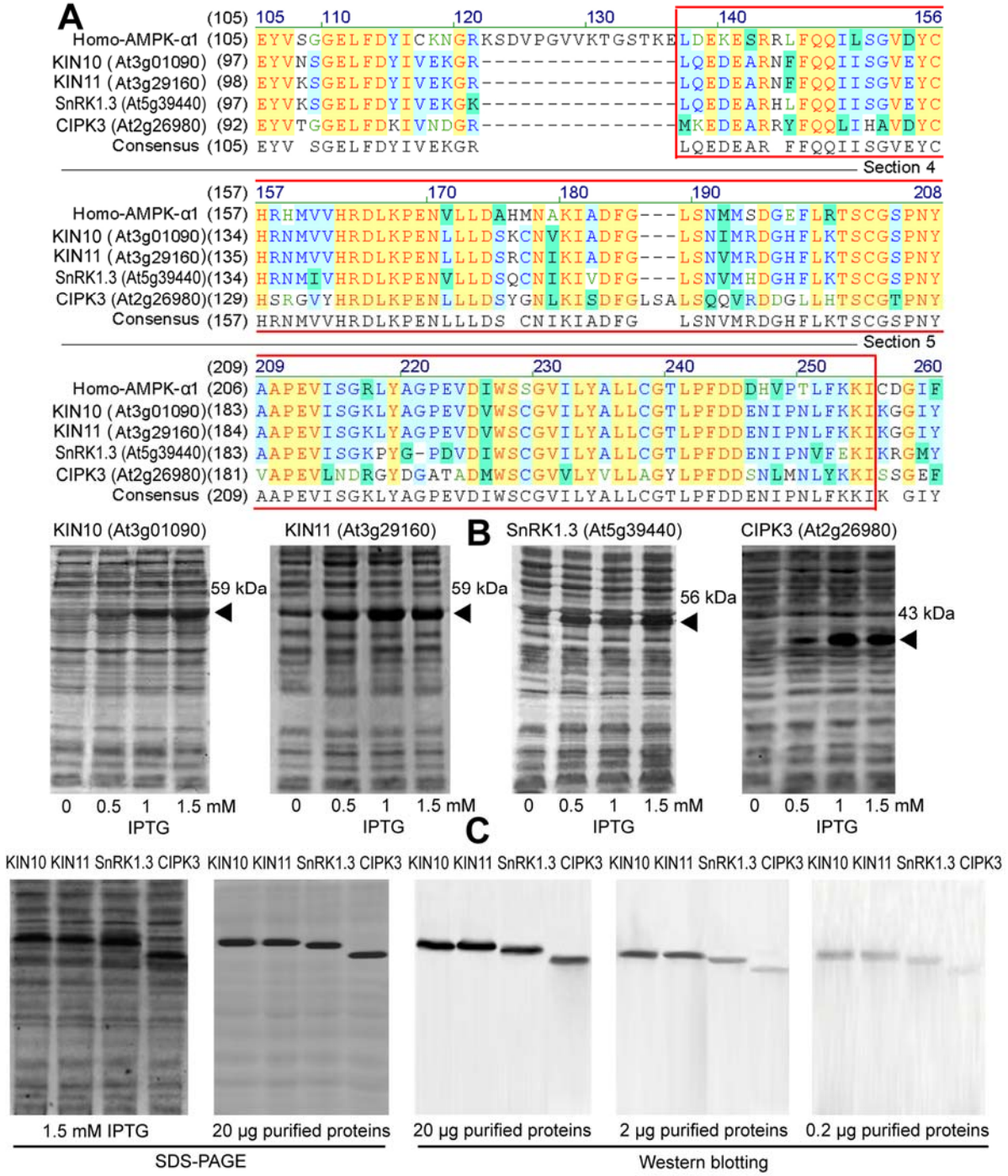


Fig. S7. Validity and specificity of AMPKα1 antibody. (*A*) Alignments of human AMPKα1 amino acid sequences (AAH48980) with four Arabidopsis homologues [KIN10 (At3g01090), KIN11 (At3g29160), SnRK1.3 (At5g39440) and CIPK3 (At2g26980)]. 137-252 amino acid sequences within the catalytic subunit (marked with a red box) of KIN10, KIN11, SnRK1.3 and CIPK3 proteins share similarities of 79.3%, 79.3%, 71.0% and 56.3% with the human AMPKα1 peptide respectively (*B*) Prokaryotic expression of Arabidopsis KIN10, KIN11, SnRK1.3 and CIPK3 proteins. The proteins were induced by 1.5 mM IPTG for 2h at 37°C. Arrows show the IPTG-induced proteins. (*C*) Western blots with the AMPKα1 antibody to the purified proteins expressed in the prokaryotic expression system.

