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# HSP90 contributes to entrainment of the Arabidopsis circadian clock via the morning loop

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## Abstract

The plant circadian clock allows the synchronization of internal physiological responses to match the predicted environment. HSP90.2 is a molecular chaperone that has been previously described as required for the proper function of the Arabidopsis oscillator under both ambient and warm temperatures. Here we have characterized the circadian phenotype of the *hsp90.2-3* mutant. As previously reported using pharmacological or RNAi inhibitors of HSP90 function, we found that *hsp90.2-3* lengthens circadian period and the observed period lengthening was more exaggerated in warm-cold entrained seedlings. However, we observed no role for the previously identified interactors of HSP90.2, GI and ZTL, in *HSP90*-mediated period lengthening. We constructed phase-response curves in response to warmth pulses to identify the entry point of HSP90.2 to the oscillator. These PRCs revealed that *hsp90.2-3* has a circadian defect within the morning. Analysis of the *cca1*, *lhy, prr9*, and *prr7* mutants revealed a role for CCA1, LHY, and PRR7, but not PRR9 in HSP90.2 action to the circadian oscillator. Overall, we define a potential pathway for how HSP90.2 can entrain the Arabidopsis circadian oscillator.

## Introduction

Many organisms have evolved an internal-timing mechanism called the circadian clock to anticipate predictable environmental changes. In Arabidopsis, the circadian clock regulates approximately one-third of genes and 36% of Arabidopsis promoters show circadian regulation by transcript accumulation (COVINGTON AND HARMER 2007; COVINGTON *et al.* 2008; STAIGER *et al.* 2013). During the process of entrainment, the internal circadian clock is reset by daily exogenous cues (*zeitgebers*), to maintain synchronization with the diurnal cycle. For most organisms, the dominant *zeitgebers* are light and temperature changes perceived at dawn (OAKENFULL AND DAVIS 2017). Light input to the clock occurs via multiple types of photoreceptors; for example, in plants, phytochrome and cryptochromes control red- and blue-light signaling to the clock (OAKENFULL AND DAVIS 2017). For temperature, however, the *zeitgeber* input pathway leading to clock entrainment remains poorly understood (BOIKOGLOU *et al.* 2011; BUJDOSO AND DAVIS 2013; ANWER *et al.* 2014).

One current model of the plant circadian clock consists of interlocked transcriptional-translational feedback loops (BUJDOSO AND DAVIS 2013; RONALD AND DAVIS 2017). At the center of these loops are the morning expressed *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELOGNATED HYPOCOTYL* (*LHY*), and the evening expressed *TIMING OF CAB EXPRESSION1* (*TOC1*, also called *PSEUDO RESPONSE REGULATOR1*, *PRR1*) (MIZOGUCHI *et al.* 2002; DING *et al.* 2007). Upon being expressed, CCA1/LHY bind to the evening-element (EE) within the TOC1 promoter and directly repress *TOC1* expression (NAGEL *et al.* 2015). At dusk, TOC1 reciprocally represses the expression of *CCA1/LHY*, generating a negative feed-back loop (GENDRON *et al.* 2012).

Morning and evening phased regulators subsequently regulate CCA1/LHY and TOC1 activity. Starting just after dawn, *PRR9/7/5* are sequentially expressed throughout the day and directly repress the expression of *CCA1/LHY* through the recruitment of the TOPLESS co-repressor (NAKAMICHI *et al.* 2010; WANG *et al.* 2013). In the evening, GIGANTEA (GI) interacts and stabilizes the F-box protein ZEITLUPPE (ZTL) in a blue-light dependent manner to promote the degradation of TOC1 and PRR5 (KIM *et al.* 2007), which supports the role of protein-protein interactions in stabilizing circadian period (SCHÖNING AND STAIGER 2005). Finally, the evening complex, composed of LUX ARRYTHMO (LUX), EARLY FLOWERING3 (ELF3), and ELF4, repress the expression of *LUX*, *GI*, *PRR7*, and *PRR9* (NUSINOW *et al.* 2011; HERRERO *et al.* 2012).

HEAT SHOCK PROTEIN90 (HSP90) is a highly conserved and abundant protein in prokaryotes and eukaryotes. The HSP90 family of proteins are involved in the assembly, maturation, stabilization, and activation of proteins (CHEN *et al.* 2005). Arabidopsis has seven HSP90 isoforms (HSP90 1-7). Of these, four display cytosolic localization (HSP90.1-4), and the remaining (HSP90.5-7) are predicted to be localized to the chloroplast, mitochondria, and endoplasmic reticulum, respectively (KRISHNA AND GLOOR 2001). HSP90.2 has been previously linked to the circadian clock through protein-protein interactions with GI and ZTL (KIM *et al.* 2011; NOREN *et al.* 2016; CHA *et al.* 2017; GIL *et al.* 2017), and alleles at this locus have pathology phenotypes (HUBERT *et al.* 2003). HSP90 and GI are reported to act as co-chaperones to promote ZTL maturation and accumulation (CHA *et al.* 2017). Inhibition of global HSP90 activity by geldanamycin (GDA) application or through specific targeting of cytosolic HSP90 isoforms caused a lengthening of circadian period (KIM *et al.* 2011). ZTL and HSP90 have recently been shown to impart

thermotolerance to the circadian clock by acting as a protein quality control system at warmer temperatures (GIL *et al.* 2017). GI and ZTL also act as a hub in the plastid control of nuclear circadian rhythms in a PRR5 and HY5 signaling pathway (NOREN *et al.* 2016). Therefore, HSP90 isoforms likely have multiple roles within the circadian oscillator.

In this study we have characterized the circadian phenotype of the *hsp90.2-3* mutant. This specific allele at *HSP90.2* was chosen given its strong "poison pill" phenotype, as the null had no pathology phenotype (HUBERT *et al.* 2003). We found that *hsp90.2-3* has a longer circadian period in both light-dark (LD) and warm-cold (WC) entrained plants. This period-lengthening effect did not require either of the previously identified circadian interacting partners of HSP90.2, ZTL or GI. Phase-response curves in response to warmth pulses were constructed and revealed that *hsp90.2-3* displayed a defect at the morning phase. Further analysis revealed that the period-lengthening effects of GDA were lost in the *cca1*, *lhy*, and *prr7* backgrounds. However, no genetic role of *PRR9* was observed, revealing functional independence between PRR9 and PRR7. Together, this work has revealed new insights regarding how HSP90 could contribute to clock function.

