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Title

Expression of circadian clock genes and diurnal oscillations of key physiological events in response to AsV and AsIII in soybean plants

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Highlights

- Arsenic affected the expression profile of key clock genes in soybean plants.
- Arsenic-response physiological events showed diurnal oscillations.
- Differences between leaves and roots suggests the existence of organ-specific clocks.
- Effects of arsenic were dependent on its chemical form, plant organ and time of the day.
- Putative circadian regulation of arsenic response could display a role in tolerance.

ABSTRACT

Soybean (*Glycine max* L.) is often cultivated in soils with a high content of arsenic (As). Since this greatly affects crop growth and yield, the response mechanisms underlying tolerance to As should be well understood. Circadian rhythms are known to play a central role in plant adaptation to stressful conditions. Therefore, our aim was to evaluate the effects of arseniate (AsV) and arsenite (AsIII) on the expression of key circadian clock genes (CG) and on the possible diurnal oscillation of physiological events induced by As in soybean plants. Leaves and roots were evaluated during light-dark phases at 4-h intervals. The changes caused by As in the expression profiles of all CGs analyzed depended on its chemical form and the plant organ involved. Overexpression of *GmLCL1*, *GmTOC1*, *GmPRR9* and *GmGI* was observed in leaves mainly upon AsV-treatment, while underexpression of *GmLCL1* and overexpression of *GmPRR9*, *GmELF4* and *GmGI* occurred in roots, mainly upon AsIII-treatment. Moreover, As seemed to have an influence on

the daily fluctuations observed in events triggered in response to the stress, such as stomatal aperture control, phytochelatin (PC) and glutathione (GSH) content, and total antioxidant, ascorbate (APx) and glutathione peroxidase (GPx) activities. In particular, the increase in the synthesis of PCs (the main As chelators) and the decrease in their precursor, GSH, were higher during the light phase, while APx and GPx activities increased during the dark phase in As-treated plants. The results suggest a link between the circadian clock and the response to As in soybean plants, since exposure to As modified CG expression and induced important changes in the diurnal oscillation of several As-response mechanisms. Circadian clock regulation, then, might play a vital role in the tolerance to As.

1. INTRODUCTION

Soybean's (*Glycine max* L.) great economic importance lies in the worldwide commercialization of its grains and sub-products (oil and meal) for multiple uses, such as food and biofuel production. Argentina is one of its main producers and exporters (Good et al., 2016), but also one of the countries where arsenic (As) contaminates a large area of the soils and groundwater used for growing it (Nicolli et al., 2012; Castro et al., 2017).

As is a highly toxic metalloid whose predominant environmental forms are arseniate (AsV) and arsenite (AsIII) (Abbas et al., 2018). When incorporated by soybean plants, it considerably reduces their growth and productivity (Bustingorri and Lavado, 2014; Armendariz et al., 2016a). Our laboratory has thoroughly studied the response to AsV and AsIII in soybean plants (Talano et al., 2013; Armendariz et al., 2016 a, b; Vezza et al., 2018, 2019) and has found that some of the most relevant physiological mechanisms involved are stomatal aperture control, antioxidant system induction, and the synthesis of phytochelatins (PCs) (the main As chelators) and their precursor glutathione (GSH). Although each study was performed at a single time of the day, possible diurnal variations in these mechanisms were not explored.

The rotation of the Earth causes periodic changes in environmental conditions, generating diurnal cycles of light and temperature reflected in light-dark phases. Plants are able to adapt to these predictable environmental changes largely thanks to the circadian clock, an endogenous timing mechanism with a periodicity of approximately 24 h. It generates rhythmic oscillations in many biological processes, in such a way that they are triggered at particular times during the day. Thus, plant metabolism is synchronized with environmental rhythms, so that fitness and growth performance are maximized (Seo and Mas, 2015).