Supplemental Figure 8

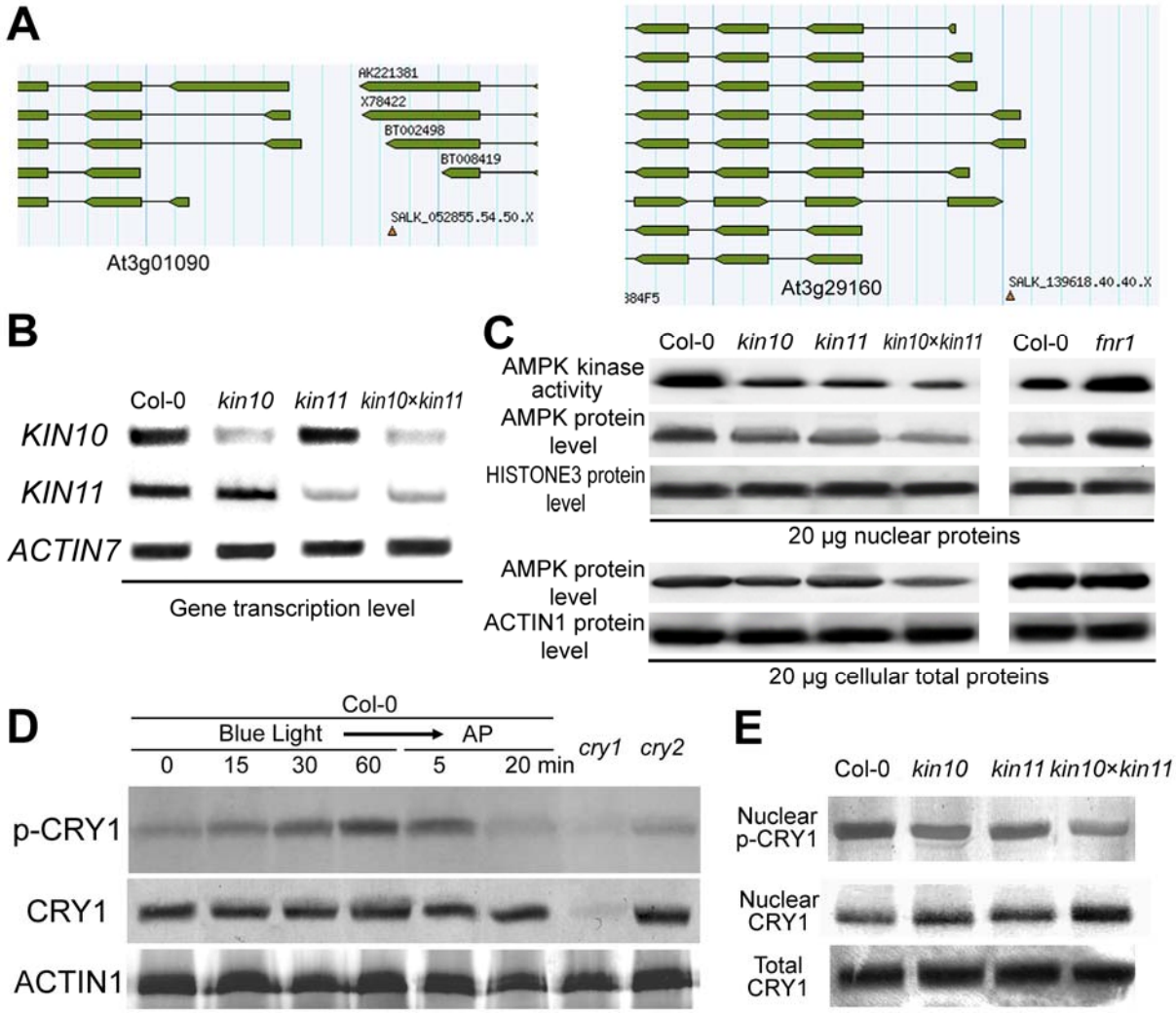
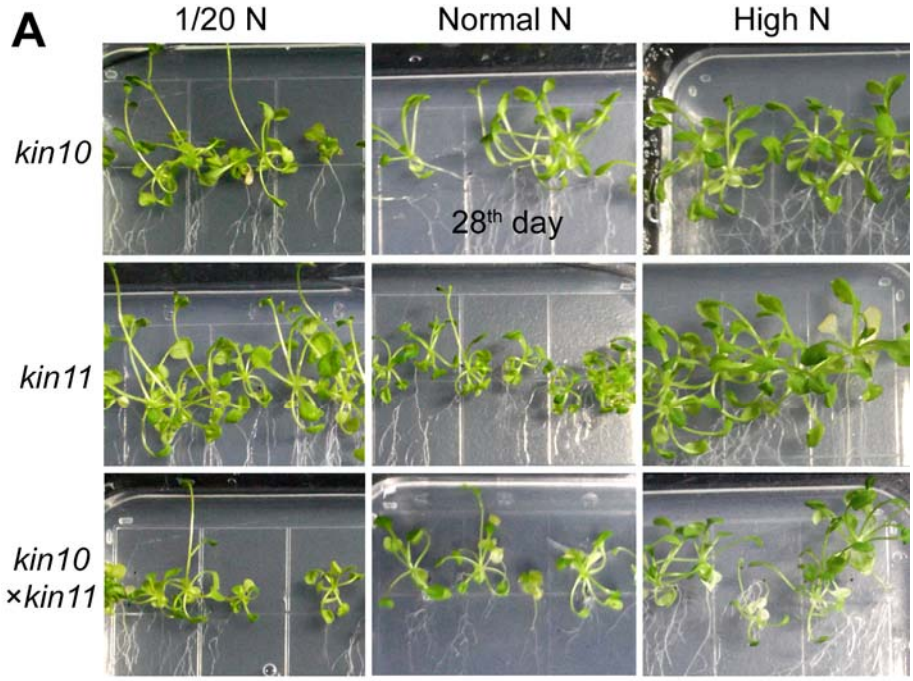


Fig. S8. Validity of CRY1 antibody and anti-phosphoserine antibody in in *kin10* mutant, *kin11* mutant and *kin10*×*kin11* double mutant. (*A*) T-DNA insertion sites of *kin10* and *kin11* T-DNA insertional mutant lines. The T-DNAs were inserted in the -300 to +50 bp region of the two genes. (*B*) *KIN10* and *KIN11* gene transcription level in *kin10* mutant, *kin11* mutant, *kin10*×*kin11* double mutant and the wild-type plants. *ACTIN7* gene was used as a loading control. (*C*) Nuclear AMPK activity, nuclear AMPK protein level and cellular total AMPK protein level of *kin10* mutant, *kin11* mutant, *fnr1* mutant, *kin10*×*kin11* double mutant and the wild-type plants. 11-day-old seedlings grown on normal N media were collected at 10:00 am in the 16/8 h light/dark cycle. (*D*) Validity and specificity of CRY1 antibody and anti-phosphoserine antibody. 11-day-old seedlings were treated with the Blue-light for 15, 30 and 60 min, and then total proteins were extracted immediately. 5 mg protein from the sample of 60-min Blue-light treatment was centrifuged and resuspended in 50 mM Tris-HCl (pH 8.5) and incubated with 0.5 units of alkaline phosphatase (AP, type VII-SA, Sigma) for 5 or 20 min at 30°C. For CRY1 phosphorylation assay, CRY1 protein from total protein extract (200 μg) was immunoprecipitated with the CRY1 antibody, and then was subjected to Western blotting with anti-phosphoserine antibody. p-CRY1, phosphorylated CRY1. (*E*) Nuclear phosphorylated CRY1, nuclear CRY1 protein level and cellular total CRY1 protein level of *kin10* mutant, *kin11* mutant, *kin10*×*kin11* double mutant and the wild-type plants. 11-day-old seedlings grown on normal N media were collected at 10:00 am in the 16/8 h light/dark cycle.