## Methods

### Plant Lines

Ws-2 and Col-0 were used as wild type (WT) either harboring *CCR2::LUC*, CCA1*::LUC*, or *CAB2::LUC* (DOYLE *et al.* 2002; FARRÉ *et al.* 2005). The luciferase containing *gi-11, ztl* alleles, *prr7-3*, *prr9-1*, *prr7-3*/*prr9-1*, *cca1-11*, *lhy-21*, *cca1-11*/*lhy-21*, and *hsp90.2-3* have all been described previously (FOWLER *et al.* 1999; HUBERT *et al.* 2003; FARRÉ *et al.* 2005; KEVEI *et al.* 2006; DING *et al.* 2007). Before circadian phenotyping of *hsp90.2-3*, it was backcrossed 6 times to *Ws-2 CCR2::LUC*, and in the BC6F2 generation a homozygous *hsp90.2-3 CCR2::LUC* line was isolated and bulked for analyses.

### Bioluminescent Assays

Seeds were surface-sterilized and plated onto MS medium with 3% sucrose and then stratified for ~3 days. After stratification, seedlings were entrained under either 12/12 light-dark cycles (with a constant temperature of 22ºC), or 12/12 cycles of 22ºC/16ºC (with constant light) for 7 days (BOIKOGLOU *et al.* 2011; ANWER *et al.* 2014). On day 6, seedlings were transferred to black 96-well Microplates with MS medium containing 3% sucrose with DMSO, and where relevant, 2 µM of GDA. Plants were superficially treated with 15uL 5mM D-Luciferin. Seedlings were then re-entrained for one day under the respective entrainment conditions before being transferred to the TOPCOUNT® (PerkinElmer). All TOPCOUNT experiments were carried out under constant blue-red light and a constant temperature of 21ºC. Data was analyzed as previously described (HANANO *et al.* 2006; HANANO *et al.* 2008; KOLMOS *et al.* 2009). All experiments were replicated and provided consistent results.

### Phase-response-curve assay

For phase-response-curve (PRC) assays, plants were grown as for luciferase assays, as described above, under 7 days LD condition (12 hours in light and 12 hours in darkness) and then transferred to a TOPCOUNT® under constant red and blue light for one full day before a 3 hours long 27°C warmth pulse. This was respectively applied every 3 hours to a given 96-well plate beginning at ZT0. Resultant data was then analyzed using *Peak Picker* in Biological rhythms analysis software system (BRASS) (SOUTHERN AND MILLAR 2005). Here the first peak after the warmth treatment was chosen for both pulsed and non-pulsed plates and the time difference of the timing of the peak between pulsed and non-pulsed populations was calculated (COVINGTON *et al.* 2001).

**Statistical Analysis**

All statistical analysis was completed using R (version 3.4.2) within the R studio software package (version 1.1). Unless stated otherwise, the sample size for determining period estimates was 48 plants.

**Data Availability**

Seeds are available upon request. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

## Results

### *hsp90.2-3* causes a lengthening of circadian period

To establish if *hsp90.2-3* has a circadian phenotype, it (HUBERT *et al.* 2003) was introgressed with Ws-2 wild-type plants (WT) harboring *CCR2::LUC* [also termed *GRP7* (KÖSTER *et al.* 2014)] to generate the *hsp90.2-3 CCR2::LUC* line. The free-running period (FRP) of *CCR2::LUC* was then analyzed under constant light (LL) after plants were either entrained to light-dark (LD) or warm-cold (WC) cycles. After either entrainment protocol, *hsp90.2-3* was found to lengthen *CCR2::LUC* FRP (Figure 1A-D), but the magnitude of period lengthening was greater under WC entrainment compared to LD entrainment (ΔLD = 0.78 hours ± 0.05, ΔWC = 0.92 hours ± 0.05, [$p < 0.05$]). *hsp90.2-3* plants also displayed a change in the amplitude of *CCR2::LUC* rhythms; the amplitude of *CCR2::LUC* rhythms increased in LD entrained plants, while the amplitude decreased in WC entrained plants (Figure 1A-B).

As previously reported (O'NEILL *et al.* 2011), GDA treatment of Ws-2 *CCR2::LUC* resulted in a similar lengthening of FRP as observed in the *hsp90.2-3* mutant (Figure 2A). To determine if multiple HSP90 isoforms signal redundantly to the circadian clock, *hsp90.2-3* mutants were treated with 2 µM GDA. For both WC- and LD-entrained plants, GDA treatment resulted in further lengthening of *CCR2::LUC* FRP compared to non-treated *hsp90.2-3* seedlings (Figure 2B). However, unlike the *hsp90.2-3* mutant, *hsp90.2-3* in combination with 2 µM GDA resulted in a more severe period lengthening under LD entrainment compared to WC entrainment (ΔLD = 1.42 hours ± 0.05, ΔWC = 1.09 hours ± 0.06, [$p < 0.001$]). This

suggests that HSP90 isoforms act redundantly within the oscillator, but may contribute independently to different entrainment pathways.

To confirm the effects of GDA on periodicity, the FRP profile of Ws-2 *CAB2::LUC* was also tested. As seen with *CCR2::LUC*, *CAB2::LUC* FRP was longer when treated with 2 µM GDA compared to WT regardless of the prior entrainment condition (Supplementary Figure 1A). The period-lengthening effect was also found to not be accession dependent, Col-0 *hsp90.2-3 GI::LUC* lines also had a longer FRP than Col-0 WT *GI::LUC* (Supplementary Figure 1B). Additionally 2 µM GDA treatment of Col-0 WT seedlings also lengthened the FRP of both morning (*CCA1::LUC*) and evening (*TOC1::LUC*) reporter genes (Supplementary Figure 1C-D). These results suggest that the *hsp90.2-3* phenotype and the effects of GDA on circadian periodicity are not dependent on the reporter gene or ecotype used.

### ***HSP90*** **circadian period lengthening genetically does not require** ***GI*** **or** ***ZTL***

HSP90.2 protein has been previously reported to interact with the circadian component GI to stabilize ZTL (KIM *et al.* 2011). To determine if the observed effects of GDA on circadian periodicity required the activity of either GI or ZTL, the FRP of *CCR2::LUC* was analyzed in the previously described *gi-11* or *ztl-21* mutants (GOULD *et al.* 2006; KIM *et al.* 2007). *gi-11 CCR2::LUC* or *ztl-21 CAB2::LUC* were entrained to either LD or WC cycles before being treated with 2 µM GDA upon release into free-running conditions. Regardless of the prior entrainment condition, GDA treatment caused a lengthening of FRP in both the *gi-11* and *ztl-21* backgrounds (Figure 3). To confirm the *ztl* result, the FRP of additional *ztl* mutants harboring *CAB2::LUC* were examined. As was observed with *ztl-21 CAB2::LUC,* in all but one instance the other *ztl* mutants displayed a lengthening of circadian period under both

LD and WC entrainment when treated with GDA (Supplementary Figure 2). The only *ztl* allele that did not show any period lengthening was *ztl-30,* but this response was only seen in LD-entrained plants (Supplementary Figure 2A). Under WC conditions, GDA treatment in the *ztl-30* background caused the same period lengthening as observed for WT and the other *ztl* alleles treated with GDA (Supplementary Figure 2B).