At the genetic level, the circadian clock is constituted by several genes that interact through transcriptional and post-transcriptional feedback loops, leading to rhythmic gene expression. Simply put, Circadian Clock Associated1 (*CCA1*) and Late Elongated Hypocotyl (*LHY*) are partially redundant

transcription factors expressed at dawn that repress the expression of late/evening-expressed genes, including Timing Of Cab Expression1 (*TOC1*), Early Flowering 3 (*ELF3*), Early Flowering 4 (*ELF4*) and Gigantea (*GI*). They also promote the expression of Pseudo-Response Regulator (*PRRs*) gene family members. Moreover, maximum expression of *TOC1* and *ELFs* occurs during the late/evening phase and represses the expression of *CCA1/LHY* and *PRRs*, respectively (Pokhilko et al., 2012; Seo and Mas, 2015). However, recent molecular biology studies have uncovered new circadian clock components and complex regulatory relationships that complicate clock modeling (Johansson and Köster, 2019).

The circadian clock acts as the master controller of plant gene expression, regulating more than 30% of the transcriptome (Johansson and Köster, 2019). The genes it regulates are involved in many relevant physiological processes, including flowering time, photosynthesis, gas exchange, phytohormone synthesis and signaling, the metabolism of iron, sulfur, nitrogen and carbohydrates, redox state and stress responses (Bendix et al., 2015; Greenham and McClung, 2015; Sosa Alderete et al., 2018). Many stress-induced genes have clock response elements in their promoters that make their diurnal regulation possible. This regulation ensures that responses are triggered at the most appropriate time during the day, to reduce the adverse effects of the stressful condition and, at the same time, optimize metabolic efficiency (Spoel and Van Ooijen, 2014). This ability is associated with stress tolerance, since mutations in circadian clock components lead to changes in the sensitivity to several adverse conditions like drought, saline, osmotic, heat or cold stress (Nakamichi et al., 2009).

Most studies about the circadian clock in plants have used the model plant *Arabidopsis thaliana*. However, knowledge about the circadian clock in plant species with agronomic importance, such as soybean, could contribute to understanding and improving their development under different environmental conditions. Even though some homologs to the *A. thaliana* circadian clock genes have been identified in soybean (Marcolino-Gomes et al., 2014; Syed et al., 2015), their response to As has not yet been investigated. Therefore, the aim of present work was to elucidate the effects of AsV and AsIII treatments on clock gene expression profiles (*GmLCL1* [homologs of *CCA1/LHY*], *GmTOC1*, *GmPRR9*, *GmELF4* and *GmGI*) as well as on the possible diurnal oscillation of physiological events involved in the response to As, such as stomatal aperture control, PC and GSH content, total antioxidant activity (TAA), and ascorbate (APx) and glutathione peroxidase (GPx) activities in leaves and roots.

2. MATERIALS AND METHODS

2.1 Plant material, growth conditions and treatments

Soybean seeds (*Glycine max* L.) cv. DM 4670 were surface-disinfected using 70% (v/v) ethanol for 1 min and 30% (v/v) sodium hypochlorite for 8 min, washed thoroughly with sterile distilled water, and germinated in Petri dishes with moistened filter paper at 25°C in the dark for 3 d.

Germinated seeds were transferred to pots containing sterile perlite, irrigated by capillarity with 1/4 Hoagland solution and maintained in a growth chamber under the following conditions: 14h light/10h dark cycle, 100 μmol m^{-2} s^{-1} light intensity and 25±2°C.

After 14 d of growth, plants in V2 phenological stage were irrigated with solutions containing 25 μM sodium arsenate (AsHNa$_2$O$_4$7H$_2$O) (SIGMA) (AsV), 25 μM sodium arsenite (NaAsO$_2$) (SIGMA) (AsIII) (treatments) or distilled water (control). After 1 d of As treatment, leaves and roots were harvested at 4-h intervals, from the beginning of the light phase (Zeitgeiber Time (ZT) 0) to the end of the dark phase (ZT24). The samples were immediately frozen, homogenized with liquid N$_2$ and kept at -80°C until needed for further assays.