Supplemental Figure 9



B

Days to bolting	1/20 N	NN	HN
Col-0	21±1.2	25±1.2	32±1.8
kin10	21±1.0	25±1.4	32±1.6
kin11	21±1.4	25±1.2	32±1.8
kin10×kin11	22±1.4	25±1.2	29±1.6

Leaf number	1/20 N	NN	HN
Col-0	8±0.4	9±0.8	10.5±0.8
kin10	8±0.6	9±0.4	10.5±0.8
kin11	8±0.6	9±0.8	10.5±0.6
kin10×kin11	8±0.4	9±0.6	10±0.8

Fig. S9. *kin10×kin11* double mutant is relatively insensitive to nitrogen levels. (*A*) 28-day-old *kin10* mutant, *kin11* mutant and *kin10×kin11* double mutant grown in MS media under different nitrogen conditions. (*B*) Flowering times of *kin10* mutant, *kin11* mutant and *kin10×kin11* double mutant grown in MS media. Days to flowering and rosette leaf number were scored (mean ± SD; $n \geq 25$ plants). 1/20 low-N condition; NN, normal N condition; HN, high N condition.

Supplemental Figure 10

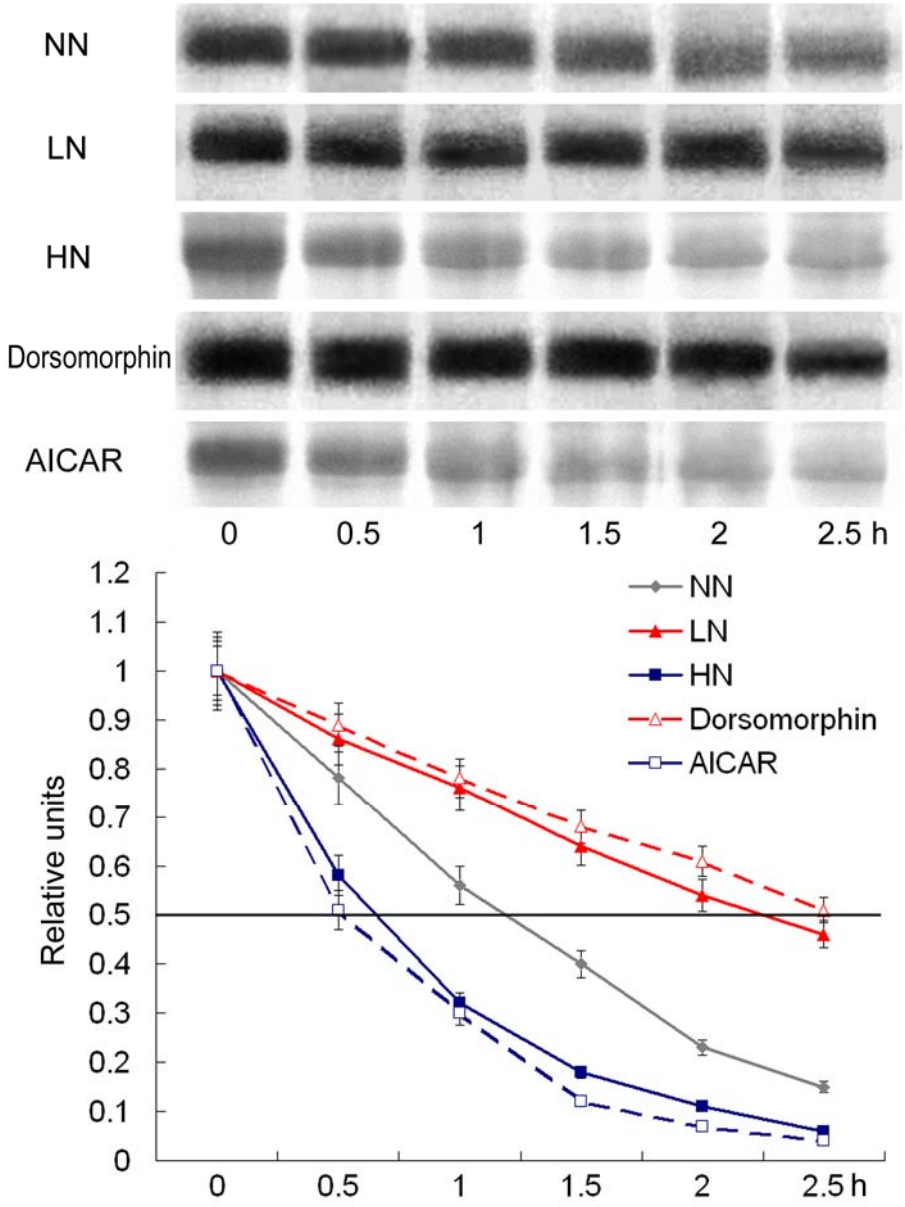


Fig. S10. Nuclear CRY1 protein half-lives of the wild-type seedlings grown under different nitrogen conditions. 11-day-old wild-type seedlings grown under 1/20 low-N (LN), normal-N (NN) or high-N (HN) conditions, or under NN condition but treated with 20 μM Dorsomorphin or 2 mM AICAR were submerged to 25 mL 0.1% DMSO alone (mock) or 300 mM cycloheximide (overlap to the medium) for 2 hr before the illumination beginning in the light-dark cycle. Then tissue was harvested into liquid nitrogen 30 min for the preparation of nuclear protein extracts and Western blotting with the CRY1 antibody. CRY1 band level of each growth condition without cycloheximide treatment (0 h) was normalized to 100% (1.0 relative unit). CRY1 half-lives of LN, NN, HN, Dorsomorphin-treated and AICAR-treated seedlings were 2.4, 1.2, 0.6, 2.5 and 0.5 h respectively. Error bars show standard deviations (n = 3).