To identify if GDA had an additive effect in the *ztl* or *gi* mutant backgrounds, Δperiod changes in response to GDA treatment were calculated (supplementary table 1 and table 2). No significance difference in period lengthening was observed for *gi-11* under LD or WC entrainment, or the majority of the *ztl* alleles entrained under LD cycles compared to Ws-2. However, under WC cycles the reverse was seen. Most *ztl* alleles had a significantly greater period lengthening effect when treated with GDA compared to GDA treated WT plants (supplementary table 2). This therefore suggests that the general period lengthening effect caused by GDA treatment did not require the presence of GI or ZTL, but HSP90 and ZTL activity could converge in a temperature-entrainment pathway.

### ***hsp90.2-3*** **has a morning phase defect**

To identify the point of entry of HSP90.2 to the circadian oscillator, a phase response curve (PRC) was generated for the WT and the *hsp90.2-3* genotypes. PRCs test the sensitivity of the oscillator to re-setting stimuli (*zeitgebers*) at different points of the day (COVINGTON *et al.* 2001). Three-hour long warmth pulses of 27˚C were applied at respective three-hour intervals to Ws-2 and *hsp90.2-3* plants throughout the day, and then the phase change in *CCR2::LUC* expression was recorded. In Ws-2, these warmth pulses elicited a phase advance of ~4 to ~7 hours

during the period from dawn to late morning (Figure 4). *hsp90.2-3* showed reduced phase advances during the same period; there was no observable phase advance at dawn, and only a maximum phase advance of ~2 hours by late morning (Figure 4). During the later afternoon, *hsp90.2-3* showed a subtler phase delay than was seen in Ws-2 (~0.5 hours and 2 hours respectively). No change in *CCR2::LUC* phase response was seen between *hsp90.2-3* and Ws-2 across the subjective night. This indicates that the likely entry point of HSP90.2 to the circadian oscillator occurs within the morning.

**CCA1/LHY and PRR7 are a hub for HSP90 Circadian Activity**

The morning loop of the Arabidopsis circadian clock is primarily composed of CCA1/LHY and PRR9/7 arranged in a reciprocal repressive loop (BUJDOSO AND DAVIS 2013; RONALD AND DAVIS 2017). To determine if the observed period lengthening effects of GDA required either CCA1/LHY, PRR9 or PRR7, the effects of GDA on FRP profiles of the respective mutant was analyzed after both light-dark and warm-cold entrainment conditions, respectively. *cca1-1, lhy-21 and cca1-1/lhy-21* mutants harboring *CCR2::LUC* displayed no period lengthening when treated with 2 µM GDA, regardless of the prior entrainment condition (Figure 5). Similarly to *cca1* and *lhy*, GDA treatment of *prr7-3 CCA1::LUC* and *prr7-3/prr9-1 CCA1::LUC* lines resulted in no observed period lengthening after either LD or WC entrainment (Figure 6). However, unlike *prr7, prr9-1 CCA1::LUC* did display a similar response to WT; GDA treatment caused a lengthening of *prr9-1* FRP and this occurred independently of the prior entrainment conditions (Figure 6A-B). As was observed in the *hsp90.2-3* mutant and in WT treated with GDA, *prr9-1* displayed a greater period lengthening when WC entrained. Together, this suggests that HSP90 requires the

237 presence of CCA1, LHY, and PRR7, but not GI nor ZTL, to lengthen circadian
238 period.

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## Discussion

HSP90.2 is a molecular chaperone previously shown to be required for proper circadian rhythms (KIM *et al.* 2011; O'NEILL *et al.* 2011; GIL *et al.* 2017). Here, we have confirmed this result by characterizing the *hsp90.2-3* mutant. This mutant was found to have a longer circadian period regardless of prior entrainment conditions, although there was a greater phenotypic defect after WC entrainment (Figure 1). Such phenotypes were not dependent on the Arabidopsis ecotype or reporter gene used (Supplementary Figure 1). Our work also supports that HSP90 isoforms function in a partially redundant manner to the circadian clock as treating the *hsp90.2-3* mutant with GDA resulted in further lengthening of circadian period (Figure 2). However, unlike the non-treated *hsp90.2-3* mutant, GDA treatment of *hsp90.2-3* resulted in a more pronounced period lengthening in LD- compared to WC-entrained plants. This suggests that HSP90 isoforms likely function redundantly in the general regulation of clock periodicity, but individual isoforms could contribute to separate light- and temperature-entrainment pathways.

Duality of function as both a core circadian component, but also separately as a contributor to the entrainment of the oscillator has now been described for ELF3, PRR9, PRR7, CCA1/LHY, and GI (FARRÉ *et al.* 2005; GOULD *et al.* 2006; DING *et al.* 2007; THINES AND HARMON 2010; DALCHAU *et al.* 2011; BUJDOSO AND DAVIS 2013; HAYDON *et al.* 2013). The contribution of HSP90 to either light or thermal entrainment of the clock could be dependent on the cellular localization of HSP90 isoforms. These isoforms in Arabidopsis are localized to different cellular compartments and would therefore have different client proteins, and indeed a host of protein interactions can be predicted in the regulation of periodicity (SCHÖNING AND STAIGER 2005; BUJDOSO AND DAVIS 2013). For example, HSP90.5 is localized to the stroma,

has a light-responsive transcript accumulation and *hsp90.5* (*cr88*) has defects in red-light perception (LIN AND CHENG 1997; CAO *et al.* 2003). The perception of red-light is critical for the entrainment of the clock (OAKENFULL AND DAVIS 2017). Therefore, HSP90 isoforms may contribute within separate light and temperature entrainment pathways in a cell localization dependent manner.

HSP90 has been previously linked to the circadian oscillator through direct interactions with GI and ZTL (KIM *et al.* 2011). Applying 2 µM of GDA to either the *gi* or *ztl* mutants resulted in lengthening of circadian period as seen with WT (Figure 3, Supplementary Figure 2), suggesting that HSP90 does not require either ZTL or GI to regulate FRP. HSP90 and ZTL have been recently shown to maintain circadian thermostability and GI is also required to maintain circadian oscillations and the precision of these oscillations under warm temperatures (GOULD *et al.* 2006; GIL *et al.* 2017). We did find that applying 2 µM of GDA to either the *ztl* or *gi* mutant increased the variance of periodicity estimates, an effect not seen in *hsp90.2-3* or with GDA treatment in either the Ws-2 or Col-0 background (data not shown). Therefore, the previously described HSP90/ZTL/GI module may act as buffering agent to maintain clock precision through the regulation of proteostasis, while HSP90.2 could function independently of the GI and ZTL module to regulate clock periodicity (Figure 7).

