Warm nights disrupt transcriptome rhythms in field-grown rice panicles

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In rice, a small increase in nighttime temperature reduces grain yield and quality. How warm nighttime temperatures (WNT) produce these detrimental effects is not well understood, especially in field conditions where the typical day-to-night temperature fluctuation exceeds the mild increase in nighttime temperature. We observed genome-wide disruption of gene expression timing during the reproductive phase in field-grown rice panicles acclimated to 2 to 3 °C WNT. Transcripts previously identified as rhythmically expressed with a 24-h period and circadian-regulated transcripts were more sensitive to WNT than were nonrhythmic transcripts. The system-wide perturbations in transcript levels suggest that WNT disrupt the tight temporal coordination between internal molecular events and the environment, resulting in reduced productivity. We identified transcriptional regulators whose predicted targets are enriched for sensitivity to WNT. The affected transcripts and candidate regulators identified through our network analysis explain molecular mechanisms driving sensitivity to WNT and identify candidates that can be targeted to enhance tolerance to WNT.

climate change impact | nighttime temperature increase | circadian regulators | global food security | diel transcriptional networks

Global climate models predict with high certainty that mean surface temperatures will increase by 1 to 4 °C by 2100 (1–3). A breakdown of these temperature trends highlights a more rapid increase in minimum nighttime temperature compared with the maximum daytime temperature at the global, regional (4), and farm (5, 6) scales. Nighttime warming is occurring globally over all land areas. While the asymmetry of warming temperatures is not globally consistent, more land areas experience greater nighttime warming than greater daytime warming (7). In contrast to the short heat-spikes predicted with increasing day temperatures, warmer nighttime temperatures (WNT) are expected to have a longer duration, impacting important growth and developmental phases of crops (8). In response to increased daytime temperatures, rice plants employ mechanisms to minimize heat-induced damage, including avoidance through transpirational cooling (9), escape through early morning flowering (10), and reproductive resilience (11). In contrast, domesticated rice has limited plasticity to overcome the impacts of WNT (8). The adverse effects of WNT on rice yield and quality have been documented across controlled environments (12, 13) and field conditions (14, 15), demonstrating the potential for economic losses (16). The limited physiological capacity of rice to respond and the larger temporal and spatial scales of WNT compared with location-specific daytime temperature increases (8) suggest that the economic losses under current and future warmer nights pose a severe threat to sustaining global rice production.

The physiological responses in rice to high nighttime temperatures include a significant reduction in pollen viability, increased spikelet sterility, and membrane damage, collectively leading to yield losses (12, 17–19). However, these investigations imposed temperatures that are higher than future predictions. Thus, a knowledge gap exists between rice responses in controlled chambers and real-world conditions. Studies using field-based heat tents demonstrated the difference between chamber and field studies (8, 14, 20–22). Previous agronomic data from field-based studies using predicted ranges of temperature change have demonstrated the impact of warmer night temperatures on the carbon balance in rice genotypes including inbreds, hybrids, popular cultivars, and landraces (14, 15, 23). The field-based studies identify accelerated senescence and higher night respiration during postflowering as a critical factor determining yield and quality losses due to increased nighttime temperature (21). Although the relationship between night respiration and sugar metabolism enzymes has been documented, particularly during the grain-filling stage (21), the mechanistic changes at the molecular level that are associated with these physiological responses are yet to be investigated.

In the field, environmental conditions are dynamic. This variation is not captured by controlled environments and only partially by field-based heat tents. Previous observations indicate that the variability of natural field conditions plays an essential role in regulating transcriptomic responses (24) and contributes to the stability of the circadian clock in rice (25). *Arabidopsis* research has demonstrated that plant responses to abiotic stress are dynamic throughout the day (26–41), supporting the need to capture stress responses at multiple time points to provide a comprehensive mechanistic understanding of rice exposed to WNT. Examination of the temporal mechanistic responses to stress in crops under field

Significance

The effects of warmer nighttime temperatures (WNT) on crops are one poorly understood dimension of climate change. WNT result from the asymmetrical increase in nighttime versus daytime temperatures. In rice, WNT reduce grain yield and quality. WNT reduce the amplitude of daily temperature cycles plants use to set their circadian clock. Therefore, we examined how WNT affect the timing of molecular activities. In field-grown plants, WNT alter the daily pattern of the transcriptome. Genes with strong rhythmic expression and those under circadian control are affected most by WNT. Many candidate regulators of the disrupted genes are circadian clock associated, emphasizing the altered timing under WNT. The pathways and mechanisms identified can assist efforts to identify lines tolerant to WNT.

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conditions is generally lacking and is the primary motivation driving our investigations.

The circadian clock ensures proper timing of the internal molecular activities with the environment (42, 43). The clock is sensitive to subtle environmental changes to ensure the internal biochemical processes are in phase with the surroundings (44). WNT may disrupt this environmental coordination. In rice and *Arabidopsis*, daily rhythms of temperature, also known as thermocycles, entrain the circadian clock and control the rhythmic expression of a portion of the transcriptome (45–47). In *Arabidopsis*, the photoreceptor PHYB connects changes in ambient temperature to the circadian clock (48–50), and changes in ambient temperatures affect the expression of the core circadian components (51). The evening clock component, ELF3, contains a prion-like domain that functions as a tunable thermosensor (52). Although these studies indicate possible mechanisms for how plants sense temperature changes, it is still not clear how the daily temperature range is perceived and integrated into the circadian clock. Moreover, the significance of a thermocycle sensitive clock, the impacts of altering the daily temperature cycles, and the effects on the expression of these temperature-responsive rhythms, particularly under field conditions, remain to be characterized. Under WNT, the daily thermocycle amplitude is reduced and could impact the expression of thermocycle-regulated transcripts.

To fill the knowledge gap on the molecular responses inducing yield losses in field conditions under WNT, we investigated how WNT levels, in-line with the Intergovernmental Panel on Climate Change (IPCC) predictions, affect the genome-wide expression patterns in the panicles of rice grown under field conditions. The specific objectives of our study were to 1) quantify the diurnal reprogramming of the rice panicle transcriptome under WNT; 2) identify major interacting molecular pathways that determine rice response to WNT; and 3) find regulators of transcriptionally responsive genes under WNT.

Results

WNT Negatively Impact Biomass and Yield. IR64, a popular high-yielding rice variety, was grown under normal nighttime temperatures (NNT) or WNT, using a field-based infrared ceramic heating system (Fig. 1). WNT treatment started at panicle initiation and continued through maturity. At 50% flowering, field-grown rice panicles were collected for transcriptional analysis throughout the 24 h cycle. WNT maintained a 2 to 3 °C increase in temperature in the 12 h night period (1800 to 0600 hours) compared with ambient temperature (Fig. 1). As previously reported using other methods of increasing the nighttime temperature (5, 15), we observed a 12.5% decrease in the grain yield under WNT (Kruskal–Wallis test P value < 0.05, Fig. 1). Total aboveground biomass (P value < 0.05), number of spikelets per panicle (P value < 0.05), and 1,000-grain weight (P value < 0.05) were also significantly affected by WNT (Fig. 1, *SI Appendix*, Fig. S1, and Dataset S1). Panicles per square meter and spikelet fertility did not change significantly with WNT (Dataset S1).

WNT Impact Transcription Patterns During the Day. Eight time points were collected throughout the 24-h diel cycle to evaluate the molecular changes associated with the observed agronomic changes in WNT-grown plants. We performed RNA sequencing (RNA-Seq) on rachis 1 to 3 from panicles at the 50% flowering stage. A total of 1,110 genes differentially expressed genes (DEGs) were identified between WNT and NNT (adjusted P value < 0.05 and log fold change > 0.5), corresponding to 6% of the 15,213 reliably detectable genes (Fig. 2, *SI Appendix*, Figs. S2–S4, and Dataset S2). In response to WNT, 415 genes were up-regulated, and 695 genes were down-regulated (Fig. 2 *A* and *B*). Most of the 415 up-regulated DEGs were identified from the daytime samples, while significantly down-regulated genes were more often detected in the nighttime (Fig. 2 *C* and *D*). The expression of all detectable genes is available at https://go.ncsu.edu/clockworkviridi_wnt.