Supplemental Figure 11

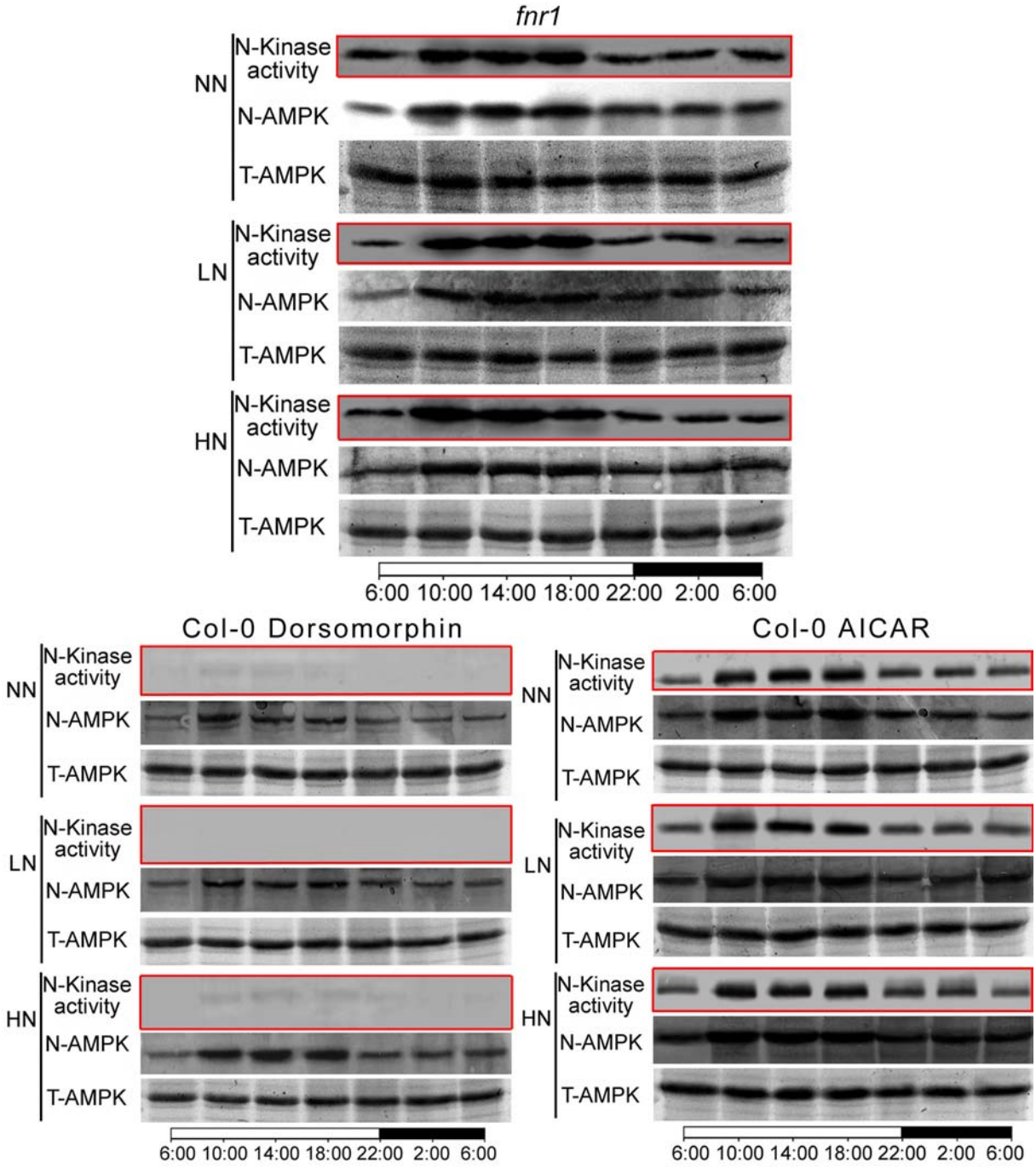


Fig. S11. Nuclear AMPK activity (N-Kinase activity), nuclear AMPK protein level (N-AMPK) and cellular total AMPK protein level (T-AMPK) of *fnr1* mutant and wild-type plants (Col-0) treated with Dorsomorphin or AICAR over a 24-hour time course. For nuclear AMPK kinase activity assay in Dorsomorphin-treated plant nuclei, 20 μM Dorsomorphin was added in the kinase reaction buffer. For nuclear AMPK kinase activity assay in AICAR-treated plant nuclei, 2 mM AICAR was added in the kinase reaction buffer. Autoradiographs of AMPK kinase-activity assay are marked with red boxes. LN, 1/20 low-N condition; NN, normal N condition; HN, high N condition.