PRCs for WT and *hsp90.2-3* plants exposed to warmth pulses were constructed to determine when HSP90.2 regulates circadian periodicity. These revealed a phase defect within the morning (Figure 4). We subsequently found that there was no lengthening of circadian period in *cca1*, *lhy*, and *cca1/lhy* plants treated with GDA regardless of the prior entrainment conditions (Figure 5). We also found that *prr7* and *prr7/9* failed to show any lengthening of FRP when treated with GDA

after either LD or WC conditions, respectively (Figure 6). Genetically one interpretation is that the timing of the entry point of HSP90.2 depends on all of *LHY*, *CCA1*, and *PRR7*. In contrast, *prr9* did show a longer circadian period when treated with GDA (Figure 6). This reveals a functional independence between PRR9 and PRR7 within the circadian oscillator. Together, these data suggest that CCA1/LHY, HSP90 and PRR7 constitute a unique morning loop that regulates general circadian oscillations regardless of the prior entrainment condition (Figure 7), and that is consistent with the morning PRC defects seen in *hsp90.2-3* plants (Figure 4).

It is not fully clear how HSP90.2 signals through CCA1/LHY and PRR7. HSP90 isoforms have a large client pool of proteins (KADOTA AND SHIRASU 2012), and many genes become misregulated when *HSP90* function is inhibited, including *PRR9* and *CCA1* (SANGSTER *et al.* 2007). However, we observed no role for PRR9 in GDA's period lengthening effect (Figure 6), and we also observed no change in *CCA1* or *LHY* expression level or pattern of expression in LD entrained plants (data not shown). Previous work also found no effect of GDA on PRR7 protein stability or gene-expression profile (KIM *et al.* 2011). This therefore suggests that the effects of HSP90 on *CCA1*/*LHY* and *PRR7* may not be fully direct and could occur upstream of these transcriptional regulators. The activity of CCA1/LHY and PRR7 is modulated by a range of morning-associated transcriptional activators, and afternoon/evening expressed transcriptional repressors (RONALD AND DAVIS 2017). Further screens of the FRP of clock mutants when treated with GDA will provide further answers for how HSP90 regulates clock periodicity. In this it is notable that physiologically and genetically temperature and light set the clock in differing ways (BOIKOGLOU *et al.* 2011; ANWER *et al.* 2014).

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## Figure legends

**Figure 1 – *hsp90.2-3* has a circadian period phenotype.** Free-running profile of *CCR2::LUC* in Ws-2 and *hsp90.2-3* seedlings under constant red-blue light (LL) after plants were prior trained to 12:12 cycles of **(A,C)** light-dark or **(B,D)** warm-cold. Plants were released into free-running conditions at ZT36. C.P.S, counts per second. **(C,D)** Mean period estimates of **(A & B)** respectively. Error bars represent SEM. *** $p < 0.001$. Significance determined using a T test. In each experiment 48 WT and *hsp90.2-3* seedlings were analysed for rhythms under each entrainment protocol. All experiments repeated at least once.

**Figure 2 – Geldanamycin treatment has an additive effect on *hsp90.2-3* circadian period phenotype.** Period estimates of the free-running period (FRP) of *CCR2::*LUC in the **(A)** Ws-2 or **(B)** *hsp90.2-3* seedlings treated with either DMSO or with 2 µM geldanamycin (GDA) under constant red-blue light (LL). For **(A)** plants were entrained under 12:12 light-dark cycles. Plants in **(B)** were entrained under the stated entrainment conditions. For both **(A,B)** GDA was applied upon transfer to free-running conditions. Error bars represent SEM. ** $p < 0.01$, *** $p < 0.001$. Significance determined via a T test. In each experiment, the FRP of 48 seedlings was examined. Each experiment was repeated at least once.

**Figure 3 – Geldanamycin lengthening of circadian period is not dependent on GI or ZTL.** Period estimates of the free-running profile of **(A)** *gi-11 CCR2::LUC*, or **(B)** *ztl-21 CAB2::LUC*. Plants were entrained under 12:12 cycles of light-dark (LD) or warm-cold (WC) before transfer to free-running conditions. 2 µM of geldanamycin (GDA) was applied upon transfer to free-running conditions. Error bars represent SEM. ** $p < 0.01$, *** = $p < 0.001$. Significance determined using a T test. In each experiment 48 seedlings of WT and the respective mutant were examined under each entrainment condition apart from *gi-11* LD (GDA) where n = 30. All experiments repeated at least once.

**Figure 4 – *hsp90.2-3* has a morning phase defect.** Ws-2 and *hsp90.2-3 CCR2::LUC* plants were entrained for 7 days under 12/12 cycles light-dark cycles before being exposed to 3 hour long pules of 27 ºC. PRCs were then constructed by plotting the observed phase shift in *CCR2::LUC* expression against the circadian time that heat pulses were administered. Positive values represent phase advances, negative values represent phase delays. *** $p < 0.001$, n.s. no significant difference. Significance determined via a T test. Error bars represent pooled standard error.

**Figure 5 – GDA fails to lengthen circadian period in the *cca1* or *lhy* mutant.** **(A,B)** Period estimates of *CCR2::LUC* profile under free-running conditions in Ws-2 (wild-type, WT), *cca1-11* and *lhy-21* mutants treated with or without 2 µM GDA.

Plants were prior entrained to LD (**A)** or WC **(B)** cycles before being released into free-running conditions. GDA treatment was applied upon transfer to free-running conditions. Error bars represent SEM. In each experiment 48 WT and mutant seedlings were examined under each entrainment condition. n.s. = no significance, *** = $p < 0.001$. Significance determined by a T test. All experiments were repeated at least once.

**Figure 6 – The effect of geldanamycin treatment on period length is disrupted in the *prr7* and *prr7/prr9* background**. Period estimates of the free-running profile of *CCA1::LUC* in the Col-0, *prr7-3*, *prr9-1* and *prr7-3/prr9-1* background. Plants were entrained under **(A)** LD, or **(B)** WC cycles before being released into free-running conditions. Plants were treated with or without 2 µM geldanamycin (GDA) upon transfer to free-running conditions. Error bars represent SEM. ** = $p < 0.01$, *** = $p < 0.001$ and n.s. = no significance. Significance determined via a T test. In each experiment 48 WT and mutant seedlings were examined under each entrainment condition. All experiments were repeated at least once.

**Figure 7 – An expanding role of HSP90 within the Arabidopsis Circadian Oscillator.** HSP90 has been previously shown to interact with ZTL to regulate both periodicity and under heat stress the stability of the oscillator. GI and HSP90 are thought to co-operatively stabilise ZTL activity. Here we have found that Hsp90 also signals independently of GI and ZTL through the morning loop components CCA1/LHY and PRR7. We did not detect a direct effect of HSP90 on regulating *CCA1/LHY* expression, and HSP90 was also found previously to not regulate *PRR7* expression. This therefore indicates HSP90 is signalling via a yet unidentified protein to regulate CCA1/LHY and PRR7 activity. Purple lines indicate an interaction (direct or indirect), red lines indicate a repressive interaction and blue lines highlight the effect of the interaction on the oscillator.