The time of sampling influences the identification of DEGs. The time point with the most DEGs was 1 h before sunrise (dawn), just before the WNT treatment ceased each day (396 DEGs, time point 23 h). Only 10 DEGs were identified at 3.5 h and 12 DEGs at sunset (dusk), the time point when the WNT treatment was initiated. Even though the increased temperature was applied only at night, many DEGs were identified in the daytime samples when the WNT and NNT conditions were identical. An eigengene, which is the vector representation of the expression of a selected group of genes across different samples, was generated for all up- and down-regulated genes (Fig. 2 *C* and *D*). The eigengene representing the expression pattern of DEGs up-regulated under WNT at any time point highlights their cyclic expression in NNT. They peak in the day in NNT, but in WNT, the timing of this peak in expression is altered (Fig. 2*C*). Functional enrichment of these WNT–up-regulated genes included terms enriched for protein posttranslational modification, signaling, carbohydrate metabolism, RNA processing, and kaurene synthesis (*SI Appendix*, Fig. S5*A*) (53). However, each time point presents a unique DEG profile and enriched functional categories (Dataset S3). In part, this appears to be due to the underlying expression variation in NNT. For example, during the morning hours when photosynthetic genes are active, DEGs were enriched for photosynthesis-related activity. No difference in expression was detected at night, likely due to their low NNT levels at those time points. Therefore, sampling at only one time point would miss the impacts of WNT on molecular functions not active at that time. Most of the 695 down-regulated genes are identified during the nighttime. The eigengene representing the down-regulated DEGs indicates that under NNT, the majority of these transcripts peak just before sunrise (dawn). WNT lower the expression amplitude of these genes and advance the phase of peak expression (Fig. 2*D*). Down-regulated genes were enriched for protein folding, photosynthesis, and heat stress (*SI Appendix*, Fig. S5*B*).

DEGs Are Enriched for Rhythmically Expressed and Circadian-Controlled Genes. The observation that many DEGs show a change in their daily expression pattern (e.g., Fig. 2) led us to speculate that the rhythmically expressed and circadian-regulated genes may have enhanced sensitivity to WNT. We evaluated whether the DEGs in WNT are enriched for rice genes identified as rhythmically expressed in a study in controlled conditions by Filichkin et al. (47). We observed a significant overlap between WNT DEGs and genes identified as rhythmic when grown in both photocycles and thermocycles in the prior study (Fig. 3*A* and *SI Appendix*, Fig. S6*A*). WNT DEGs are under-represented for noncycling genes in photocycles and thermocycles (P value < 2.93×10^{-30}). The WNT DEGs were enriched for genes with a peak expression at night, between Zeitgeber times (ZT) 11 to 21 h (Fig. 3*A*). Genes with peak expression at ZT 19 (6 h after dusk) showed the strongest enrichment for DEGs in WNT (P value < 8.55×10^{-5}). For example, *LOC_Os10g41550* encodes a beta-amylase with rhythmic expression peaking before dawn in the chamber-grown Nipponbare rice seedlings (Fig. 3*B*). We observe a similar expression peak before sunrise (dawn) of the corresponding gene, *MH10t0431700*, in our field-grown IR64 panicles in NNT. However, in WNT, the expression pattern is delayed, with a peak expression at sunrise, when expression levels are already decreasing in NNT. The 24 h expression pattern of beta-amylase in NNT has a higher correlation to chamber-grown seedlings in photocycles and thermocycles (0.95) than the same tissue in WNT (0.62). WNT DEGs are also overrepresented in transcripts rhythmically expressed in seedlings grown in only photocycles or thermocycles (Fig. 3*C* and *SI Appendix*, Fig. S6*B*).

In addition to rhythmic expression in the presence of photocycles or thermocycles, the WNT DEGs were enriched for circadian-regulated genes with thermocycle-entrained expression. Genes were considered circadian regulated if the rhythmic expression

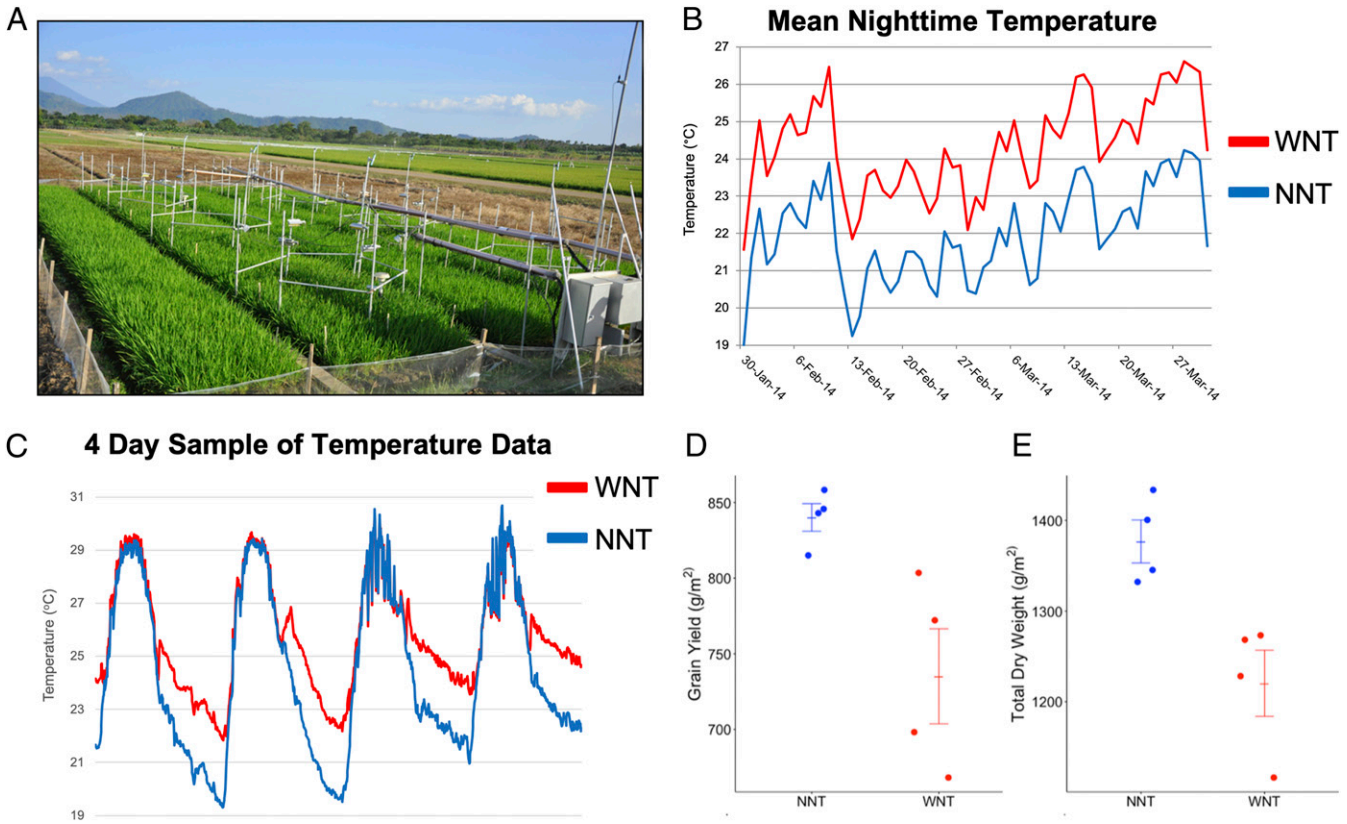


Fig. 1. Experimental setup of WNT field samples and impacts on agronomic performance. (*A*) WNT samples were contained within the ring of ceramic heaters that maintained increased temperatures only at night. (*B*) Temperature data from the period when the heaters were turned on. (*C*) Closeup of 4 d of treatment showing that the diel temperature range in both NNT and WNT exceeds the nighttime temperature difference between NNT and WNT. (*D* and *E*) The effects of the WNT treatment (WNT, red) compared with NNT (blue) on (*D*) grain yield (g/m^2) and (*E*) average 1,000-grain weight. A total of 12 plants were sampled from each of four plots per treatment. Error bars indicate ± SE (n = 4).

persisted in constant conditions after entrainment in ref. 47. The WNT DEGs showed enrichment for circadian regulation only when compared with transcripts entrained in the presence of thermocycles. After entrainment with either thermocycles alone or with both photocycles and thermocycles, WNT DEGs were enriched in the genes that maintained a rhythmic expression pattern when released to constant conditions (P value < 0.05) (Fig. 3*D*). However, WNT DEGs were not enriched in the circadian-regulated transcripts after entrainment with photocycles alone (Fig. 3*D*), indicating that WNT DEGs are enriched for genes under the control of the thermocycle clock based on the data in ref. 47. For example, *LOC_Os10g41550* (the beta-amylase gene in Fig. 3*B*) is rhythmically expressed when entrained by thermocycles alone but not by photocycles alone.

WNT Alter Temporal Expression Patterns. The enrichment of the WNT DEGs for genes previously identified as rhythmic in diel and circadian conditions suggests that WNT potentially disrupt the overall rhythmic expression of transcripts throughout the day. Therefore, we evaluated the daily dynamics of expression for all 15,213 detectible transcripts, even those not identified as DEGs. All transcripts were categorized as dynamically expressed across the 24-h period or not dynamically expressed in these diel conditions using JTK cycle (54) (Fig. 4*A* and Dataset S4). The period of dynamically expressed genes was similar in both NNT and WNT (*SI Appendix*, Fig. S7).

