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# Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene *CCA1*

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Understanding how nutrients affect gene expression will help us to understand the mechanisms controlling plant growth and development as a function of nutrient availability. Nitrate has been shown to serve as a signal for the control of gene expression in *Arabidopsis*. There is also evidence, on a gene-by-gene basis, that downstream products of nitrogen (N) assimilation such as glutamate (Glu) or glutamine (Gln) might serve as signals of organic N status that in turn regulate gene expression. To identify genome-wide responses to such organic N signals, *Arabidopsis* seedlings were transiently treated with ammonium nitrate in the presence or absence of MSX, an inhibitor of glutamine synthetase, resulting in a block of Glu/Gln synthesis. Genes that responded to organic N were identified as those whose response to ammonium nitrate treatment was blocked in the presence of MSX. We showed that some genes previously identified to be regulated by nitrate are under the control of an organic N-metabolite. Using an integrated network model of molecular interactions, we uncovered a subnetwork regulated by organic N that included *CCA1* and target genes involved in N-assimilation. We validated some of the predicted interactions and showed that regulation of the master clock control gene *CCA1* by Glu or a Glu-derived metabolite in turn regulates the expression of key N-assimilatory genes. Phase response curve analysis shows that distinct N-metabolites can advance or delay the *CCA1* phase. Regulation of *CCA1* by organic N signals may represent a novel input mechanism for N-nutrients to affect plant circadian clock function.

circadian | gene networks | glutamate | metabolism

Nitrogen (N) is an essential nutrient and a metabolic signal that is sensed and transduced resulting in the control of gene expression in plants. Studies using nitrate reductase (NR) mutant plants have shown that nitrate can serve as a metabolic signal for inorganic N that regulates gene expression in *Arabidopsis thaliana* and other plant species (1–4). There is also ample though less direct evidence that the assimilated forms of N such as Glu or Gln may also serve as signals that regulate gene expression in plants (5, 6). The ability of plants to sense and respond to levels of inorganic and organic N-metabolites provides a mechanism to balance the availability of organic N resources within the plant with the need for N uptake. Because nitrate uptake, reduction, and its assimilation into organic form require energy, a mechanism that activates this N-assimilatory pathway based on sensing levels of organic N available in the plant is an efficient way to control N-use efficiency (3). In plants, the transcription of genes involved in the uptake and assimilation of inorganic N is induced when levels of organic N are low. Conversely, the uptake and reduction of inorganic N are shut off when levels of organic N are high (reviewed in ref. 7).

Recent microarray studies have shown that nitrate can cause changes in the expression of a large number of genes in *Arabidopsis* (1, 2). Treatment of *Arabidopsis* seedlings with low levels of nitrate has been shown to increase the levels of mRNA for hundreds of genes within minutes of exposure. The nitrate-responsive genes

include nitrate transporters, NR and nitrite reductase, putative transcription factors, and stress responses genes, as well as genes whose products play roles in glycolysis, iron metabolism, and sulfate uptake (1, 2). In a related study, N-starved plants underwent a transcriptome/metabolome analysis 30 min and 3 h after nitrate treatment (4). The expression of nitrate transporters (at 30 min) preceded the induction of amino acid biosynthetic genes and the repression of amino acid breakdown genes (at 3 h). In addition, increases in amino acid levels were observed, consistent with the changes in expression of the cognate amino acid biosynthesis genes. Putative nitrate-responsive regulatory factors including transcription factors, protein kinases/phosphatases, and trehalose and hormone metabolic genes were also identified in that study. Recently, using a NR-null mutant, it was shown that nitrate, and not a product of nitrate reduction and assimilation, regulates the expression of genes involved in energy production, metabolism, glycolysis, and gluconeogenesis (1).

Nitrogen metabolism genes can be regulated by negative feedback of the products of N-assimilation. For example, the expression of the ammonium transporter gene ammonium transporter 1 (*AMT1.1*) is repressed in treatments with high levels of inorganic N. It has been shown that this repression is blocked by methionine sulfoximine (MSX), a nonmetabolizable analog of Glu that irreversibly inhibits glutamine synthetase and hence blocks N-assimilation into Gln (5). Thus, it appears that organic forms of N may regulate the uptake of N in plants. In addition, the genes encoding asparagine synthetase 1 (*ASN1*) and 2 (*ASN2*) are differentially regulated by organic and inorganic N sources. Organic N treatments were shown to positively regulate levels of *ASN1* mRNA (6), whereas *ASN2* gene expression appears to be responsive to inorganic N sources and not a downstream metabolite (1). Together, these studies prompt a model in which both inorganic and organic N sources can each regulate plant gene expression affecting N uptake, reduction, and assimilation.