2.2 Analysis of circadian clock gene expression

The expression of circadian clock genes, including *GmLCL1*, *GmTOC1*, *GmPRR9*, *GmELF4* and *GmGI* (Table S1), was evaluated in leaves and roots by quantitative real-time PCR (qPCR). Total RNA was isolated using Trizol Reagent (Invitrogen) following the manufacturer's instructions. Absorbance was measured at 260 nm to estimate RNA yield and the purity by the 260/280 nm ratio. Then, the RNA (1 μg) was treated with DNase (Promega) for 30 min at 37°C. MMLV reverse transcriptase (Epicentre) and random hexamer primers (Biodynamics) were added for the synthesis of cDNA, following the manufacturer's indications. For amplification by qPCR, a mix was prepared with 1 μl of the cDNA, 400 nM forward-reverse primers (Table 1) and 7.5 μl of Itaq Universal SYBR Green supermix 2x (Biorad) in a total volume of 15 μl. The cycling conditions were 95°C for 3 min and 40 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. The standard curve linearity and PCR efficiency were optimized for each pair of primers. The normalized expression (NE) was calculated using the following formula: NE = $2^{-\Delta Ct}$, where ΔCt=Ct experimental$-$Ct normalizer. Elongation factor 1B (*GmEF1B*) gene was used as a reference gene, since it has been identified as stably expressed under many developmental stages and stressful conditions, as well as in different plant tissues (Wang et al., 2012; Marcolino-Gomes et al., 2014). The primers used in the present work were previously described by Marcolino-Gomes et al. (2014) (Table S1).

2.3 Stomatal aperture analysis

To analyze stomatal aperture, an acrylic layer (synthetic colorless nail coating) was brushed onto the abaxial side of fully expanded leaves. The dried acrylic layer was carefully extracted to obtain epidermal impressions, which were mounted with glycerol/water 1:10 for microscopic analysis (D'ambrogio de

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Argüeso, 1986). The histological preparations were observed and photographed using a Zeiss Axiophot microscope equipped with image capture and digitization (AxioVision 4.3, with an AxioCam HRc 200x magnification camera). The images were analyzed using the Image-ProPlus program. Finally, the ostiole area was calculated as a measure of stomatal aperture.

2.4 GSH and PC determination

To determine the levels of GSH and PCs, non-protein thiols (NPT) were extracted by homogenizing plant tissue (200 mg) with 5% sulfosalicylic acid (1 mL) and centrifuging it at 10,000 rpm and 4°C for 30 min. Total GSH was determined in supernatants of leaves and roots through the method described by Anderson (1985). For this, a reaction mixture was prepared using 143 mM sodium phosphate buffer (pH 7.5), 6.3 mM EDTA, 0.248 mg mL^{-1} NADPH and 6 mM DTNB [5,5´-Dithiobis (2-nitrobenzoic acid)], and incubated at 30°C for 10 min. The reaction was started by adding the previously obtained supernatant (10 µL in the case of leaves and 30 in the case of roots) and the GSH reductase enzyme (GR) (1.3 U). GSH content was plotted against changes in absorbance at 412 nm. Reference standards of 20-150 µM GSH were prepared and analyzed in the same way to obtain a calibration curve.

To determine PC concentration in roots, NPT was quantified following De Vos et al. (1992), with some modifications. Supernatant (300 µL) was mixed with 600 µL of 143 mM sodium phosphate buffer (pH 7.5) and 20 µL of 10 mM DTNB. After 2 min, absorbance was measured at 412 nm and NPT concentration was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. Finally, PC concentration was calculated as: PCs=NPT-GSH.

2.5 Total antioxidant activity

Total antioxidant activity (TAA) was determined as the free radical-scavenging activity of DPPH (1,1-diphenyl-2-picrylhydrazyl), as described by Brand-Williams et al. (1995). Leaves and roots (200 mg) were homogenized separately with 50% (v/v) methanol (1 mL), incubated in ice for 2 h and then centrifuged at 10,000 rpm and 4°C for 15 min. The supernatant (15 µL from leaves and 80 µL from roots) was mixed with 60 µM DPPH methanol solution and incubated in the dark for 30 min at room temperature. Then, absorbance was measured at 515 nm before and after incubation. Finally, TAA was calculated and expressed as: DPPH inhibition (%)=[(IA-FA)/IA]x100, where IA is the initial absorbance and FA is the final absorbance (after incubation).