Supplemental Figure 12

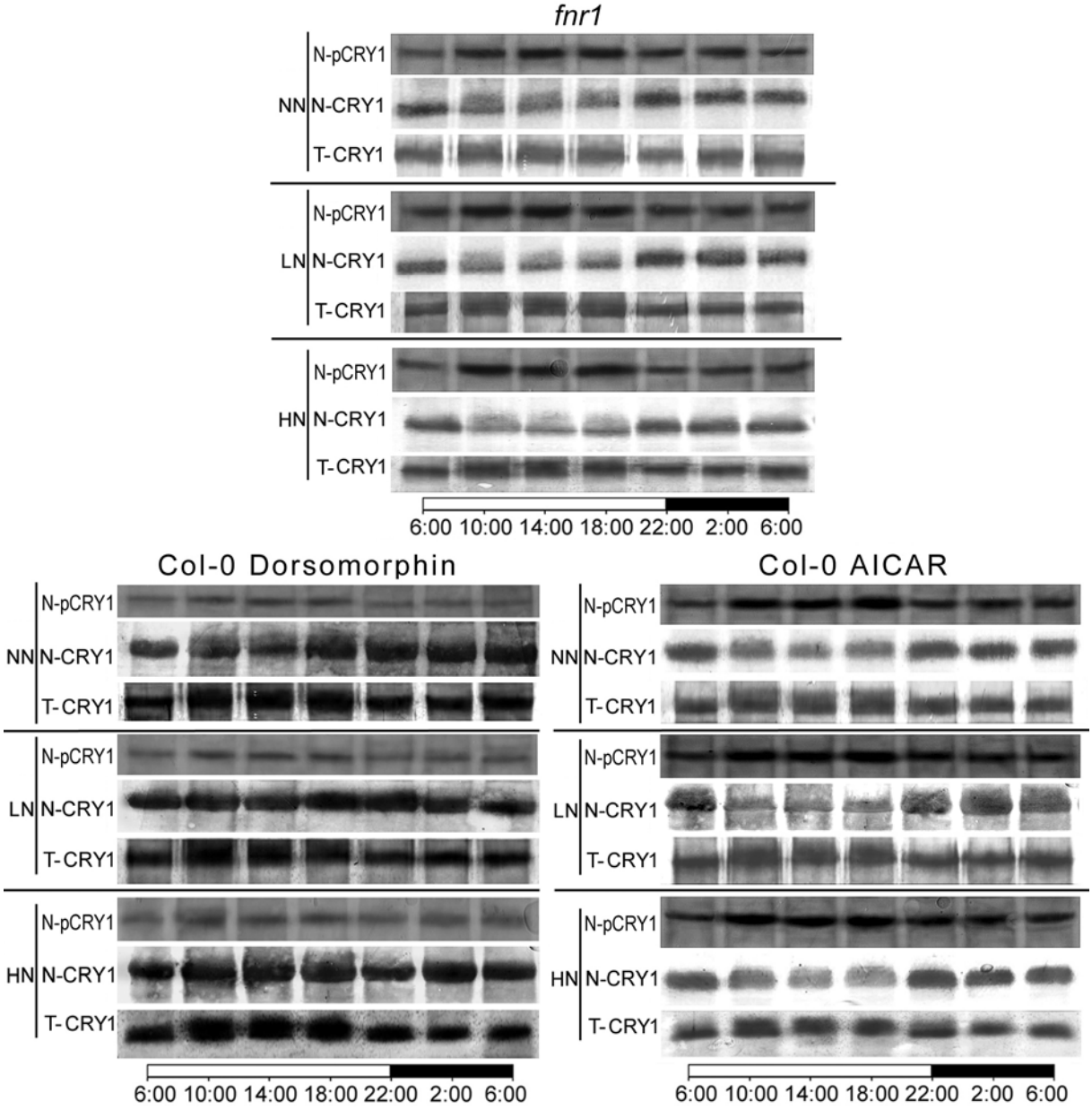


Fig. S12. Nuclear phosphorylated CRY1 (N-pCRY1), nuclear CRY1 protein level (N-CRY1) and cellular total CRY1 protein level (T-CRY1) of *fnr1* mutant and wild-type plants (Col-0) treated with Dorsomorphin or AICAR over a 24-hour time course. 20 μM Dorsomorphin or 2 mM AICAR was added in the medium for wild-type plants. LN, 1/20 low-N condition; NN, normal N condition; HN, high N condition.