In this study we used a genomic approach to identify gene networks whose expression is regulated by Glu or Glu-derived metabolites (organic N) in plants. Network analysis of the genes that responded to organic N revealed that transcription control of gene expression is important for a subnetwork of metabolic genes involved in the synthesis and degradation of asparagine (Asn). The metabolic gene network discovered in this analysis provides mo-

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lecular evidence for regulation of N-use at the level of gene expression. Moreover, the transcription factors regulated by organic N associated with this network provide a mechanistic link between circadian clock function and N-assimilation in plants.

## Results

**Inorganic Versus Organic N Responses.** To uncouple gene responses to inorganic N from those elicited by downstream products of inorganic N-assimilation we performed treatments of *Arabidopsis* seedlings with combinations of inorganic N (nitrate and ammonium), organic forms of N (e.g., Glu and Gln), and MSX, an inhibitor of glutamine synthetase (8) [supporting information (SI) Fig. 5]. Genes regulated by inorganic N signals should be unaffected by MSX treatment. By contrast, genes responding to a downstream organic N signal should fail to show induction by inorganic N treatments if Glu/Gln synthesis is blocked by MSX. This block of induction by MSX should be relieved by Glu treatment. Following this rationale, 2-week-old *Arabidopsis* seedlings grown on low concentrations of N (1 mM  $\text{NO}_3^-$ ) were transferred to media containing 40 mM  $\text{NO}_3^-$  and 20 mM  $\text{NH}_4^+$  (referred to as “Nms”). Seedlings were then harvested after a 2-h treatment time. This treatment was carried out alone (Nms) or in the presence of 1 mM MSX (Nms+MSX) or 1 mM MSX and 10 mM Glu (Nms+MSX+Glu). The Nms treatment consists of the same N source found in standard MS salts, which is the established standard amount of N for plant growth (9). A concentration of 1 mM MSX has previously been established as effective in blocking the N repression of *AMT1.1* in *Arabidopsis* seedlings and in decreasing levels of internal organic N (5). We chose a concentration of 10 mM for Glu treatments because this has been shown to be effective in the regulation of N-assimilatory genes while not being high enough to be detrimental to plant growth or development (6). To evaluate the effect of MSX alone, plants were exposed to growth media that contained MSX. To control for the effect of the plant transfer to distinct media, plants were transferred onto media plates without any of the treatment factors. This latter control was used as the baseline for the microarray experiments described below.

To evaluate our experimental design, we determined the mRNA level of genes shown to be responsive to organic N (*ASN1*) or inorganic N (*ASN2*) by reverse transcription followed by real-time quantitative PCR (RT-qPCR). This analysis showed that the *ASN1* mRNA level was induced 3.5-fold by the Nms treatment as compared with the control (SI Fig. 6A, compare Nms to the control). This induction of *ASN1* mRNA was blocked when MSX was present (SI Fig. 6A, Nms+MSX versus Nms). Importantly, when exogenous Glu or Gln was added, *ASN1* levels were induced regardless of the presence of MSX (SI Fig. 6A, compare Control to Nms+MSX+Glu and Nms+MSX+Gln). These results indicate that the induction of *ASN1* is due to Glu or a downstream metabolite, as shown previously (10, 11). In addition, the control treatments showed that MSX alone does not induce expression of *ASN1* or *ASN2* (SI Fig. 6). The addition of Glu or Gln partially blocked the induction of *ASN2* by the Nms treatment (SI Fig. 6B), consistent with the negative regulation by amino acids seen previously (10). Conversely, the induction of *ASN2* mRNA by Nms was insensitive to MSX addition (SI Fig. 6B), suggesting that the induction was mediated by an inorganic N source. This finding was consistent with previous data, which indicate that *ASN2* gene expression correlates with ammonium levels (12).

**Global Genomic Responses to Organic and Inorganic N Signals.** To investigate global gene expression changes that are mediated by Glu or a Glu-derived metabolite, we monitored the plant transcriptome using the ATH1 Affymetrix gene chip. Total RNA was extracted from plants treated with Nms, Nms+MSX, or Nms+MSX+Glu as described above; two biological replicates per treatment were performed. The Nms+MSX+Gln treatments were not analyzed with microarrays because Gln and Glu responses were similar in our