2.6 Activity of antioxidant enzymes

The activity of GSH-dependent antioxidant enzymes, such as ascorbate (APx) and glutathione peroxidase (GPx) was analyzed. Leaves and roots were homogenized separately with an extraction buffer (1:7 w/v) [50 mM potassium phosphate buffer (pH 7.8), 0.5 mM EDTA and polyvinylpolypyrrolidone (PVPP)] and then centrifuged at 10,000 rpm and 4°C for 30 min to obtain the supernatant that was used to analyze enzymatic activity. Total APx activity was determined by tracking the decrease in absorbance at 290 nm due to the oxidation of L-ascorbic acid ($\mathcal{E}_{290nm}$ 2.8 M^{-1} cm^{-1}) (Hossain and Asada, 1984). For this, 0.2 mM ascorbic acid and 0.2 or 0.4 mM H_2O_2 were used as substrates. One unit (U) of APx was defined as the amount of enzyme needed to produce the oxidation of 1 μmol of ascorbic acid after 1 min of reaction. Total GPx activity was determined by tracking the decrease in absorbance at 340 nm due to the oxidation of NADPH ($\mathcal{E}_{340nm}$ 6.2 M^{-1} cm^{-1}) (Flohe and Gunzler, 1984). Thus, 0.24 U GR, 0.1 mM GSH, 0.15 mM NADPH and 0.15 mM H_2O_2 were used in the reaction mixture. One U of GPx was defined as the amount of enzyme needed to produce the oxidation of 1 μmol of NADPH after 1 min of reaction.

2.7 Statistical analysis

A factorial arrangement was used with three different treatments (without As [control], AsV and AsIII) and at seven different points in time (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20 and ZT24). Data were analyzed using the InfoStat program (v. 2012e; InfoStat, National University of Cordoba, Argentina). To calculate significant differences, an ANOVA statistical analysis was performed, followed by Duncan's test with a significance level of 0.05 ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Analysis of clock gene expression profiles in soybean plants

The present work analyzed the expression of key circadian clock genes in soybean, such as *GmLCL1*, *GmTOC1*, *GmPRR9*, *GmELF4* and *GmGI*, which are orthologs of those from *A. thaliana* (Pokhilko et al., 2012; Marcolino-Gomes et al., 2014). At least two *LHY/CCA1*-like homologs have been found in soybean, but since their corresponding orthologs in *A. thaliana* cannot be identified, they are named *GmLCL1* and *GmLCL2* (Marcolino-Gomes et al., 2014). In this study we evaluated the expression of *GmLCL1*.

As shown in Fig. 1 and 2, all the circadian clock genes we analyzed conserved a rhythmic expression pattern consistent with the circadian oscillations of homologous genes from *A. thaliana* (Pokhilko et al., 2012) and different soybean varieties (Liu et al., 2009; Marcolino-Gomes et al., 2014; Syed et al., 2015; Locke et al., 2018). *GmLCL1* and *GmTOC1* displayed expression peaks at dawn (Fig. 1A, 2A) and dusk (Fig. 1B, 2B), respectively, while expression of *GmPRR9*, *GmELF4* and *GmGI* peaked mainly during the light phase (Fig. 1C-E, 2C-E). These circadian expression profiles were observed in the leaves and roots of