Even genes that maintain a dynamic expression pattern in both NNT and WNT (66%, 4,112 genes), have waveform differences between the two conditions. When ordered by the phase of the peak of expression in NNT, ZT 10.5 is the time point with the most transcripts at their maximal expression (Fig. 4*A*). In WNT, this pattern shifts, and ZT 7.0 is the time point when the largest number of transcripts peak in expression, with fewer transcripts peaking at ZT 10.5. In NNT, most nighttime peaking transcripts peak at ZT 23, while a similar number of transcripts are at their peak expression level in the ZT 12, 14, and 17.5. However, in WNT, most nighttime peaking transcripts peak at ZT 14. The number of transcripts peaking at ZT 17.5 and 23 is reduced in WNT compared with NNT.

To evaluate how these changes affected the overall distribution of expression, we classified genes as either morning-phased (peaks at dawn) or evening-phased (peaks at dusk). In NNT conditions, the morning and evening phase distribution was relatively equal (ratio of the morning- to evening-phased genes = 0.94, Fig. 4*B*). However, in WNT, more genes peak in the morning (ratio = 1.58, Fig. 4*B*). To explore how these peak expression changes manifest between NNT and WNT, we plotted the time of peak expression in NNT compared with WNT using a Circos plot (55) (Fig. 4 *C*–*E*). The phase of the maximum expression differs between WNT and NNT for 16.5% of the genes that maintain a dynamic expression pattern in both conditions. The effects were not limited to one time point but rather a distributed effect on transcripts peaking throughout the day. WNT resulted in both delayed (Fig. 4 *C* and *E*) and advanced (Fig. 4*D*) expression.

The shifts in peak expression are observed more often in select time points. More than 50% of the genes in NNT that peak at dawn, 10.5, 12, 17.5, or 23 h have a shifted peak of expression in WNT. For example, *MH03g0450600*, a chlorophyll a/b binding protein, peaks in

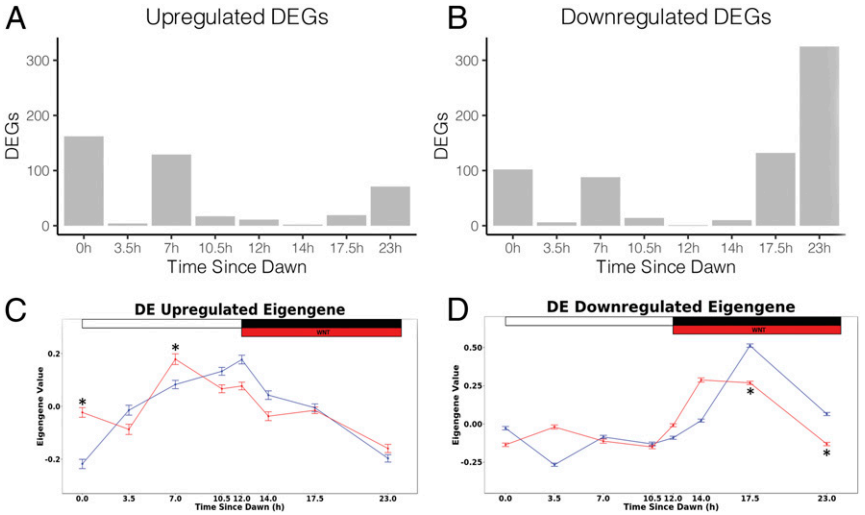
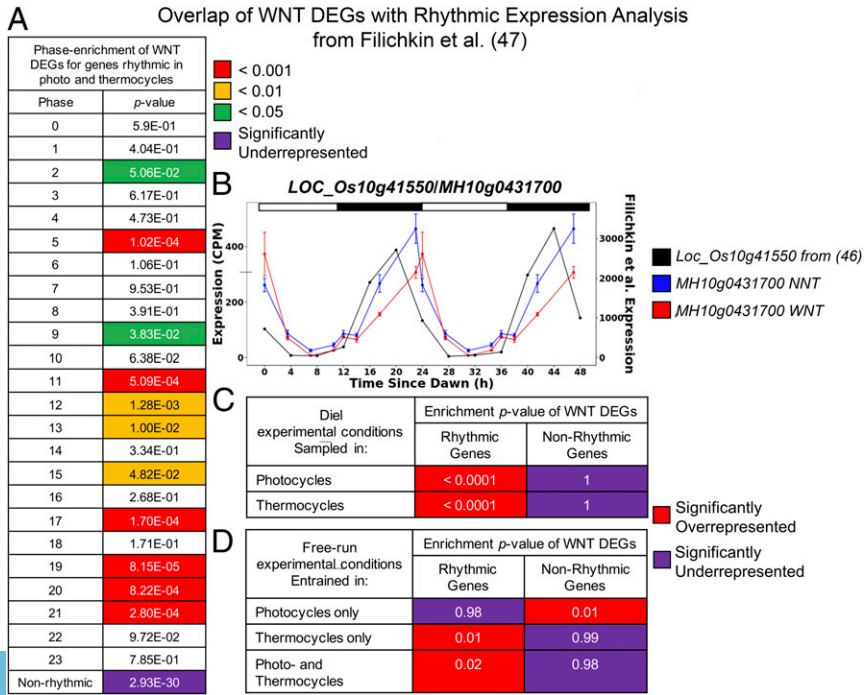


Fig. 2. DEGs in response to WNT. DEGs identified between WNT and NNT at each time point (false discovery rate < 0.05 and logFC > 0.5). Time points indicate sample time in hours after sunrise (dawn). Bargraphs of DEGs (*A*) up-regulated and (*B*) down-regulated at each time point. The Eigengene representations of all (*C*) up-regulated and (*D*) down-regulated DEGs in WNT (red) and NNT (blue). White/black bar indicates day/night period, respectively. The red bar indicates when WNT plants were exposed to WNT. An asterisk indicates that the difference between the WNT and NNT eigengenes is significant (P value < 1×10^{-10}) based on a one-sided t test for the direction tested (WNT expression is higher for up-regulated genes and lower for down-regulated genes). Error bars indicate ± SE ($n = 4$).

expression in NNT at 3.5 h after dawn but is delayed to 7 h in WNT (Fig. 4*C*). Genes with this delay in peak expression from 3.5 h in NNT to 7 h in WNT are enriched for components of the photosynthetic machinery (Fig. 4*C*). Genes that peak just before dawn at 23 h in NNT show an advance in their peak of expression in WNT. *MH07g0175300*, also known as *Os*Nramp5, a metal transporter associated with disease resistance, peaks in expression at 17.5 h in NNT but at 14 h in WNT (Fig. 4*D*). These genes are functionally enriched for processes involved in biotic stress, protein phosphorylation, and RNA splicing (Fig. 4*D*). The expression of *MH12g0102300*, which encodes a GDP-L-galactose phosphorylase, peaks at 23 h in NNT and plateaus in the early daytime hours. In WNT, higher expression of *MH12g0102300* persists into the daytime hours, changing the peak timing of expression (Fig. 4*E*). Genes with a similar disruption that delayed peak expression from 23 h to dawn in WNT were enriched for carbohydrate and fatty acid metabolism (Fig. 4*E*).

We identified genes that were dynamically expressed either only in NNT or only in WNT. We used a stricter cutoff to select transcripts that were not dynamically expressed to avoid transcripts that just missed the significance cutoff in the other condition. Of the 6,248 genes with a robust dynamic pattern in NNT, 960 showed a substantial loss in dynamic expression in WNT (*SI Appendix*, Fig. S8*A* and Dataset S4). Genes that lost daily dynamic expression in WNT were enriched for protein synthesis, including ribosomal protein synthesis (Dataset S5). The 748 genes that were dynamically expressed only in WNT conditions were enriched for DNA synthesis and cell cycle (*SI Appendix*, Fig. S6 *C* and *D* and Datasets S4 and S5).