**Supplementary Figure 1 – The *hsp90.2-3* and GDA period lengthening effects are not accession or reporter gene specific.** Period estimates of the free-running profile of **(A)** *CAB2:LUC* in the Ws-2 background, **(B)** *GI::LUC* in the Col-0 and Col-0 *hsp90.2-3* background, and **(C)** *CCA1:LUC* and **(D)** *TOC1:LUC* in the Col-0 background. Plants were entrained under light-dark (LD) or warm-cold (WC) cycles before being released into free-running conditions. ** $p < 0.01$ and *** $p < 0.001$ Where stated, plants were treated with or without 2 µM geldanamycin upon transfer to free-running conditions. Error bars represent SEM.

**Supplementary Figure 2 – Geldanamycin lengthens free-running period in a range of *ztl* mutants.** The period lengthening effect of geldanamycin on *CAB2::LUC* period was seen in both Ws-2 and *ztl* mutants entrained under **(A)** light-dark and **(B)** warm-cold cycles. Geldanamycin was applied upon transfer to free-

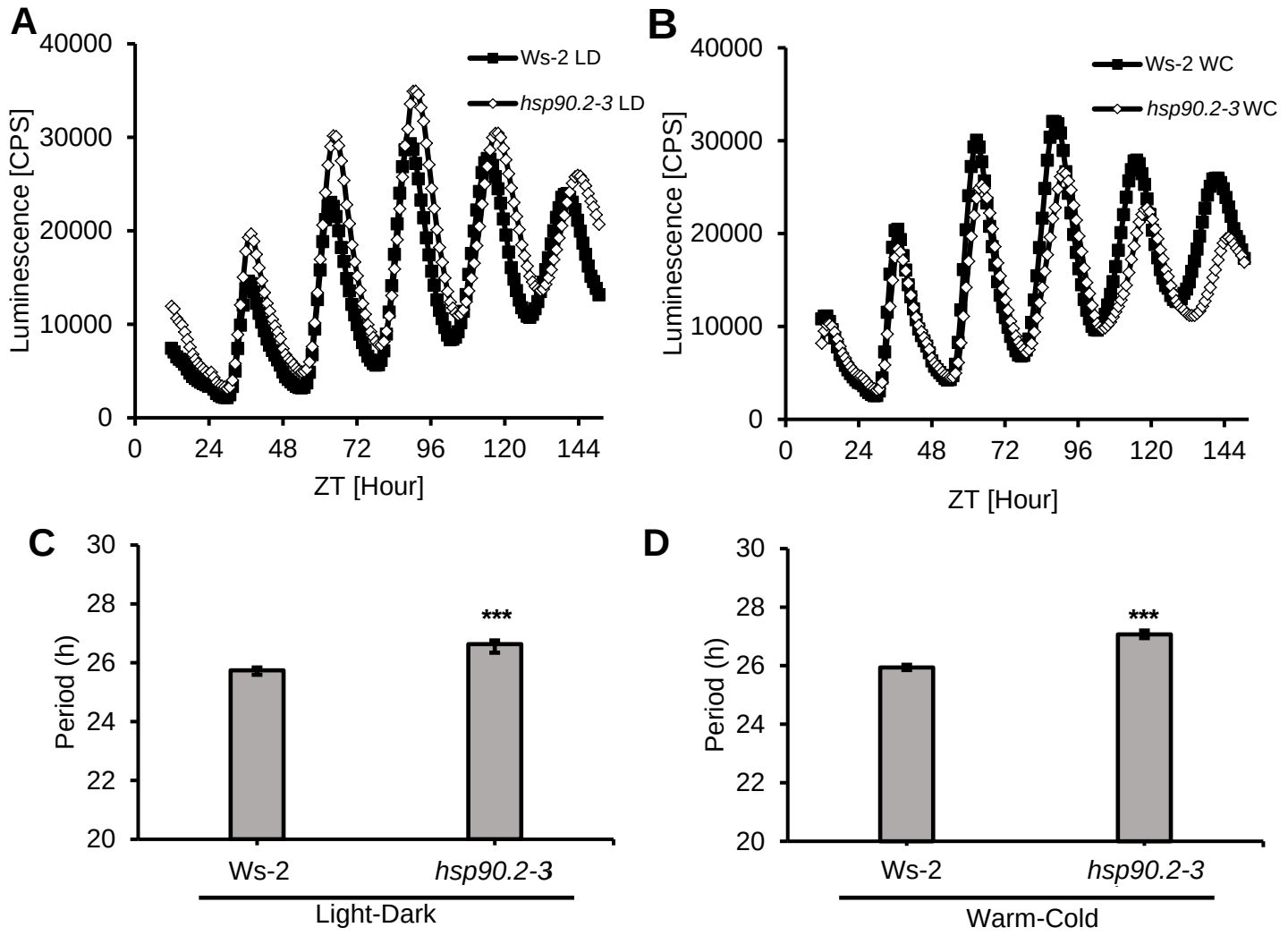
running conditions. In each instance, 48 individual seedlings were examined. For **(A)**, Ws-2 data is the same as that of supplementary figure 1A and for **(A, B)** the *ztl-21* data is the same as figure 2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. no significant difference. Significance determined by T test. Error bars represent SEM.

**Supplementary Table 1 – The average change in period length when treated with 2 µM geldanamycin in Ws-2 *CCR2::LUC* and *gi-11 CCR2::LUC* lines**. Significance was determined with a one-tail T test. n.s. highlights no significance, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. LD, light-dark cycles. N = 48 rhythmic plants, aside from *gi-11* GDA treated entrained under LD where n = 30 rhythmic plants.

| Genotype | Entrainment Condition | ΔPeriod Difference | Significance |
|---|---|---|---|
| Ws-2 *CCR2::LUC* | LD | 1.27 ± 0.09 | |
| *gi-11 CCR2::LUC* | LD | 1.32 ± 0.10 | n.s |
| Ws-2 *CCR2::LUC* | WC | 1.51 ± 0.11 | |
| *gi-11 CCR2::LUC* | WC | 1.29 ± 0.09 | n.s |

**Supplementary Table 2 – The average change in period length when treated with GDA in the Ws-2 and *ztl* mutants backgrounds harboring *CAB2::LUC***. Significance was calculated with a one-tail T test. N.S. highlights no significance found, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. LD: light-dark, WC: warm-cold. n = 48 rhythmic plants.