**Table 1. N responses**

Response	Treatment			Genes	Genes per class
	Nms	Nms+MSX	Nms+MSX+Glu		
Class A, inorganic N	D	D	D	100	159
	I	I	I	59	
Class B, inorganic N and Glu	D	D	NC	30	48
	I	I	NC	15	
	D	D	I	3	
Class C, internal Glu	D	NC	NC	194	334
	I	NC	NC	56	
	NC	I	I	49	
	NC	D	D	33	
	D	I	I	2	
Class D, external Glu	NC	NC	D	89	126
	NC	NC	I	37	
Class E, internal/external Glu	NC	I	NC	55	164
	NC	D	NC	36	
	D	NC	D	47	
	I	NC	I	22	
	I	D	I	3	
Class F, opposite internal/external Glu	NC	D	I	1	3
	D	NC	I	2	
Total	I	NC	D	1	834

Patterns of expression based on their response to the Nms, Nms+MSX, and Nms+MSX+Glu treatments. Genes were categorized into six classes based on these patterns. D, decreased; I, induced; NC, no change.

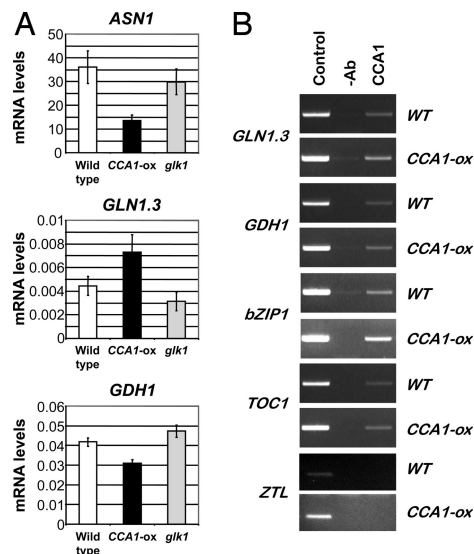
hands (SI Fig. 6 and data not shown). RNA was labeled and hybridized to the microarrays, the raw intensity values were normalized, and the data were filtered as described in *Materials and Methods*. A gene was kept in the data set only if its expression was reproducible and reliable across the three different treatments (Nms, Nms+MSX, and Nms+MSX+Glu). A total of 5,904 genes were identified that passed these stringent quality control criteria. To verify the microarray results, we analyzed the mRNA levels of selected genes by RT-qPCR including the *TAZ* zinc binding (At4g37610) and *bZIP* (At5g49450) transcription factors with results similar to the microarray data (SI Fig. 7). As expected, the genomic experiments verified the previous observation that *AMT1.1* and *ASN1* are regulated by organic N. In addition, our results identified additional genes regulated by organic N as described below.

Genes were categorized based on their response to the treatments using the Affymetrix change calls: induced (I), no change (NC), or decreased (D). Each gene was assigned a three-part code (e.g., I-NC-I), which corresponds to the gene expression response in the Nms, Nms+MSX, and Nms+MSX+Glu treatments, respectively. We found 21 unique patterns of response (SI Table 2). The largest pattern was NC-NC-NC, representing 5,070 genes not affected by the treatments. We grouped the remainder 20 patterns (834 genes) into six classes that summarize the types of N responses observed: class A, inorganic N; class B, inorganic N with Glu feedback; class C, organic N with no exogenous Glu rescue; class D, exogenous Glu; class E, exogenous and endogenous Glu; and class F, exogenous and endogenous Glu with opposite effects (Table 1).

We compared the genes regulated in our experiments to published results (1). This previous study identified 595 genes that responded similarly to nitrate treatment in both a NR-null mutant and wild-type plants. Because the mutant plants cannot assimilate nitrate, the responses observed were attributed to the action of nitrate as a signal and not a downstream metabolite. Eighty of these 595 genes showed consistent and reliable responses in our experiments. Surprisingly, only 17 of these genes were found regulated by inorganic N signals in both studies (SI Table 3). Among these, we