For genes identified as WNT DEGs, the direction of the change in peak expression between NNT and WNT is dependent on the peak expression time in NNT (Fig. 4*F*). DEGs that peaked early in the daytime hours (ZT 0 and ZT 3.5) had an overall delay in peak expression in WNT, indicated by increased peak expression density



Phase-enrichment of WNT DEGs for genes rhythmic in photo and thermocycles	
Phase	*p*-value
0	5.9E-01
1	4.04E-01
2	5.06E-02
3	6.17E-01
4	4.73E-01
5	1.02E-04
6	1.06E-01
7	9.53E-01
8	3.91E-01
9	3.83E-02
10	6.38E-02
11	5.09E-04
12	1.28E-03
13	1.00E-02
14	3.34E-01
15	4.82E-02
16	2.68E-01
17	1.70E-04
18	1.71E-01
19	8.15E-05
20	8.22E-04
21	2.80E-04
22	9.72E-02
23	7.85E-01
Non-rhythmic	2.93E-30

Diel experimental conditions Sampled in:	Enrichment *p*-value of WNT DEGs	
	Rhythmic Genes	Non-Rhythmic Genes
Photocycles	< 0.0001	1
Thermocycles	< 0.0001	1

Free-run experimental conditions Entrained in:	Enrichment *p*-value of WNT DEGs	
	Rhythmic Genes	Non-Rhythmic Genes
Photocycles only	0.98	0.01
Thermocycles only	0.01	0.99
Photo- and Thermocycles	0.02	0.98

Fig. 3. Rhythmic and circadian-regulated transcripts have increased sensitivity to WNT. Comparison of WNT DEGs with published data (47) examining the diel rhythmic and circadian regulated expression of rice transcripts. (*A*) The enrichment of WNT DEGs compared with the phase of peak expression for rice transcripts when grown in photocycles and thermocycles. Enrichment is colored by P value < 0.001 (red), < 0.01 (orange), < 0.05 (green), and underrepresented (purple, P value < 0.05). (*B*) Expression pattern of transcript, *LOC_Os10g41550* (MH10g0431700) in both photocycles and thermocycles (black), NNT, (blue), and WNT (red). Enrichment of WNT DEGs in sets of transcripts identified as rhythmic or non-rhythmic from plants grown in (*C*) photocycles only or thermocycles only, and (*D*) after entrainment in both photocycles and thermocycles, thermocycles only, or photocycles only.

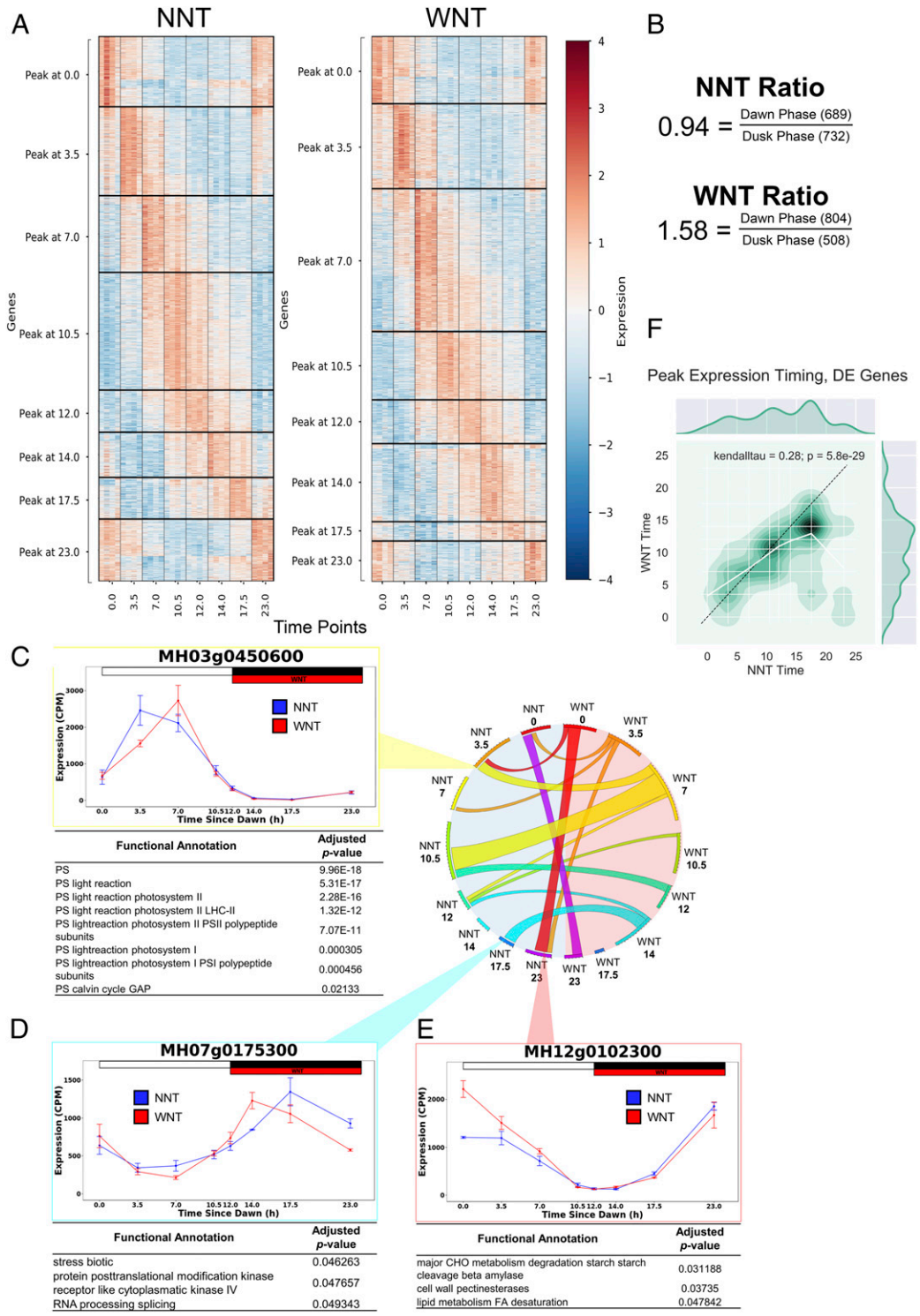


Fig. 4. Patterns of expression are disrupted by WNT. (*A*) Expression-level heatmap of genes identified by JTK cycle as dynamically expressed in both NNT and WNT. (*B*) WNT alter the ratio of morning to evening peaking genes. Circos plot of genes that are dynamically expressed in both NNT and WNT that change in the timing of their peak expression is shown. The phase of peak expression in NNT is anchored on the left half of the plot (blue), and the phase of peak expression in WNT is on the right (red). The colored bar at each time point indicates the number of transcripts with peak expression at that time point. Ribbons connect the NNT expression peak to the new WNT peak. The width of the ribbon indicates the number of genes. Select transcripts from three phase changes are highlighted in *C*–*E*. (*C*) Expression pattern of MH03g0450600, expression peaks at 3.5 h in NNT (blue), and shifts to 7 h in WNT (red). MapMan ontology enrichment is shown for all genes with this 3.5 to 7 h phase shift. (*D*) The expression pattern of MH07g0175300 expression peaks at 17.5 h in NNT (blue) and advances to 14 h in WNT (red). Functional enrichment for genes with this shift (light blue). (*E*) Expression pattern of MH12g0102300, peaks at 23 h in NNT, and the peak expression is delayed until sunrise (dawn) in WNT. Functional enrichment for genes with this shift (red). (*F*) Density plot showing changes in peak expression timing between NNT and WNT for WNT DEGs. The black line indicates a perfect positive correlation. The white line indicates the actual correlation between the peak of expression of DEGs in NNT and WNT. The density graphs on each side indicate the number of WNT DEGs peaking at each time point in each condition.

above the black line of perfect positive correlation. In contrast, genes that peak in the later daytime period (ZT 7 through ZT 12) show little change in peak expression between NNT and WNT. Most DEGs peak in expression after dusk in NNT, between ZT 14 and 17.5. Many of these night-peaking transcripts show a phase advance, peaking earlier in WNT than NNT indicated by the increased density below the line of perfect positive correlation. However, there is a large dispersion of peak expression in WNT of these night-peaking DEGs, indicating that these transcript's expression is no longer tightly coordinated in the nighttime period. These results suggest that these DEG calls may be due to the change in the expression pattern at night. The effect of WNT was stronger on genes that peak in NNT after ZT 12, consistent with our previous observation of more DEGs post ZT 12 (Fig. 2*B*), higher enrichment of rhythmically expressed transcripts in the nighttime period (Fig. 3*A*), and genes that lose or gain dynamic expression after ZT 12 (*SI Appendix*, Fig. S8 *A* and *B*).

Identifying Regulators of WNT-Perturbed Targets. To shed light on potential regulators of these altered expression patterns, we built gene regulatory networks (GRNs) to identify regulators that are either themselves perturbed or show perturbed expression of their targets. GRNs can be used to both identify candidate regulators of WNT-affected transcripts and understand the global changes in expression patterns. We used two independent GRN construction approaches in parallel (*SI Appendix*, Fig. S10*A*). First, we independently constructed a regulatory network from publicly available transcriptome data (56–58) (External Data Network). We built a second network only from our time course data (Internal Data Network). Refer to *SI Appendix* for details of the network construction methods. We evaluated the quality of the network connections and established a threshold for regulatory connections based on overlap with prior data (*SI Appendix*).