| Genotype | Entrainment Condition | ΔPeriod Difference | Significance |
|---|---|---|---|
| Ws-2 *CAB2::LUC* | LD | 0.98 ± 0.08 | |
| *ztl-21 CAB2::LUC* | LD | 0.89 ± 0.06 | n.s |
| *ztl-23 CAB2::LUC* | LD | 1.31 ± 0.08 | ** |
| *ztl-24 CAB2::LUC* | LD | 0.81 ± 0.06 | n.s |
| *ztl-25 CAB2::LUC* | LD | 1.08 ± 0.06 | n.s |
| *ztl-26 CAB2::LUC* | LD | 1.21 ± 0.10 | n.s |
| *ztl-28 CAB2::LUC* | LD | 2.06 ± 0.25 | *** |
| *ztl-29 CAB2::LUC* | LD | 1.35 ± 0.07 | *** |
| *ztl-30 CAB2::LUC* | LD | 0.64 ± 0.2 | n.s |
| Ws-2 *CAB2::LUC* | WC | 0.97 ± 0.06 | |
| *ztl-21 CAB2::LUC* | WC | 1.29 ± 0.08 | *** |
| *ztl-23 CAB2::LUC* | WC | 1.47 ± 0.10 | *** |
| *ztl-24 CAB2::LUC* | WC | 1.16 ± 0.11 | n.s |
| *ztl-25 CAB2::LUC* | WC | 1.57 ± 0.10 | *** |
| *ztl-26 CAB2::LUC* | WC | 1.45 ± 0.14 | *** |
| *ztl-28 CAB2::LUC* | WC | 1.17 ± 0.07 | ** |
| *ztl-29 CAB2::LUC* | WC | 1.31 ± 0.09 | *** |
| *ztl-30 CAB2::LUC* | WC | 1.18 ± 0.07 | ** |

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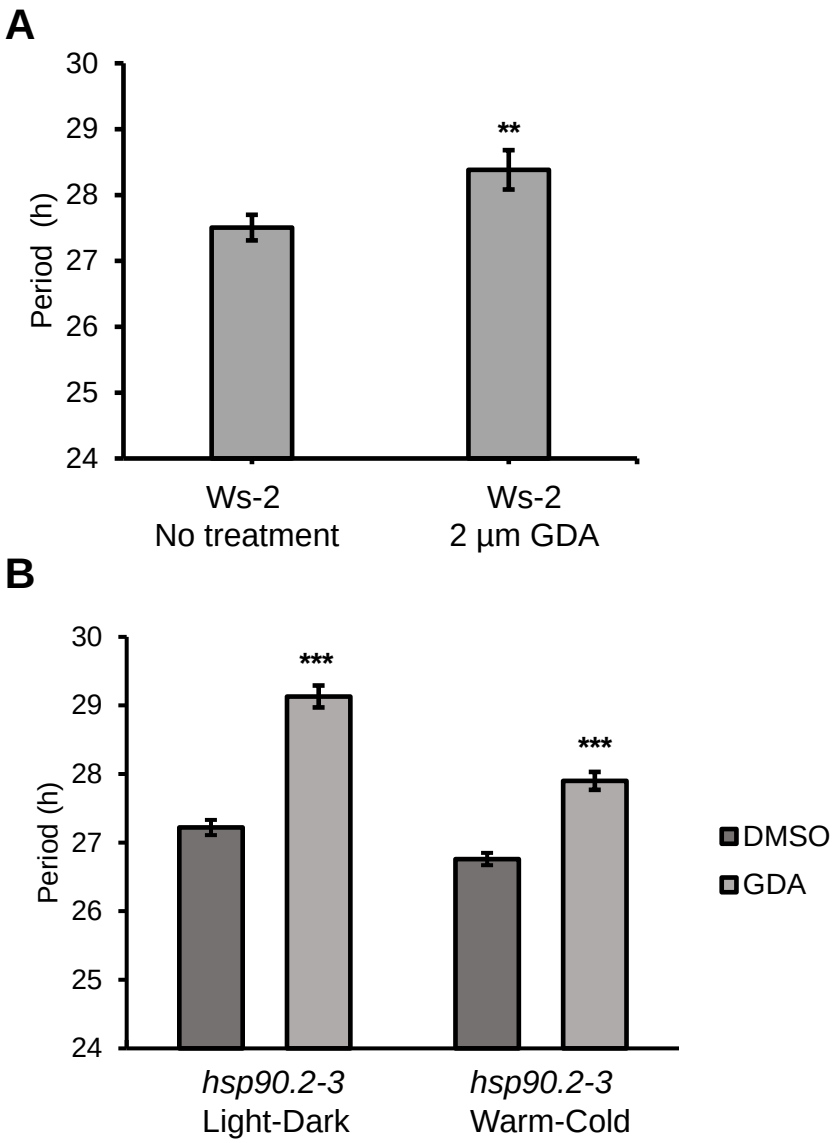


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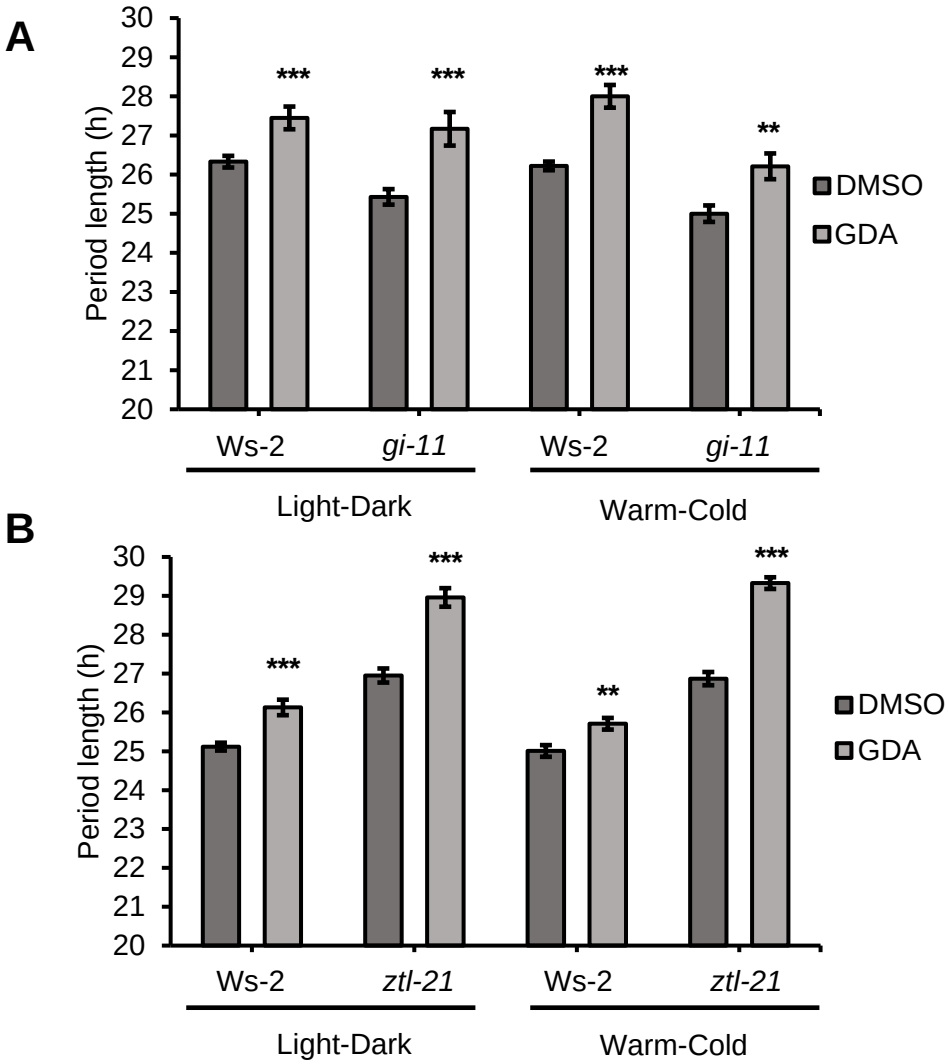