Supplemental Figure 13

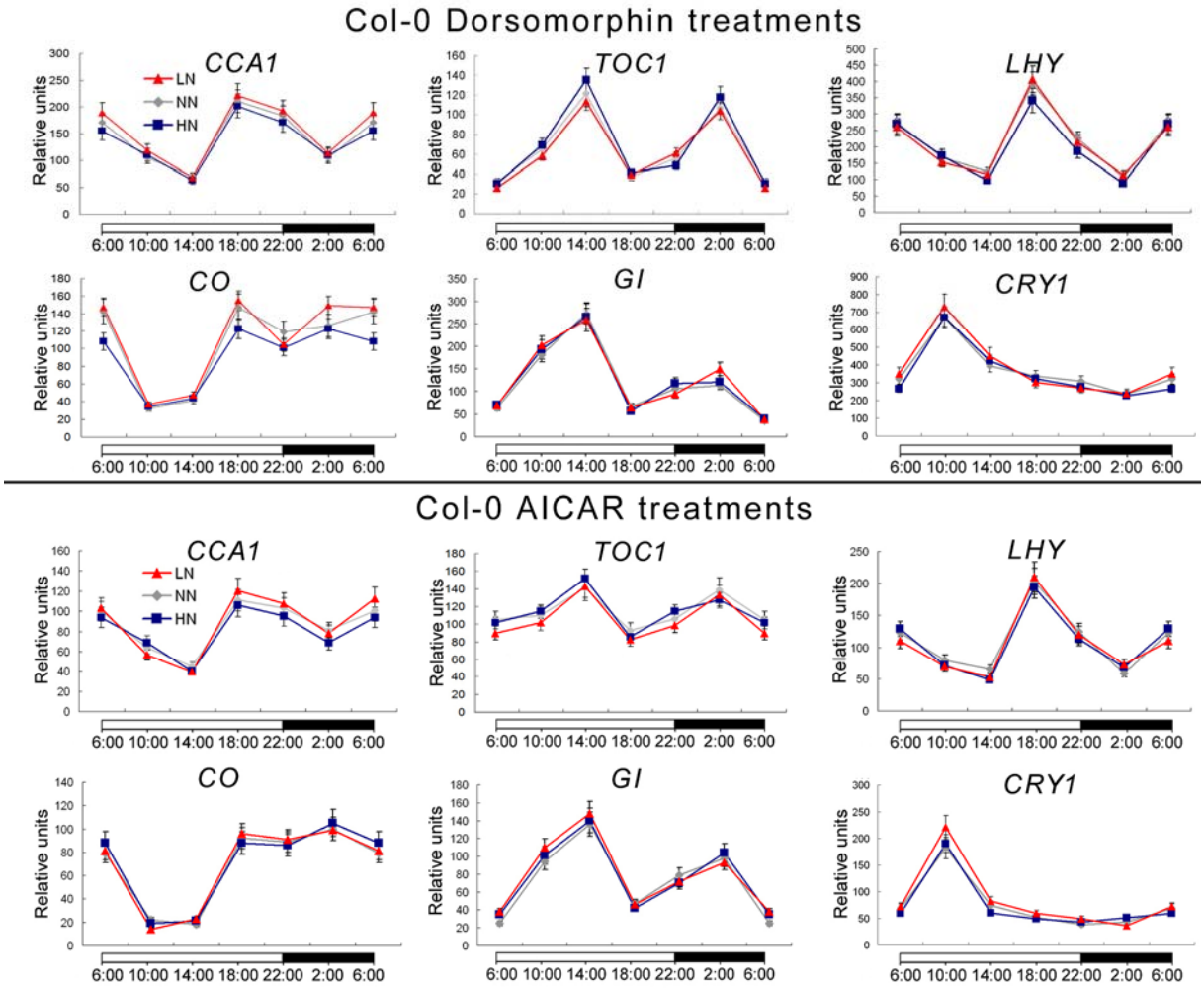


Fig. S13. mRNA abundances of *CCA1*, *TOC1*, *LHY*, *CO*, *GI*, *FNR1* and *CRY1* in Dorsomorphin-treated or AICAR-treated wild-type plants (Col-0) over a 24-hour time course. The specific gene expression levels are represented as the percentages relatively to *ACTIN7* expression levels. Error bars show standard deviations (n = 5). LN, 1/20 low-N condition; NN, normal N condition; HN, high N condition.

Supplemental Figure 14

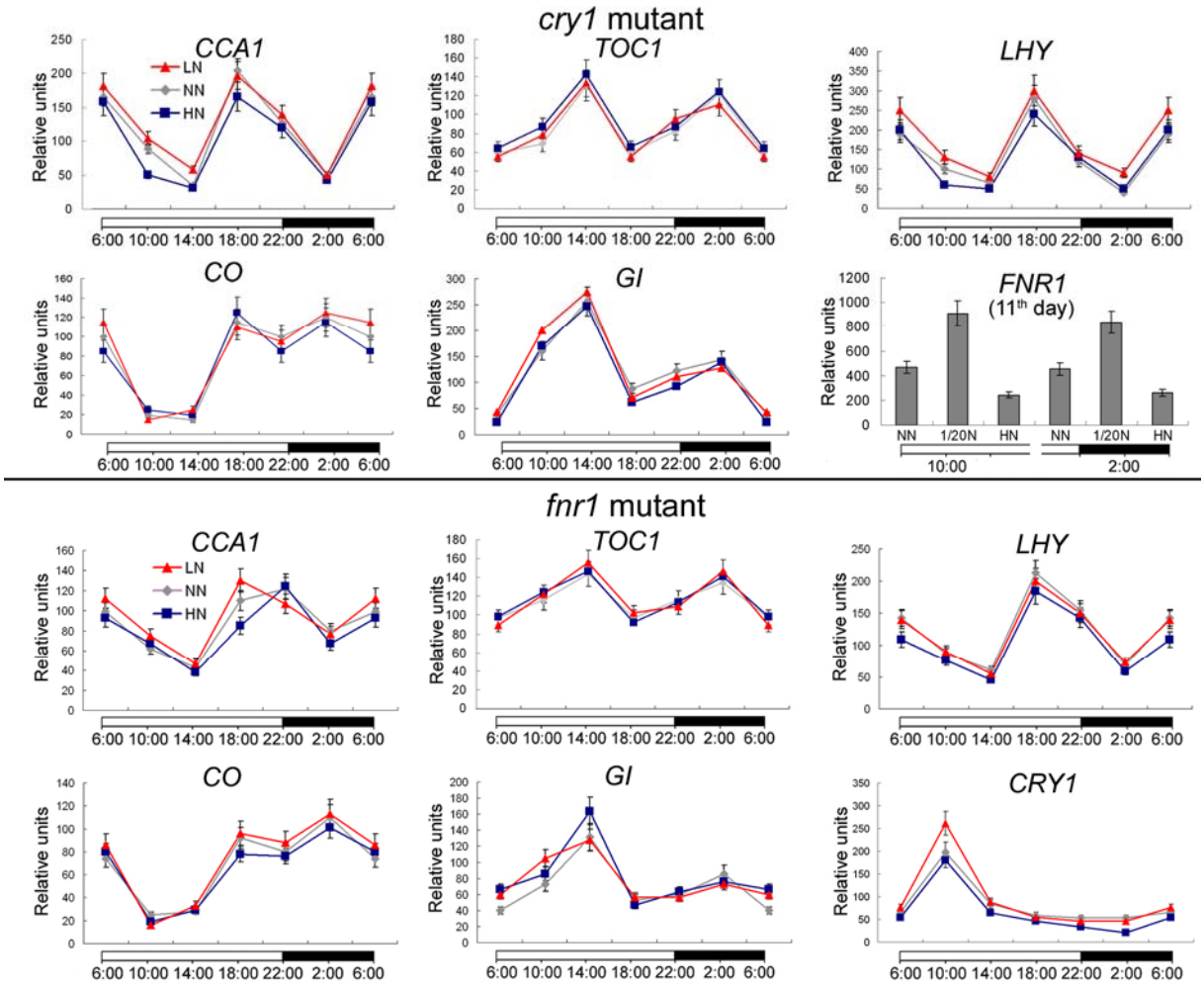


Fig. S14. mRNA abundances of *CCA1*, *TOC1*, *LHY*, *CO*, *GI*, *FNR1* and *CRY1* in *cry1* mutant and *fnr1* mutant over a 24-hour time course. The specific gene expression levels are represented as the percentages relatively to *ACTIN7* expression levels. *FNR1* is not a circadian transcript. Therefore, samples from only two time-points (2:00 and 10:00) were collected. Error bars show standard deviations (n = 5). LN, 1/20 low-N condition; NN, normal N condition; HN, high N condition.