We identified the TF (transcription factor) regulators whose targets, predicted from the external data set, had disrupted expression under WNT. TFs with targets enriched for WNT DEGs were considered regulators of WNT-sensitive genes. The targets of 25 TFs were enriched for WNT DEGs (P value cutoff < 0.01) (*SI Appendix*, Fig. S10*B*). Most of these predicted regulators of WNT-sensitive targets had a strong dynamic pattern of expression (JTK Q-value < 6×10^{-15}), and many were related to circadian clock components (Table 1). Three of the predicted top TF regulators, BBX24, PIF4, and PRR95, are orthologs of evening complex targets in *Arabidopsis* (59). Of the TF regulators with high expression in panicle tissue, 43 target genes have only one TF, and 35 target genes were shared between two or more TFs. Many of the target genes are related to photosynthesis and carbohydrate metabolism (Fig. 5). In the Filichkin et al. (47) data, 30 of the 78 targets are circadian regulated after entrainment in thermocycles alone, indicating the targets of these predicted regulators of WNT-sensitive transcripts are enriched for thermocycle-entrained genes (PHASER[56] P value < 0.01).

A distinct pattern of TF and target grouping was apparent for the targets predicted to be regulated by more than one of these top candidate TFs (Fig. 5). Group 1 consisted of targets regulated by Zf-CCCH54, EDH4, and bZIP71, Group 2 consisted of targets regulated by bZIP1, PRR95, CXC2, BBX24, PIF1, ZEP1, and DOF2, and Group 3 targets had regulators from both groups. Homologs of many Group 2 regulators and targets have been previously associated with the circadian clock in *Arabidopsis*. For example, *MH03g0287000*, a Group 2 target with sequence similarity to LNK1 in *Arabidopsis*, a known thermocycle-regulated clock component (60). The *Arabidopsis* homolog of *Os*PIF1, *At*PIF3, has been previously identified as thermo responsive (61, 62). *At*PRR7 and *At*PRR9, the same family as *Os*PRR95, are regulators of temperature compensation (63). BBX24 homologs are associated with circadian regulation (64–66). Consistent with our observations of the sensitivity of cycling transcripts to WNT and the altered pattern of the transcripts themselves, the predicted regulators of the WNT DEGs suggest a link between the circadian clock and the altered expression under WNT.

Networks of Transcriptional Responses Are Altered in WNT. To compare the effects of WNT on the predicted connections between transcriptional regulators, we employed the edge finding pipeline from McGoff et al. (67). This approach, the Internal Data Network, identified regulatory edges from the NNT and WNT expression data independently. Unlike the network derived from the external data, this approach enables a direct comparison between the edges in the NNT and WNT constructed networks. This approach is computationally expensive; therefore, we focused our analysis on 368 genes, of which 356 were transcriptional regulators (*SI Appendix*).

WNT perturbed the regulators of 89 of the 368 genes that had regulators predicted with high confidence (Target Rank Edge Probability [TREP] ≤ 3, BE ≤ 0.25). The perturbed edges spanned the entire circadian cycle. For example, Fig. 6 shows the predicted regulators of circadian genes in rice that showed detectable expression levels in the panicle tissue. In NNT, several predicted regulators of circadian clock genes peak at 17.5 h after dawn, but in WNT, no predicted regulators of circadian genes peak at this time in the network (Fig. 6 *A* and *B*). Comparing the nodes in Fig. 6*A* to Fig. 6*B*, alterations at all time points are observed. Consistent with the External Network approach, BBX24 and PIF1 were identified as regulators with targets that have altered expression under WNT. In NNT, BBX24 is one of three predicted regulators of MH06t0053500, a putative ELF3 ortholog (Fig. 6*C*). The three candidate ELF3 regulators are all BBX-like transcription factors, BBX24, MH02g0081800, and MH02g0592600. In turn, three TFs pass the threshold as regulating BBX24, an HSF-like TF, a MYB-family TF, and a putative HY5 ortholog. In WNT, in which the BBX24 peak of expression shifts to ZT 7, three different TFs are identified as predicted regulators of BBX24, a WRKY30-like ortholog, an SPL12-like ortholog, and a BBX-family TF. In WNT, the ELF3 ortholog is no longer a target of BBX24, and BBX24 is instead predicted to regulate an HSF-family member. The Internal Data Networks generated directly from our Indica RNA-Seq data can also identify regulators not present in the microarray-based studies (56–58). For example, MH06g0689300, a Squamosa promoter-binding protein-like (SPL) family member, is identified in the Internal Data Network as a regulator of WNT DEGs and is not on the rice microarray. Overall, the Internal Network analysis indicates that WNT disrupt the temporal coordination between genes in the panicle, consistent with the observed effects on gene expression patterns and the regulators of WNT-sensitive DEGs identified from the External Data Network.

Validating Targets of TFs that Respond to Increasing Nighttime Temperature. Two independent network approaches identified overlapping regulators of WNT-sensitive gene expression. In total, 13 of the top 25 regulators identified using the External Data Network were also tested in the Internal Data Network. Six of these 13 were identified as regulators of WNT-sensitive targets in both networks (Table 1, Fig. 7*A*, and Dataset S6.)

To evaluate the predicted regulators of WNT-sensitive transcripts, we grew IR64 rice in field-based tents (14, 15), using a gradient of nighttime temperatures (24, 26, 28, or 30 °C). We performed RNA-Seq from the panicle tissue of plants grown in each of these night temperatures collected at the dawn and dusk time points. We examined the effect of this gradient of nighttime temperatures on the expression of the TFs and their predicted targets (Fig. 7 *B*–*D*). Of the TFs predicted to regulate WNT DEGs, the expression of *PIF1*, *PRR95*, *BBX24*, *SPL*, and *DOF2* TFs themselves responded to increasing nighttime temperatures (Fig. 7 and *SI Appendix*, Fig. S11). For example, *PIF1* expression is significantly reduced at dawn under 28 °C and 30 °C nighttime temperature conditions (Fig. 7*B*).

Table 1. Inferring regulatory networks to identify WNT-perturbed targets

TF	Peak exp (CPM)	Peak exp time point	JTK *Q*-value	Description
MH04t0504500*	582.33	3.5	2.14×10^{-109}	BBX24
MH09t0440700	409.62	7	2.19×10^{-48}	Two-component response regulator-like PRR95
MH04t0463500	359.24	7	5.88×10^{-83}	Zeaxanthin epoxidase OSZEP1
MH03t0017800	316.43	23	6.19×10^{-48}	CCCH-type zinc finger protein Ehd4
MH03t0690900*	269.91	3.5	1.42×10^{-65}	IF1 Phytochrome-interacting bHLH factors-LIKE
MH09t0199300	245.3	NA	1.00	bZIP transcription factor OsbZIP71
MH08t0029600	232.38	Dawn ^	3.25×10^{-10}	Zinc finger CCCH domain-containing protein 54
MH12t0389100*	204.27	10.5	5.65×10^{-16}	Ocs element-binding factor 1 BZIP
MH03t0074500	176.29	3.5	2.60×10^{-73}	Cyclic dof factor 2
MH07t0089300	15.45	23	8.02×10^{-41}	TSO1-like CXC 2
MH07t0542400	91.57	3.5	3.08×10^{-54}	Cyclic dof factor 2
MH03t0731600	85.57	NA	1.27×10^{-04}	PHD finger protein ALFIN-LIKE 3
MH08t0489700*	81	14	1.08×10^{-48}	WRKY transcription factor OsWRKY30
MH03t0093500	75.01	NA	1.00×10^{-5}	Chitin-inducible gibberellin-responsive protein 2
MH11t0062400	70.39	NA	2.66×10^{-1}	Hypothetical protein
MH01t0115900	53.77	23	1.41×10^{-31}	BES1/BZR1 homolog protein 4
MH03t0080100	38.31	Dawn ^	1.50×10^{-9}	GCN5-related *N*-acetyltransferase
MH04t0394400	35.69	NA	1.00	ERF114
MH02t0546000	32.35	3.5	1.58×10^{-36}	Scarecrow-like protein 8
MH08t0380600*	28.7	NA	1.55×10^{-6}	WRKY transcription factor OsWRKY69
MH02t051 0500	24.14	NA	1.00	Homeobox-leucine zipper protein HOX24
MH01t0514300*	20.13	Dawn	1.19×10^{-24}	ABRE-binding factor OsBZip8
MH03t0099900	16.96	NA	1.00	Homeobox-leucine zipper protein HOX12
MH03t0268900	12.26	NA	1.00	Heat stress transcription factor B-4d
MH01t0528100	6.79	NA	1.00	NAC domain-containing protein 68

The significant regulators of WNT DEGs identified from the external data source. TFs are sorted by their peak expression (CPM) in NNT. Gene IDs in bold text indicate genes that were included in both network approaches. Gene IDs with an asterisk were also identified by the network derived from the internal data source. Peak Expression Time Points with a ^ indicate that although close, the gene did not meet the strict cutoff for cycling.