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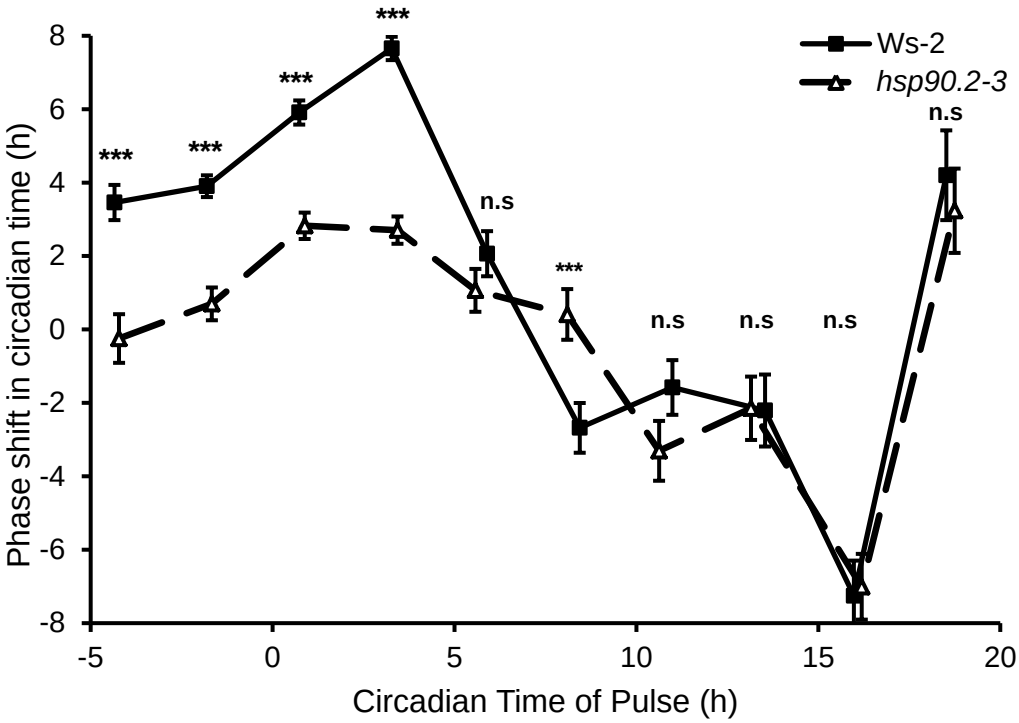


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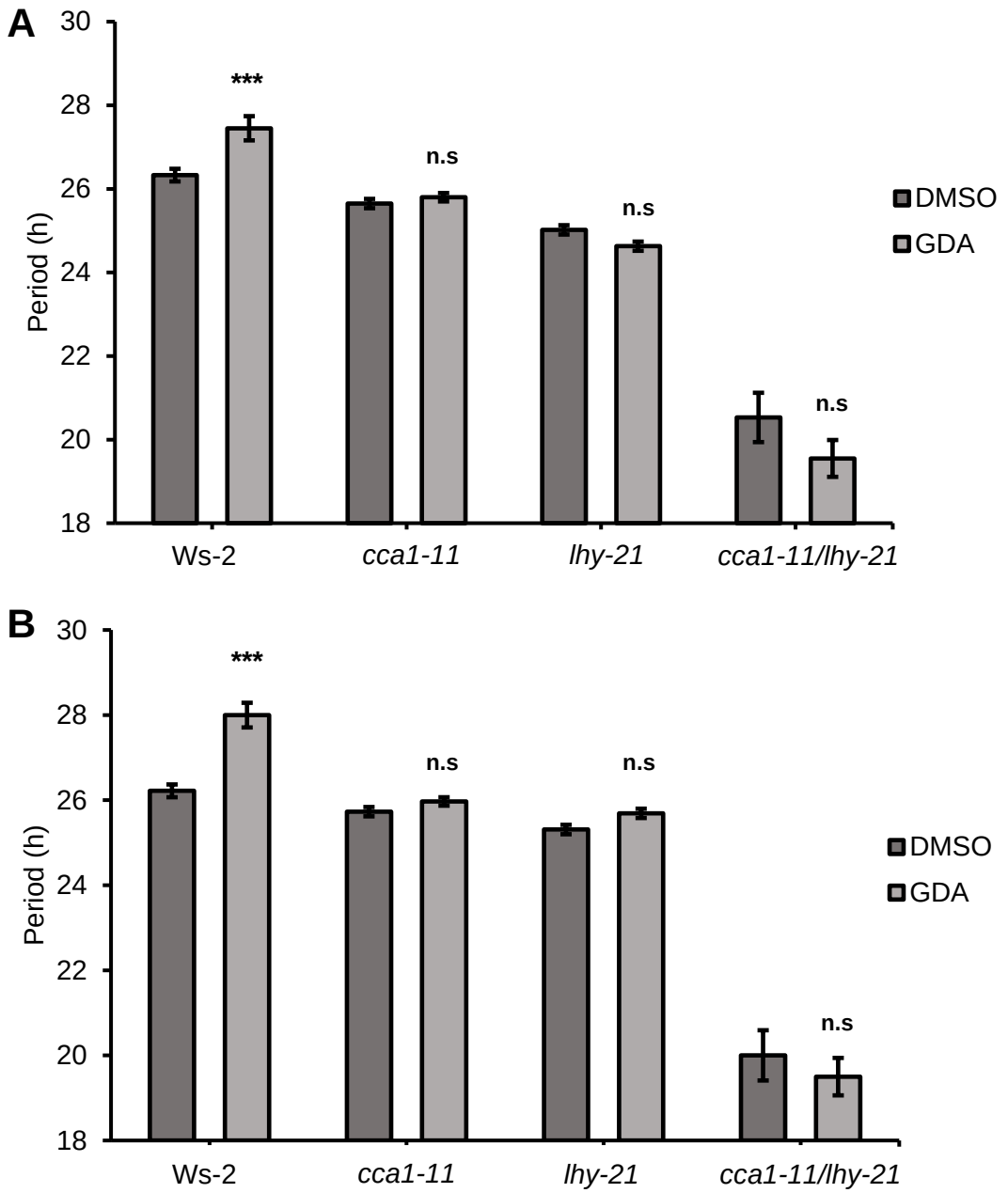