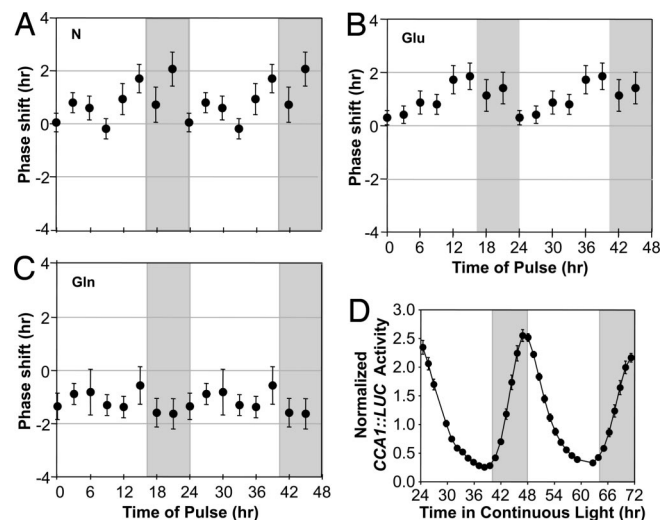


**Fig. 2.** Altered mRNA levels of target genes and binding of CCA1 protein to target gene promoter regions validate predicted regulation by CCA1. (A) RT-qPCR was performed on *CCA1-ox*, *glk1* knockout, and wild-type plants to determine mRNA levels for *ASN1*, *GLN1.3*, and *GDH1*. Three biological and two technical replicates were carried out for each sample. mRNA levels were normalized to clathrin (*At4g24550*). The mean  $\pm$  standard error of the mean is shown. (B) ChIP assays to show binding of CCA1 to *GLN1.3*, *GDH1*, *bZIP1*, *TOC1*, and *ZTL* gene promoter regions. Control, input DNA control (no IP); –Ab, IP without antibody; CCA1, IP with the CCA1 antibody.

redundancy in the function of *CCA1* and *GLK1* in regulating *ASN1*, *GDH1*, and *GLN1.3* gene expression. *CCA1-ox*, *glk1*, and wild-type plants were grown for 2 weeks as above, and samples were collected in the morning (3 h after dawn). Total RNA was extracted from whole seedlings, and RT-qPCR was performed to determine mRNA levels for *ASN1*, *GLN1.3*, and *GDH1* in the three genotypes. As shown in Fig. 2, all three genes tested showed altered expression patterns in the mutant lines used (as determined by analysis of variance,  $P \leq 0.05$ ), which were consistent with the predicted network model shown in Fig. 1. In addition, *bZIP1* mRNA level was also repressed in *CCA1-ox* (SI Fig. 8). *ASN1*, *GDH1*, and *GLN1.3* mRNA levels were not altered in the *glk1* line, with the exception of a small increase in *GDH1* mRNA levels. This is probably because of the redundant function of *GLK1* and *CCA1* in regulating the expression of the tested genes. In contrast, and as predicted by the model shown in Fig. 1, *ASN1* and *GDH1* levels were decreased in the *CCA1-ox* line. Also consistent with the predictions of the model, *GLN1.3* mRNA levels were increased as compared with wild type in the *CCA1-ox*.

The network model predicts that the effect of CCA1 on the expression of the target genes will be direct. To test this hypothesis, we used ChIP assays using a CCA1 antibody (Fig. 2B). As controls, we demonstrated that the ChIP assays could detect binding of CCA1 protein to a region of the *TOC1* promoter, a known target of CCA1, but was not able to detect the *ZTL* promoter, which has no circadian oscillation at the mRNA level. Consistent with the model for CCA1, ChIP assays in both wild-type and *CCA1-ox* lines were able to confirm binding of CCA1 to the promoter regions of *GLN1.3*, *GDH1*, and *bZIP1* promoters. These results support the model and indicate that CCA1 regulates expression of *bZIP1*, *GDH1*, and *GLN1.3* genes directly.

**N-Nutrient Signals Act as Input to the Arabidopsis Circadian Clock.** CCA1 is a key component of a negative feedback loop at the center of the *Arabidopsis* circadian clock (16, 17). Because our results showed that N treatments affected CCA1 expression, we hypoth-



**Fig. 3.** Exposure of seedlings to pulses of inorganic and organic N shifts the phase of the circadian clock. (A–C) Plots of the phase shift of *CCA1::LUC* expression in response to a 4-h pulse of inorganic N (20 mM  $\text{KNO}_3$ /20 mM  $\text{NH}_4\text{NO}_3$ ) (A), 10 mM Glu (B), or 10 mM Gln (C) against the time at which the pulse was administered to wild-type seedlings. Pulses were administered at 3-h intervals spanning one complete circadian cycle, and data were collected over the next six cycles. Phase shifts are double-plotted to emphasize the circadian pattern of the response. Phase advances (the peak in expression occurring earlier) are plotted as positive values, and delays are plotted as negative values. (D) *CCA1::LUC* expression of control (untreated) seedlings. In all panels, the entraining photoperiod (16:8) is indicated by the vertical white (light) and gray (dark) bars. The mean  $\pm$  standard error of the mean is shown.

esized that N might serve as an input capable of affecting the circadian clock function. To test this hypothesis, we provided pulses of inorganic or organic N at intervals spanning a circadian cycle and determined the effects on the phase of the oscillation in *CCA1::LUC* expression. Each treatment resulted in stable phase shifts indicating that N status serves as an input to the circadian clock (Fig. 3 and SI Fig. 9). Inorganic N and 10 mM Glu treatments conferred slight phase advances whereas 10 mM Gln conferred only delays. The Nms and Glu pulses did not affect the period, but the Gln pulse shortened the period as determined by one-way analysis of variance and Dunn's multiple-comparison tests. Thus, the clock regulates a number of steps in N metabolism, such as NR expression and activity (18) and *ASN1* expression [this and previous studies (19)]. In turn, N status feeds back to the clock, at least in part through its effect on CCA1 expression.