Supplemental Tables

Table S1. SSH results by Method 1 [(Sample4 – Sample3) – (Sample2 – Sample1)].

Gene	Location	Function	Frequency	Verification
FNR1	**At5g66190**	ferredoxin-$NADP^+$ reductase	37/100	Yes (5.2)
CRY1	**At4g08920**	Cryptochrome1, blue light photoreceptor	43/100	Yes (4.8)
RNP1	**At3g14100**	RNA recognition motif-containing protein	3/100	Yes (3.7)
SEP1	At4g34190	Stress enhanced protein 1	2/100	Yes (3.1)
RHA2B	At5g18550	RING-H2 finger protein 2B	4/100	Yes (2.7)
RPS5	At2g41840	Ribosomal protein S5 family protein	4/100	Yes (3.2)
LHCB2.3	At3g27690	light-harvesting complex II chlorophyll a/b binding protein	2/100	Yes (4.4)
PK	At2g07180	Protein kinase superfamily protein	2/100	Yes (2.8)
TUB4	At5g44340	Tubulin beta chain 4	1/100	Yes (4.9)
GRP2	At4g38680	Glycine rich protein 2	2/100	Yes (3.6)

50 random positive clones for each PCR product were sequenced. SSH was repeated once and the results were merged. Numbers in the "Verification" column indicate the ratio of expression level at 1/20 N condition after floral transition to expression level at Normal N condition before floral transition. Genes indentified in both Method 1 results and Method 2 results are shown in boldface.

Table S2. SSH results by Method 2 [(Sample4 – Sample2) – (Sample3 – Sample1)].

Gene	Location	Function	Frequency	Verification
ARA6	At3g54840	Ras-related small GTP-binding family ARA6	2/100	Yes (2.5)
GAPC2	At1g13440	glyceraldehyde-3-phosphate dehydrogenase	7/100	Yes (3.3)
CYCB2;3	At1g20610	cyclin-B2-3	2/100	Yes (4.0)
P4K2	At1g26270	phosphatidylinositol 4-kinase type 2-beta	2/100	Yes (3.0)
MRN17.7	At5g22840	serine protein kinase	3/100	Yes (2.3)
HPR	At1g68010	hydroxypyruvate reductase	2/100	Yes (2.7)
GPP1	At4g25840	glycerol-3-phosphatase 1	5/100	Yes (3.1)
DegP10	At5g36950	protease Do-like 10	4/100	Yes (2.2)
SNG1	At2g22990	serine carboxypeptidase I	3/100	Yes (3.5)
ACX1	At4g16760	peroxisomal acyl-coenzyme A oxidase 1	9/100	Yes (5.1)
PABN1	At5g51120	polyadenylate-binding protein 1	1/100	Yes (2.9)
CRY1	**At4g08920**	Cryptochrome1, blue light photoreceptor	21/100	Yes (4.8)
RNP1	**At3g14100**	RNA recognition motif-containing protein	3/100	Yes (3.7)
MQB2.19	At5g62860	F-box associated ubiquitination effector	3/100	Yes (2.8)
PHD	At3g08020	PHD finger-containing protein	2/100	Yes (3.9)
FNR1	**At5g66190**	ferredoxin-$NADP^+$ reductase	18/100	Yes (5.2)
AAE5	At5g16370	AMP-binding protein	3/100	Yes (3.6)
K16L22.5	At5g41770	cell cycle control crooked neck protein-like	5/100	Yes (4.1)
WRKY45	At3g01970	SIT4 phosphatase-associated family protein	3/100	Yes (2.6)
XYISO	At5g57655	xylose isomerase	2/100	Yes (2.9)

50 random positive clones for each PCR product were sequenced. SSH was repeated once and the results were merged. Numbers in the "Verification" column indicate the ratio of expression level at 1/20 N condition after floral transition to expression level at Normal N condition before floral transition. Genes indentified in both Method 1 results and Method 2 results are shown in boldface.

Table S3. Flowering times of *fnr2* mutant, *cry2* mutant and wild-type plants grown in MS media.

Days to bolting	1/20 N	NN	HN	Blue	H Fe & S
Col-0 Ctrl	21±1.2	25±1.2	32±1.8	22±1.4	20±1.2
Col-0 Dorsomorphin	21±1.1	22±1.0	24±1.2	21±1.2	20±1.0
Col-0 AICAR	27±1.4	30±1.6	32±1.8	28±1.6	27±1.6
fnr2	25±1.4	30±1.8	35±1.8	25±1.0	25±1.4
cry2	29±1.8	38±2.2	51±2.8	22±1.2	23±1.2

Leaf number	1/20 N	NN	HN	Blue	H Fe & S
Col-0 Ctrl	8±0.4	9±0.8	10.5±0.8	8±0.4	7.5±0.6
Col-0 Dorsomorphin	8±0.4	8±0.4	9±0.6	8±0.4	7.5±0.6
Col-0 AICAR	9.5±0.6	10±0.8	10.5±0.8	9.5±0.6	9.5±0.8
fnr2	9±0.6	10±0.6	11±0.8	9±0.6	9±0.6
cry2	10±0.8	12±1.0	14±1.2	8±0.4	8±0.4

20 μM Dorsomorphin (AMPK inhibitor) or 2 mM AICAR (AMPK agonist) was added in the medium for wild-type plants (Col-0). Days to flowering and the rosette leaf number were scored (mean ± SD; $n \geq 25$ plants). Ctrl, control plants without Dorsomorphin or AICAR treatment; 1/20 N, 1/20 low-N condition; NN, normal N condition; HN, high N condition; Blue, blue-light treatment; H Fe & S, high Fe & S treatment (0.4 mM $FeSO_4$).