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soybean plants (Fig. 1, 2) although with some differences between both organs. *GmPRR9* and *GmGI* peaked at ZT12 in leaves (Fig. 1C, 1E) and at ZT8 in roots (Fig. 2C, 2E). Similarly, *GmELF4* peaked near ZT12 in leaves (Fig. 1D), while its maximum expression was detected between ZT12 and ZT16 in the roots of control plants (Fig. 2D). In addition, the relative expression of all circadian clock genes evaluated was higher in leaves than in roots (Fig. 1, 2). These findings suggest the existence of different organ-dependent clocks in the same plant, as was discussed in more detail by Endo (2016) and Inoue et al. (2018). In this sense, a recent study performed by Li et al. (2020) showed different circadian dynamic in the expression of core oscillators and their target genes as well as in clock component protein-protein interactions between shoot and root of *Arabidopsis* plants. Organ-specific clocks were also identified in other plant species, such as maize, even at tissue and cellular level (Wang et al., 2011). However, the relationship between different clocks are still unknown. The way the root clock runs has been described as a simplified version of the shoot clock, which could largely be due to differences in how much each organ is exposed to light, with root clocks being entrained by low-intensity light (Bordage et al., 2016). Moreover, recent evidence has shown that plant clocks are potentially hierarchical. In other words, there appear to be signaling mechanisms synchronizing the clock in different organs, and the shoot clocks might be able to influence the root clocks, possibly through light signals travelling from shoot to root (Nimmo, 2018). However, the extent to which the circadian clocks in different organs are coupled and the nature of the underlying coupling mechanisms are so far unknown. To the best of our knowledge, existing reports only deal with the soybean clock in shoots (Marcolino-Gomes et al., 2014; Syed et al., 2015; Locke et al., 2018), so this study is the first to simultaneously evaluate the expression patterns of clock genes in soybean leaves and roots. The different rhythmic properties observed for the clocks of leaves and roots could enable the temporal and spatial separation of the biological processes they regulated, so that they take place at the optimal time of the day and in the appropriate organ as well.

3.2 Effects of As treatments on clock gene expression

The effects of AsV and AsIII on circadian clock gene expression were evaluated next. The diurnal expression of several clock genes underwent significant changes in the leaves and roots of As-exposed plants with respect to the control. Such changes were dependent on the plant organ and the chemical form of As involved (Fig. 1, 2). In leaves, there was an upregulation of *GmLCL1*, *GmTOC1*, *GmPRR9* and *GmGI* (Fig. 1A-C, E), while in roots, As treatment produced a down-regulation of *GmLCL1* (Fig. 2A) and an upregulation of *GmPRR9*, *GmELF4* and *GmGI* (Fig. 2 C-E). Some differences were also detected between AsV and AsIII treatments. For example, the expression peaks of *GmPRR9* and *GmGI* at ZT12 were 30 and 35% higher, respectively, in the leaves of AsV-treated plants than in those treated with AsIII (Fig. 1C, E).

On the other hand, expression levels for these same genes were higher in the roots of AsIII-treated plants than in those treated with AsV. In this organ, *GmPRR9* expression differed 38% at ZT8 between AsIII and AsV treatments (Fig. 2C), while *GmGI* expression differed 41% at ZT4 and 17% at ZT8 (Fig. 2E). These organ-dependent variations induced by As support the hypothesis about the existence of organ-specific clocks, which might not only run at different rhythms, but also be independently entrained by the same stressful condition, making precise responses to environmental cues possible.

An essential function of the clock is to direct downstream biological processes. The clock genes affected by As have been associated with several of the physiological and biochemical processes involved in the response to As in soybean plants, as elucidated in our previous research (Vezza et al., 2018, 2019). Several studies point towards a regulation of abscisic acid (ABA) biosynthesis and signaling by the TOC1 and PRRs transcripts (Legnaioli et al., 2009; Nakamichi et al., 2009), which could mediate the stomatal closure observed in As-treated soybean plants. In addition, ELFs family members could modulate water transport and aquaporin expression in roots (Takase et al., 2010) and, therefore, As uptake. Expression of the *GI* gene, may be sensitive to changes in sugar levels (Müller et al., 2014; Endo, 2016), so the gene could act as a connector between environmental signals that modify sugar metabolism, such as stress by As, and the circadian clock. Nevertheless, since most functional studies about clock genes have been performed in *A. thaliana*, findings could be different in other plant species, as suggested by Müller et al. (2014). The fact that soybean is a polyploid species further complicates the matter: because it has more than one ortholog clock gene with those from *A. thaliana*, these extra genes could respond differently to the same stressful condition, as observed under drought and flooding conditions (Syed et al., 2015). In this work, the two chemical forms of As affected the expression of key clock genes in soybean, which means they could also have an impact on the daily oscillations of defense mechanisms triggered in response to As stress. Some of these mechanisms are evaluated below.