## Discussion

We used genomic and pharmacological approaches to distinguish organic from inorganic N responses in *Arabidopsis* seedlings. The majority of the genes regulated by the N treatments used in our study (81%) responded to organic N signals. Among the genes regulated by organic N, we distinguished two classes of genes: (i) genes that responded only to external Glu application and (ii) genes that responded to internal and external sources of Glu (Table 1). The difference in these two expression patterns raises the possibility that there are different mechanisms for sensing internally produced cellular Glu versus extra cellular Glu. The other possibility is that the differences in internal versus external Glu responses observed in our study reflect distinct threshold responses to Glu levels. There is precedence for internal and external Glu-sensing mechanisms in other organisms. Bacteria regulate ammonium assimilation via a mechanism involving PII, a sensor that measures levels of  $\alpha$ -keto-glutarate and Glu (20). Plants contain a PII protein that is localized to chloroplasts (21), a potential sensor of internal levels of Glu. By contrast, extracellular Glu is sensed by Glu receptors in animal





change calls were consistent in at least three of the four comparisons. This filtering resulted in a set of 834 genes that were detected and responded consistently in our experiments. We used custom-made S-PLUS and PERL functions to analyze the data.

**Network Analysis.** For network analysis we used our existing network model of plant gene interactions (13). In addition, we predicted protein:DNA interactions as follows: The consensus sequence for transcription binding sites from curated databases (36, 37) were searched in 1,500 bp of upstream sequence using the DNA pattern search tool from the RSA tools server with default parameters (38). The search was performed in both strands of DNA, the upstream region was not allowed to overlap with the coding region of the upstream gene, and motif matches were not allowed to overlap. A motif was considered overrepresented if it was present in an upstream sequence more than three times the standard deviation above the mean occurrence in all of the upstream sequences in the genome. A protein:DNA interaction was predicted when the upstream sequence of a gene contained an overrepresentation of the regulatory motif for that transcription factor and the expression of the transcription factor and putative target gene was highly ( $\geq 0.7$  or less than or equal to  $-0.7$ ) and significantly ( $P \leq 0.01$ ) correlated. These predictions are available in [SI Data Set 1](#). Similar regulatory predictions for other microarray data sets can be generated with the VirtualPlant system ([www.virtualplant.org](http://www.virtualplant.org)) using the “Gene Networks” tool.

**ChIP Assays.** Immunoprecipitations (IP) were performed as previously described (39). Briefly, 2-week-old wild-type and CCA1-ox plants were collected at the beginning of the light cycle and immediately fixed in 1% formaldehyde for 15 min in a vacuum at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Nuclei were prepared for chromatin

isolation. The isolated chromatin was sonicated 10 times for 20 s each at 100% power (Diagenode Bioruptor) in an ice water bath. A small aliquot of sheared chromatin was removed to serve as control. The diluted chromatin was used for IP with the CCA1 antibody and one control IP without antibody. [SI Table 6](#) lists primer sequences used for amplification of the CCA1 binding sites in each of the genes tested. PCR amplifications included 95°C for 2 min followed by 36 cycles of 95°C for 15 s, 58°C (for *bZIP1*, *TOC1*, and *GDH1*) or 60°C (for *ZTL* and *GLN1.3*) for 30 s, and 72°C for 30 s.

**Circadian Phase Response Curves.** CCA1::LUC seedlings were entrained on MS basal medium plus 0.5% sucrose and 1 mM KNO<sub>3</sub> for 8 days in 16/8-h light/dark (100–150  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ), after which seedlings were moved into continuous light. At 3-h intervals, seedlings ( $n = 16$  per treatment) were transferred to fresh solid medium plus 2 ml of liquid Nms or medium containing 10 mM Glu or 10 mM Gln for 4 h, then rinsed in liquid entrainment medium three times for a total of 30 min and transferred individually to the wells of 96-well microtiter plates containing fresh solid media for luciferase activity measurements, which were determined with a Packard TopCount scintillation counter as described (40). The period and phase of rhythms after the pulses were determined by fast-Fourier transform nonlinear least-square analysis (41). The phase shifts were calculated as described (30).

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