At dusk, *PIF1* is not expressed. Activated targets of PIF1, predicted by the External Network analysis, positively correlated with PIF1 expression at dawn ($r = 0.96$) with significantly reduced expression at 28 °C and 30 °C. Repressed targets of PIF1 negatively correlated with *PIF1* expression ($r = -0.79$) at dawn and had significantly increased expression at 30 °C. The correlation in expression between *PIF1* and the PIF1-predicted targets that are also WNT DEGs is high for both activated targets ($r = 0.97$) and PIF1-repressed targets ($r = -0.98$) (Dataset S7).

In contrast to *PIF1*, *PRR95* is only expressed at dusk. At 26, 28, and 30 °C, WNT increase *PRR95* expression (Fig. 7*C*). Targets predicted to be activated by PRR95 at dusk positively correlated with *PRR95* expression ($r = 0.96$) and significantly higher expression with the increasing nighttime temperatures. At the dusk time point, targets predicted to be repressed by PRR95 correlated negatively with *PRR95* expression ($r = -0.92$), and their expression was reduced under increasing nighttime temperatures. *PRR95* expression is highly correlated with the predicted PRR95 targets

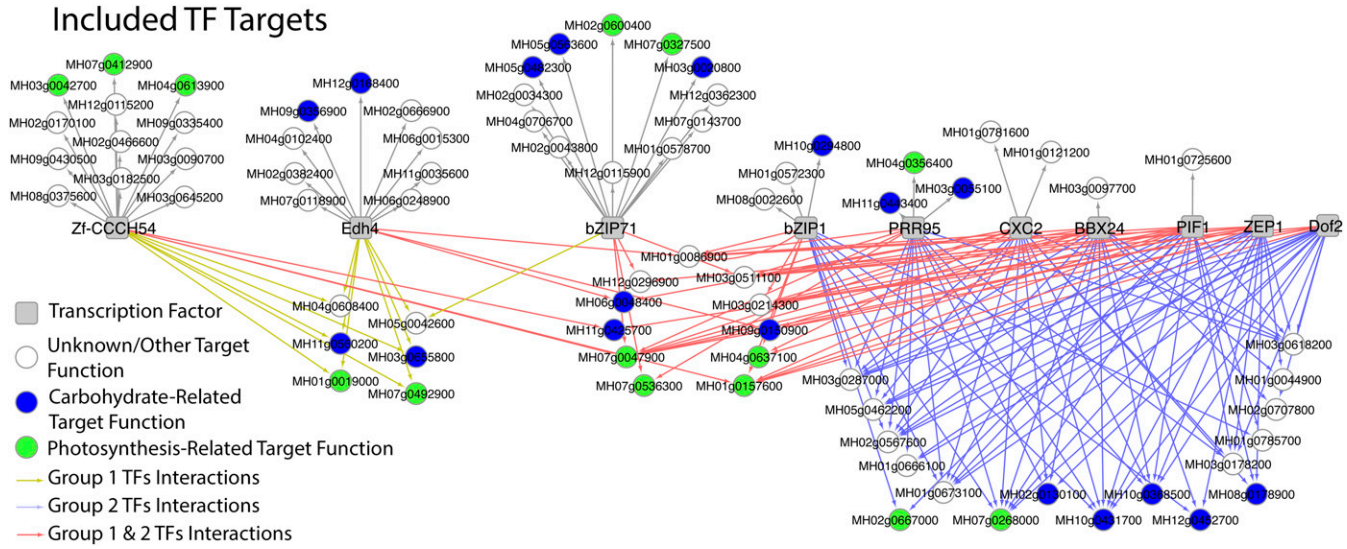


Fig. 5. Overlap of the TF regulators and DEG targets. TF (gray), unknown/other targets (white), photosynthesis-related target (green), and carbohydrate metabolism (blue) are connected by group 1 edges (yellow), group 2 edges (light blue), and group 1/2 edges (red).

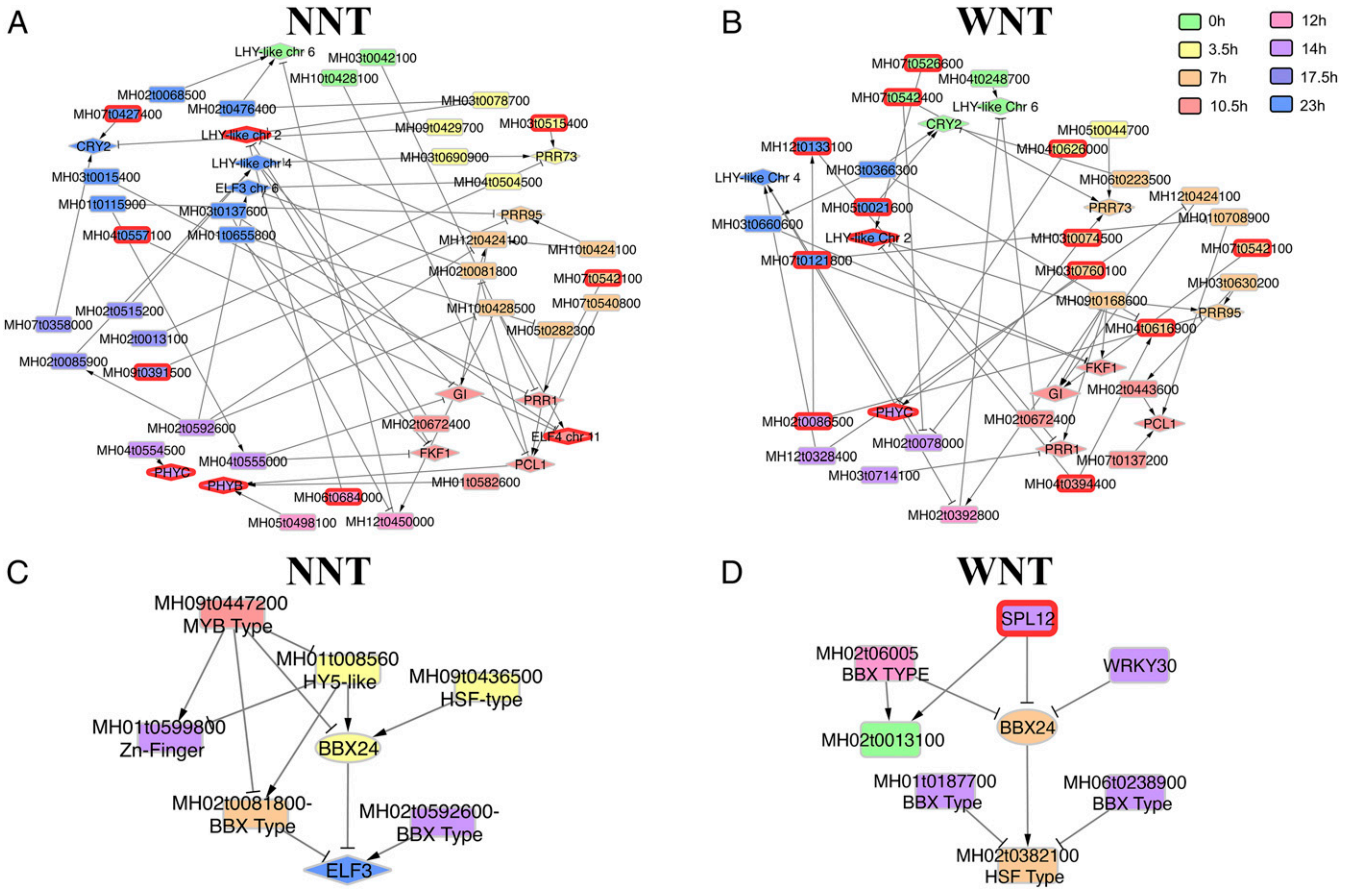


Fig. 6. Distinct NNT and WNT networks from internal data. A network of Circadian/Circadian-related genes and the direct regulators of these circadian genes with Target Rank Edge Probability (TREP) ≤ 3 and Baseline Error (BE) ≤ 0.25 using (*A*) NNT or (*B*) WNT Internal Data. Nodes colored by peak expression (0 h, green; 3.5 h, yellow; 7 h, orange; 10.5 h, dark pink; 12 h, pink; 14 h, purple;17.5 h, dark blue; and 23 h, blue). Diamond-shaped nodes are orthologs of canonical circadian clock genes. Pointed arrows indicate predicted positive regulation of the target, and flat-headed arrows indicate negative regulation. Nodes with a red border are nodes with the regulators identified as perturbed by WNT. (*C* and *D*) Subnetwork of direct input and output from the BBX24 node (indicated by oval shape). Predicted BBX24 connections in (*C*) NNT and (*D*) WNT. TFs predicted to coregulate BBX24 targets are shown. Targets regulated by two or more of the predicted BBX24 regulators are also shown. Colors, node shapes, node border colors, and arrows follow the same style as in *A* and *B*.

that are WNT DEGs at dusk (activated $r = 0.94$, repressed $r = -0.87$, Fig. 7*C* and Dataset S2). Even though the External Network was generated from data not considering WNT stress (56–58), the expression of the TFs predicted to regulate WNT-sensitive targets showed >0.7 absolute correlation with their targets (Table 1, Fig. 7, *SI Appendix*, Figs. S10 and S11, and Dataset S6, https://go.ncsu.edu/clockworkviridi_wnt). The targets of SPL, identified only in the Internal Data Network, since it is not on the microarray, predicted to be positively regulated were highly correlated with *SPL* expression both in dawn and dusk in the gradient experiment (dawn $r = 0.85$, dusk $r = 0.99$, Fig. 7*D*).