3.3 Physiological processes related to As response in soybean plants

3.3.1 Diurnal variation of physiological processes

In addition to clock gene expression, we assessed the diurnal variation of physiological processes involved in the response to As (Vezza et al., 2018, 2019). Stomatal aperture in leaves, PC content in roots, and GSH content, TAA, APx and GPx activities in leaves and roots were analyzed at 4-h intervals for 24 h (Fig. 3, 4). PC content was not evaluated in the leaves of control or As-treated plants since PCs were previously undetectable there, due to the scarce translocation of As (Vezza et al., 2019). The synthesis of PCs is activated by metals/metalloids, as Ogawa et al. (2011) suggested and Vazquez et al. (2009) demonstrated in soybean plants exposed to As and Cd.

Almost all the physiological processes evaluated exhibited diurnal variations under control conditions (Fig. 3, 4), with the exception of GPx in leaves, whose activity did not change across the 24 h (Fig. 3E). Stomatal aperture was observed throughout the light phase in leaves (Fig. 3A) and PC content peaked at ZT8 in roots (Fig. 4A). It is known that stomatal aperture is one of the processes under circadian control. Disrupting the circadian clock caused by overexpression of *CCA1/LHY* resulted in loss of ability to open stomata in *Arabidopsis* plants (Hassidim et al., 2017). Besides, *elf3* and *toc1* mutant also shows defects in stomatal aperture (Zhang et al., 2013 and references therein). In contrast, the involvement of circadian control in the regulation of many biological processes is still unknown, especially those related to specific responses to stress, such as PC synthesis. The potential crosstalk between PC synthesis and clock genes is an interesting aspect that should be deepened in further studies due to PCs role in the detoxification of metals/metalloids.

As observed for clock gene expression, different diurnal profiles were found in leaves and roots for those events examined in both. Under control conditions, the highest value for GSH was observed at ZT8 in the two organs, though peak amplitude was higher in roots than in leaves (Fig. 3B, 4B). TAA peaked during the late dark phase (ZT20-24) in leaves (Fig. 3C), and during the late light phase (ZT12) in roots (Fig. 4C). In terms of antioxidant enzyme activity in control leaves, APx had a maximum peak during the light phase (ZT8) and a significant increase during the dark phase (ZT20) (Fig. 3D), while the profile for GPx was non-oscillatory throughout the diurnal cycle (Fig. 3E). The highest enzymatic activity in control roots, on the other hand, was observed at the end of the dark phase (ZT24) (Fig. 4D, E). In *A. thaliana*, the generation of reactive oxygen species (ROS) is regulated by the circadian clock, and mutations in clock genes affect the transcriptional regulation of ROS homeostasis regulon (Guadagno et al., 2018; Poór et al., 2018). Thus, ROS rhythms could determine the temporal activity of the antioxidant system, including GSH, TAA, APx and GPx. Moreover, circadian clock regulatory elements were identified in promoters of genes corresponding to antioxidant enzymes in other plant species, such as rice and wheat (Menezes-Benavente et al., 2004; Tyagi et al., 2018). However, due to the diversity and complexity of metabolic pathways related to antioxidant system, its components could have widely varying diurnal profiles depending on environmental conditions, plant species and organ, without apparent general patterns (Poor et al., 2018). According to those findings, the diurnal variations in physiological processes observed in this work could be the result of a regulation mediated by the circadian clock. The different profiles in leaves and roots, furthermore, could indicate that this regulation is organ-specific, thus supporting the existence of different clocks in the plant, although complementary studies are necessary to prove these assumptions particularly in soybean plants.