Table S4. Correlation analyses among NADPH / $NADP^+$, AMP / ATP, nuclear AMPK activity, nuclear CRY1 phosphorylation level and nuclear CRY1 protein level in wild-type plants grown under 1/20 low-N (LN), normal-N (NN) or high-N (HN) conditions.

	LN		NN		HN		R^2	R^2
	Light	Dark	Light	Dark	Light	Dark		
NADPH / $NADP^+$	1.6	5.4	1.0	3.3	0.7	1.5	} 0.93	
AMP / ATP	2.6	1.7	3.8	2.0	4.5	3.1		} 0.83
nuclear AMPK activity	60	20	100	30	180	60	} 0.87	
nuclear phosphorylated CRY1	50	15	100	25	200	80		} 0.88
nuclear CRY1 protein level	50	180	25	100	10	35	} 0.99	
TOC1 transcript	90	32	110	63	145	100		

Extreme values of each parameter (relative units) during the day or at night are shown. Then the correlation coefficients (R) between each two parameters were calculated by using the logistic regression model.

Table S5. Adaptors and primers used for SSH.

Adaptor & Primer	Sequence
CDS-PT	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTT
AD-G	AAGCAGTGGTATCAACGCAGAGTACGGGGG
AD-PCR	AAGCAGTGGTATCAACGCAGAGTAC
Adaptor 1	5’-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3’ 3’-GGGCCCGTCCA-5’
Adaptor 2R	5’-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3’ 3’-GCCGGCTCCA-5’
PCR Primer 1	CTAATACGACTCACTATAGGGC
Nested PCR Primer 1	TCGAGCGGCCGCCCGGGCAGGT
Nested PCR Primer 2R	AGCGTGGTCGCGGCCGAGGT

Table S6. List of primers for characterizing Arabidopsis genes.

Gene	Location	L primer	R primer
LFY	At5g61850	TCATTTGCTACTCTCCGCCGCT	CATTTTTCGCCACGGTCTTTAG
FT	At1g65480	CGAGTAACGAACGGTGATGA	CGCATCACACACTATATAAGTAAAACA
ACT7	At5g09810	ATCCCTCAGCACCTTCCAAC	ACCCGATACTTAAATAATTGTCTCAT
CRY1	At4g08920	AGGTGGTGGCTCAAGAACAG	CCAAGGGAAGAACTTTAGATGG
FNR1	At5g66190	TTTACCTTCCTCCAAGTCATCC	TCTTCAGCAACTCCCACAGC
RNP1	At3g14100	GGAGGTCCGTGTCCAACGAG	CTGTGCGGCGGCTACATTCT
SEP1	At4g34190	CCTTATCTCAAGTGTCTGCGTCTC	GCTGCTCTGTTTCCTCCTCTAC
RHA2B	At5g18550	GATGTGGCGGTTAGGGTTGC	TTTGAGACTGAAGCGATGGATA
RPS5	At2g41840	CTCACGCAAAGCAATACATCG	AGCCTTGGACGCCAGGAAAT
LHCB2.3	At3g27690	TGCGTCGTACCGTCAAGTCT	CTCCCAACATTGCCCATCTAC
PK	At2g07180	GGGACTTACGGATACGCTGCTC	TTCGCTCTTGCTTCTACCACTTT
TUB4	At5g44340	GCGAAAGGTCATTACACCGAAGG	CAGGGAAACGAAGACAGCAAGT
GRP2	At4g38680	CGCAAAGGCTCCGTCAAGTGG	CCGTCGGGTCCAGAAACATCG
GAPC2	At1g13440	TCAGTGGAAGCACCATGAGC	GCAAGAGGAGCAAGGCAGTT
ACX1	At4g16760	CACGGATGTAACTGAAAGACTGG	CCGAGATGTTTGTGGAGGAGA
HPR	At1g68010	TACTTTCGATTTCAAACCACATAA	CCAGCTCCAATAACTCCAACA
GPP1	At4g25840	CAGCAAGTCTCCGATTTGTAGC	GAGTCACTGATTCCACTTTCGTC
DegP10	At5g36950	ATATTTGCGGGCTTCGTATT	CTCCGTTCACTTTATTCACTTGTAG
ARA6	At3g54840	GCCTCGTTCTTGTCCCAAAC	AAGCTCCATTCCATCCTCAGTAG
CYCB2;3	At1g20610	ACGACGGCAACAACAGGACC	TCAACCGCAGCCAAAGGATT
P4K2	At1g26270	AGATGCTGCGGAGGGAACTT	TTCCACCTTTGACAACTGACCAC
MRN17.7	At5g22840	GGGAATGCTTGTTGGACTTA	AGATCACGAGCTGATTGACG
SNG1	At2g22990	GGTATTGGTGAGGACGAGAA	ATGCCTGCTTAGCCACTTTT
PABN1	At5g51120	GCGGCTGAGAAGGAAGAAGT	TGAACCGTGTTGATGATGATAAG
MQB2.19	At5g62860	GAGGTCCAATGAAGCAGCAA	TGACACCAGCGATGCAATAC
PHD	At3g08020	TTTAAGGGCTGCTGCTGGTT	GAGGCTTGCTACACTTTATTCTCACTA
AAE5	At5g16370	GACCACTCAAGATGGAGCAAAT	GGACATGGGAACAGCAAACTG
K16L22.5	At5g41770	CGGAAAGATACAGAAGTCAAGC	CAAACTCAGCAAACGCCACA
WRKY45	At3g01970	AAGAAATGGAGGATAGGAGGTG	GATTACAGGGCAGCCGAGAC
XYISO	At5g57655	ATGCTCAAACTGGATGGGATA	CTCAAAGATGCGAGGGAAAC
KIN10	At3g01090	GAGGATGAGGCGAGGAACTT	GCCGATGGCTAACAGGTGAA
KIN11	At3g29160	AATAGATTTGGCAATAATGGAGTGG	ACCTCGGGAGCAGCGTAGTT