Discussion

The asymmetric warming between day and night is a critical variable to consider for sustaining crop productivity in future climate scenarios. The physical phenomenon of a thinner planetary boundary layer at night leads to larger nighttime effects on the surface air temperature (68). WNT negatively affect larger agricultural productivity areas, compared with the localized impacts of increasing daytime temperatures (8). Prior studies have tested the effects of increased nighttime temperatures in different genetic backgrounds, in chamber or greenhouse conditions, exposing plants to nighttime temperatures (17–19, 69) that are greater than those predicted by IPCC or other climate models (1, 70). However, a modest 2 °C increase in nighttime temperature reduces yield between 0 and 45.3% depending on year and variety (71). We observed that WNT levels similar to IPCC models have a significant yield decrease (12.5%) in field-grown rice of the popular IR64 variety.

WNT Alter the Timing of Fundamental Biochemical Processes. The term thermoperiodicity describes the differential impacts of day and night temperature changes on plants (61, 72). Thermoperiodicity and the negative impacts of WNT have been observed in crops and ornamental species (62, 63, 73–75). Mechanisms for why a mild increase in nighttime temperature has such substantial consequences in contrast to a similar increase in daytime temperatures are not understood. Photosynthesis, transpiration, and respiration are temperature-sensitive processes that contribute to yield. However, only respiration occurs consistently during the day and night. An increase in nighttime respiration has been associated with high nighttime temperatures (17, 18, 75–80). Therefore, increases in dark respiration are often considered as the primary mechanism for the observed yield decrease. However, the increase in maintenance or nighttime respiration may not fully explain the difference in yield (23, 63). The impacts of high nighttime temperature during the vegetative stage can be compensated by active photosynthetic machinery (80). In addition, the response of dark respiration under high nighttime temperature has only been documented in the leaf tissue in rice (21, 80, 81) or wheat (82), with no reports on panicle tissue.

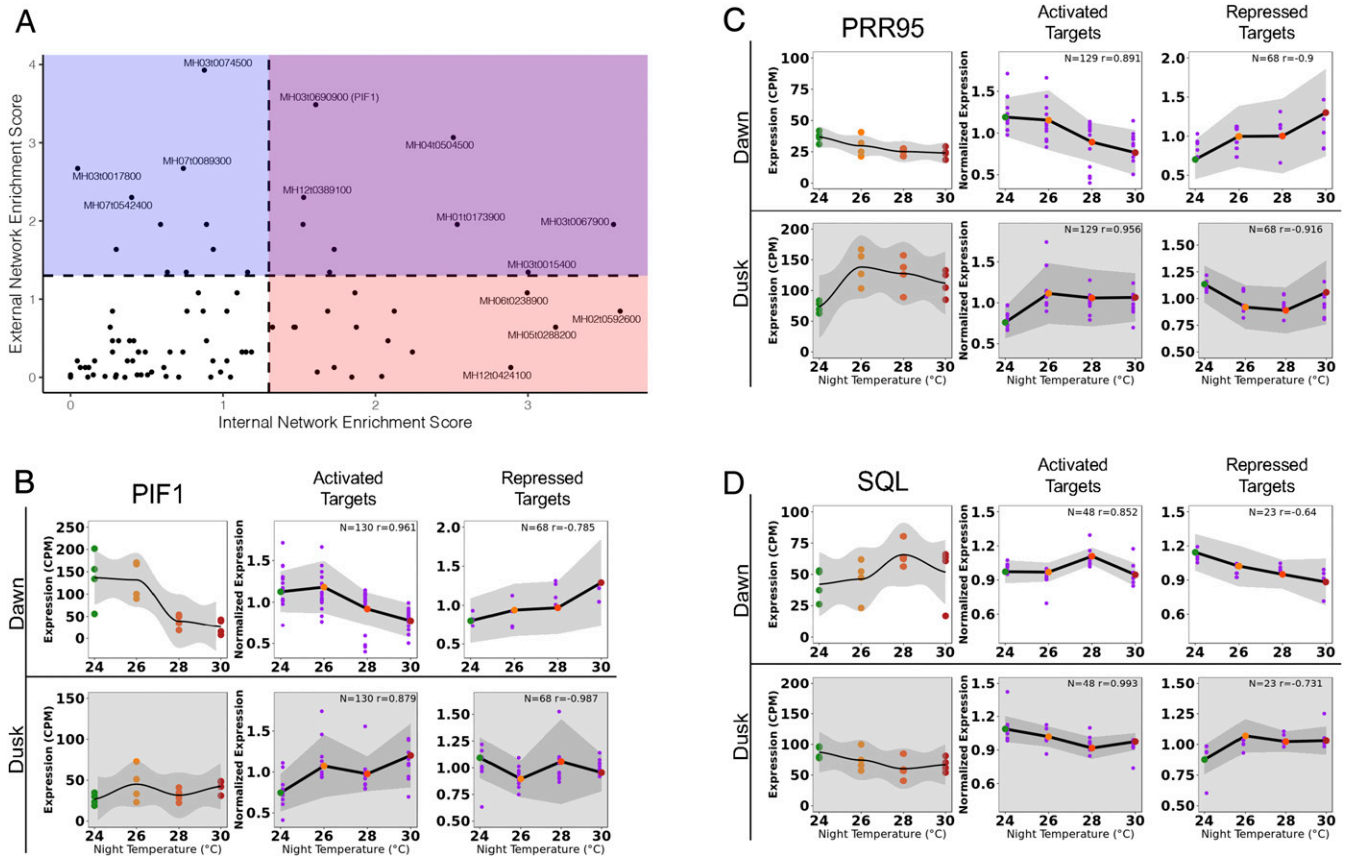


Fig. 7. Evaluation of regulators of WNT responses in an independent experiment. (*A*) Plot of enrichment of TF targets that are WNT-sensitive using targets identified by the External (*y*-axis) and Internal Network (*x*-axis). Enrichment Score is the $-\log_2(P \text{ value})$ of the hypergeometric test of the overlap between WNT DEGs and the predicted TF targets. Dotted lines are $-\log_2(0.05)$ (*B–D*). Expression (CPM) of (*B*) *PIF1*, (*C*) *PRR95*, and (*D*) *SQL* at sunrise (dawn, white) and sunset (dusk, gray) under nighttime temperatures of 24, 26, 28, and 30 °C. All PIF1 targets and PIF1 targets that are also DEGs (purple) are separated into activated and repressed targets at sunrise (dawn, white) and sunset (dusk, gray) time points. Line represents mean normalized expression (gene expression divided by mean gene expression) of all targets at 24 °C (green), 26 °C (dark yellow), 28 °C (orange), and 30 °C (red). The shaded region represents the SD. (*C*) All PRR95 targets and (*D*) all SQL targets are shown as in *B*.

Increasing nighttime temperatures have been associated with positive and negative effects on the next day's photosynthesis (79, 83–86). The protective role of light-driven electron flow is not available in the dark, resulting in increased reactive oxygen species production and photosystem II damage (87). Therefore, the photosystems of many plants are more sensitive to heat-induced damage in the dark than in the light. This light-dependent sensitivity persists across a variety of species (88). Our findings that WNT delay the early morning transcript level increase in many photosynthetic genes may indicate that altered timing of photosynthesis may compound the effects of increased respiration rates. Additionally, if these gene expression changes are reflective of the physiological timing, this delayed induction and subsequent overexpression later in the day could result in time-of-day–dependent differences in photosynthetic activity between control and WNT plants. Thus, the time of day the activity is measured could explain the conflicting reports of the effects of WNT on photosynthesis.

Select Time Points Provide Phase-Specific Markers for WNT Sensitivity. By examining transcripts at multiple time points, we observe changes in the timing of expression a single time point would have missed. Many photosynthesis-associated transcripts have delayed expression early in the day, changes that would have been missed at a later time point. Any single time point captures at most 36% of the total WNT-sensitive DEGs. However, examining the DEGs at dawn and 1 h before dawn (23 h) captures 59% of the total DEGs. Three time points (dawn, 7 h, and 23 h) capture 79% of the total DEGs. Although such informative time points are likely to vary by species and condition, these results indicate that identifying select time points could provide a representative view of daily variation, thus reducing costs in large-scale temporal experiments.