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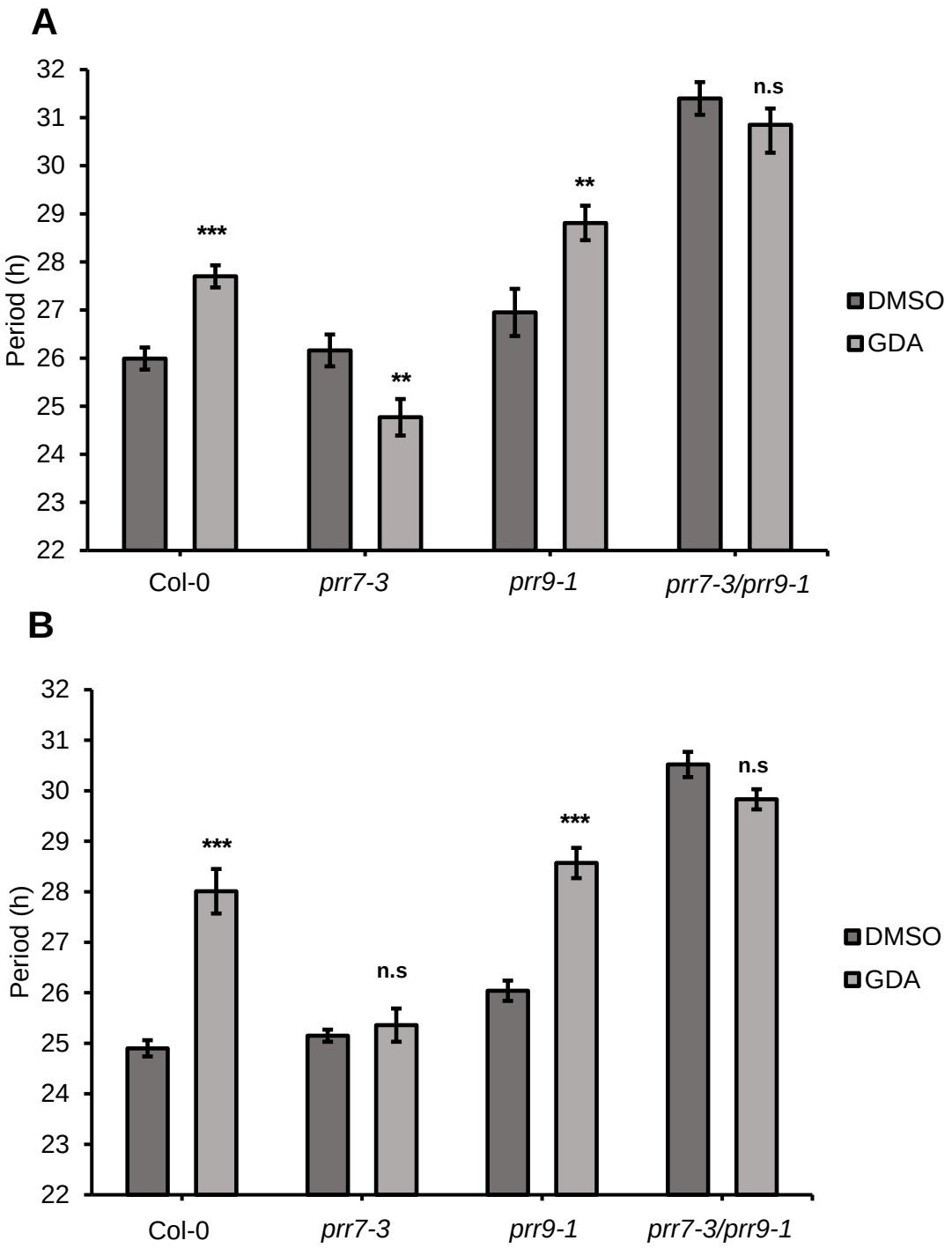


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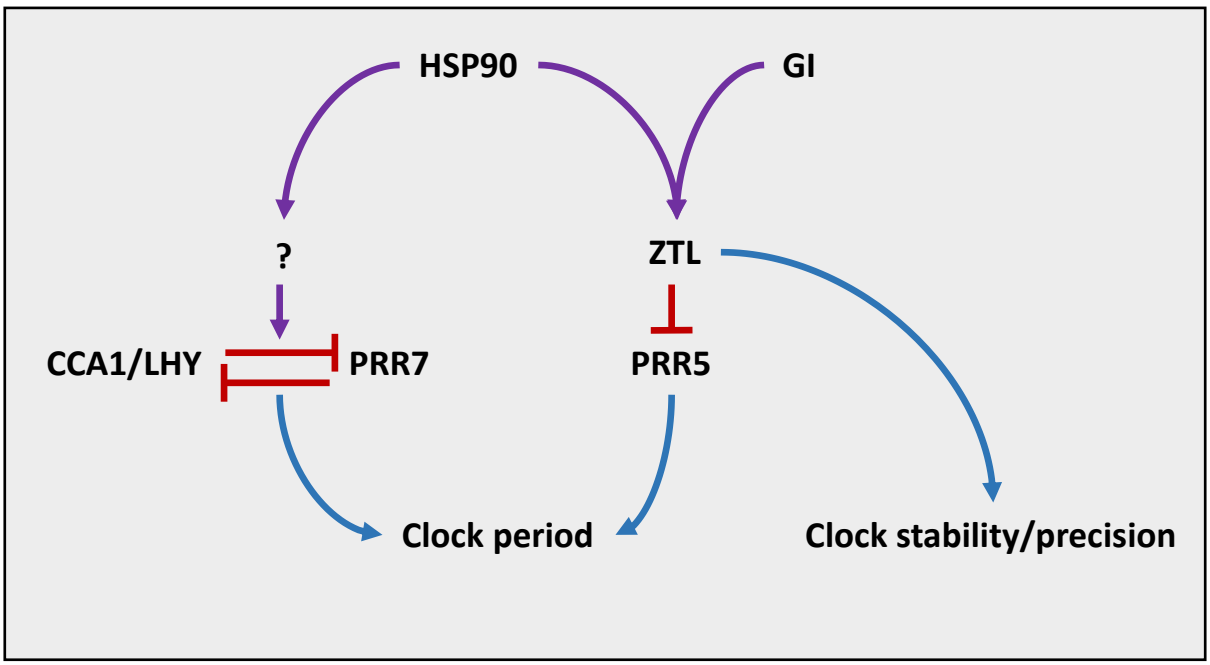


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