Thermocycles May Function in the Proper Temporal Coordination of Molecular Activities. While WNT can increase the rate of nighttime biochemical activities, another aspect of WNT is that they reduce the daily temperature range (DIF). This day-to-night temperature amplitude or thermocycle is beneficial to overall crop productivity (89). Many crops have reduced yield under constant light conditions (90), yet constant light damage can be reduced by providing a thermocycle with warm days and cooler nights in tomato and potato (91–93). In tomato, net photosynthetic rates drop when grown in constant light but can be recovered by providing a recurring, daily temperature drop for just 2 h, suggesting that the temperature change is a cue to establish diel rhythms (94). There is a linear relationship between DIF reduction and adverse effects on morphology (e.g., reduced biomass) and physiology (e.g., increased nighttime respiration) in maize (75). A large DIF could reduce the harmful effects of daytime heat on photosynthesis, emphasizing the importance of the amplitude of the daily temperature cycle.

In *Arabidopsis* and rice, thermocycles can entrain the circadian clock and control up to 30% of the transcriptome (45–47). In field conditions, we could not evaluate whether the WNT effects

persisted in constant light and temperatures. However, we observed many of the WNT DEGs were circadian regulated based on prior research identifying circadian-regulated genes in rice (47). WNT DEGs are enriched for circadian-regulated transcripts when entrained by thermocycles alone or both light and temperature cycles in combination but not when entrained by photocycles alone (Fig. 3). The enrichment for WNT DEGs in transcripts identified as thermocycle-entrained suggests that WNT disrupt the cues needed for proper timing of these transcripts. The thermocycle-entrainable transcripts are mostly distinct from those entrained by photocycles (46, 47). The functional significance of thermocycle-entrained genes has not been established in any plant species. However, if the negative impacts of WNT are due in part to the altered expression of thermocycle-entrained genes, understanding the roles of thermocycles in plant signaling and responses will be a critical component to anticipating the impacts of asymmetric changes in temperature patterns (68).

Disruption of the Circadian Clock and Phytochrome Signaling by WNT. In field conditions, we observe dynamic expression of most transcripts, consistent with prior observations in vegetative tissue (25, 95). Our results in the rice panicle show that WNT globally disrupt the timing of gene expression in field conditions. Even after weeks of growth in WNT, the transcriptional profiles differed between WNT and NNT panicles. Therefore, we propose an additional mechanism for the detrimental impacts of WNT. By disrupting the global patterns of gene expression, WNT disrupt the synchrony of molecular activities within the plant and the coordination with the external environment. The disrupted phase relationship between the timing of gene expression, downstream molecular events, and environmental factors results in reduced productivity. Although not well studied in rice, in *Arabidopsis*, plants with a clock that is out of phase with their environment show decreased rates of growth and photosynthesis (42, 96, 97). In barley, the rhythmic expression of photosynthetic parameters showed allelic variation that suggests adaptation to local environments (98). Therefore, the observed disruption of expression timing could contribute to the overall decrease in yield and biomass (Fig. 1).

How the DIF is perceived and integrated into the circadian clock is unknown. Our GRN analysis identified 26 TFs with targets disrupted by WNT. Many of these TFs are known circadian clock regulators, associated with the circadian clock, or are themselves expressed rhythmically in rice (47). We identified PIF1, a basic helix-loop-helix TF and Phytochrome-Interacting Factor, as a candidate regulator of WNT-sensitive transcripts. In *Arabidopsis*, the *Os*PIF1 homologs, *At*PIF4 and *At*PIF5, are regulators of thermoresponsive growth (99–101) and interact with circadian clock components (102, 103). *At*PIF4 up-regulates the auxin pathway, activating growth in response to increasing temperatures (104, 105). Under increasing temperatures, the expression of *At*PIF4 and *At*PIF5 is regulated by *At*PHYB. *At*PHYB interacts with *At*ELF3, part of the evening complex (102, 106, 107). Loss-of-function mutations in the evening complex mimics the *phybde* loss-of-function knockouts under warmer temperatures (59, 108). These interactions indicate that the photoreceptor PHYB connects changes in ambient temperature to the circadian clock in *Arabidopsis* (48–50). Although the connections between phytochromes and increasing temperature are not as well studied in rice, if WNT affect the rate of interconversion between active and inactive forms of PHYB in rice, this could disrupt the output from the circadian clock through PHYB. The observed disruption of the dynamic expression of thermocycle-entrained genes could be a consequence of the impact on this PHYB signaling pathway. In *Arabidopsis*, the higher-order phytochrome mutants show disrupted timing of metabolite accumulation. In the quadruple loss-of-function mutant in *Arabidopsis* phytochromes, *phyABDE*, the plants accumulate sugars and amino acids to a higher level during the day and mobilize the sugars faster at night. Thus, the reduced biomass of the phytochrome mutants is due to altered timing of photosynthesis and growth (109). WNT-grown rice plants also show a significant reduction in biomass (Fig. 1*E*) and alterations in grain quality (110–114), suggesting changes in sugar mobilization.

The plant circadian clock is dynamically plastic to changes in the environment, contributing to carbon homeostasis (44). Feedback from the metabolic status, in part through endogenous sugar levels, can dynamically adjust the circadian oscillator, depending on when the altered metabolism is perceived. These dynamic responses may explain contrasting shifts in expression we observe. For example, the delay in morning expressed photosynthetic transcripts and the advance in the expression of genes with nighttime peak levels (Fig. 4) may reflect temporally varying WNT-induced changes in carbon use and mobilization.

We also identified PRR95 as a candidate regulator of WNT-sensitive genes. PRR95, a Pseudo Response Regulator (PRR) family member, is a circadian clock component in rice (115, 116). In *Arabidopsis*, expression of the PRRs responds to temperature changes. Additionally, *At*PRR7 and *At*PRR9 are important for temperature compensation, the clock's ability to maintain a 24-h period across a range of temperatures (117). Here, we find that WNT alter the expression levels of *PRR95* (Fig. 7) and the predicted PRR95 targets. In our nighttime temperature gradients, we observed a correlation between *PIF1* and *PRR95* expression levels and the expression of their predicted targets. This coexpression across a decreasing DIF supports the predicted regulatory role of PIF1 and PRR95 on these WNT-sensitive transcripts and the effects of the thermocycle amplitude, potentially through the circadian clock or phytochrome signaling, on gene expression.

We identified other candidate regulators of WNT DEGs associated with the circadian clock. BBX24 is a CONSTANS-like transcription factor, which in *Arabidopsis* is an activator of PIF activity (66). EDH4 is essential in the environmental coordination of flowering (118). The *Arabidopsis* BBX24 ortholog has been identified as a target of the evening complex, along with the orthologs of PIF1 and PRR95. Only 11 *Arabidopsis* evening complex targets have orthologs detectibly expressed in the panicle, yet three were WNT DEGs (hypergeometric enrichment P value < 0.001). Although the limited overlap makes comparison difficult, the enrichment, combined with the established roles of PHYB and the evening complex in thermosensing and the discovery of *At*ELF3 as a prion-based thermosensor, suggests a role for the evening complex in the altered WNT expression patterns (49, 50, 52). In *Arabidopsis*, the warm temperature–responsive genes that were direct evening complex targets appear to be expressed earlier in the night (59). In contrast, the evening expressed WNT DEGs peak toward the end of the night, consistent with the predicted signal cascade downstream of the evening complex, which could include BBX24, PIF1, and PRR95. Our findings suggest that WNT disrupt the temporal expression patterns by affecting the circadian clock or circadian-related components. However, we cannot distinguish whether the effects are due to the reduced DIF's effect on the circadian clock entrainment, the downstream effects of increased nighttime respiration, or temperature effects on transcription, translation, or other biological activities necessary for proper temporal coordination.

In conclusion, growth under WNT disrupts the temporal expression patterns in the panicle. Multiple indicators suggest that this is through altered regulation of the circadian clock or circadian-regulated outputs. It is unclear whether the observed effects are a direct effect of WNT on the circadian clock machinery or an indirect consequence of increased nighttime respiration or altered metabolite transport. However, our results suggest that altered timing is a vital phenotype to monitor in WNT responses. The candidate regulators and their WNT-sensitive targets identified here can be

used as markers to monitor WNT effects across environments and genotypes and identify mechanisms to improve WNT tolerance in anticipation of future weather patterns.

Materials and Methods

Stress Treatment. WNT were imposed using an infrared heating facility with ceramic heaters (Fig. 1 and *SI Appendix*). Field-based tents (14) were used to impose 24, 26, 28, and 30 °C night temperature treatments for the validation experiments. Two replicate tents were randomly allotted for each of the treatments.

Data Availability. Transcript level data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession nos. GSE135847 and GSE126650). The expression of individual genes can be visualized at http://go.ncsu.edu/clockworkviridi_wnt.

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