3.3.2 Effects of As treatments on diurnal oscillation of physiological processes

As exposure affected all the physiological processes evaluated. As observed for clock gene expression, these effects depended on the chemical form of As, the plant organ and the time of the day. Exposure to both AsV and AsIII significantly reduced stomatal aperture in leaves (Fig. 3A). This As-induced response could have an important function in decreasing water flow and, therefore, preventing the entry of the metalloid into the plant (Rucińska-Sobkowiak, 2016; Vezza et al., 2018). Nevertheless, As has been found to enter and accumulate in soybean leaves and roots, since stomatal closure is not complete (Armendariz et al., 2019). This entry induces different response mechanisms, including the activation of the antioxidant system (Armendariz et al., 2016a; Vezza et al., 2019). In this sense, GSH content significantly increased at ZT8 in the leaves of plants treated with both chemical forms of As, but remained high up until the dark phase (ZT16-20) only upon exposure to AsIII (Fig. 3B), considered to be the most toxical form (Armendariz et al., 2016a). To estimate the cellular redox state upon As-treatment, we also analyzed TAA, which is attributed to non-enzymatic antioxidant compounds. In the leaves of plants treated either with AsV or AsIII, there was a clear TAA peak during the light phase at ZT8 and ZT12, respectively, whereas a significant decrease could be observed during the dark phase (ZT24) in comparison to the control (Fig. 3C). APx and GPx activities increased markedly during the dark phase in the leaves of As-treated plants, mainly when AsIII was used (Fig. 3D, E). These results suggest a temporary coordination of the physiological processes evaluated.

As explained before, PCs were only detected in the roots of As-treated soybean plants. The highest levels were observed at ZT8, with slightly higher values under AsIII treatment (Fig. 4A). PCs play a key role in the tolerance to As because they are able to act as chelants, decreasing the amount of free metalloid and, consequently, its interaction with cellular components and its translocation to aerial parts (Yadav 2010; Vezza et al., 2019). This first pathway to As detoxification is of greater relevance in roots, since this is the organ where As is uptaken and accumulated cellularly in the first place. The temporary profile for PC synthesis could be related to the dynamics of As uptake in plants. The metalloid can be incorporated apoplastically, following the water flow, and then enter the symplastic pathway through aquaporins, as is the case for AsIII, or phosphate transporters, as is the case for AsV (Zhao et al., 2009; Mukhopadhyay et al., 2014). The water flow is largely driven by the hydrostatic forces that transpiration generates in the stomata. This occurs during the day, as indicated by the greater stomatal aperture observed at this time (Fig. 3A). Maximum water flow rates in xylem vessels were recorded around ZT6 in different plant species, such as maize and rice (Lopez et al., 2003; Sakurai-Ishikawa et al., 2011), and had a correlation with the gene expression profile of aquaporins from the PIP family, which peaked between ZT2 and ZT6 and decreased to basal values during the dark phase (Lopez et al., 2003; Sakurai-Ishikawa et al., 2011). The

activity of phosphate transporters might also be regulated by the circadian clock, since it was observed to increase during the light phase by Wang et al. (2014). These events suggest that the As uptake rate oscillates diurnally, likely reaching its maximum level during the light phase. In this case, the time of the day in which PC synthesis increases (ZT8) (Fig. 4A) appears to coincide with the one when there is greater As incorporation, which is supported by the fact that PC synthesis is induced by As, as discussed above (Ogawa et al., 2011).

GSH content in the roots of As-treated plants was the same or less than that in control plants at most of the day times evaluated (Fig. 4B). A remarkable difference was observed at ZT8, where treatments with AsV and AsIII caused a significant reduction in GSH content. Under AsIII treatment, GSH content seemed to conserve a diurnal profile although at an advanced phase with respect to the control, because it peaked at ZT4 instead of ZT8. This decrease in GSH levels, mainly at ZT8, coincided with the time of the day in which PC synthesis is at its highest (Fig. 4A). PCs are known to be synthesized from GSH (Yadav, 2010), something which could increase the consumption of this metabolite. Moreover, the GSH content profiles did not match those observed for TAA (Fig. 4C), suggesting the involvement of other antioxidant compounds. These could play an important role in the response to As, since there were changes in TAA in the roots of As-exposed plants, in comparison to untreated plants (Fig. 4C). Maximum TAA peaks were observed during the light phase, at ZT0 and ZT8 for AsV treatment and ZT4 in the roots of AsIII-treated plants (Fig. 4C). TAA also increased with respect to the control at ZT24 under As treatment (Fig. 4C). These changes in the oscillatory profile of TAA could mean that As affects the temporary regulation performed by the circadian clock.

The activity of antioxidant enzymes (APx and GPx) decreased at ZT0 or showed no significant changes during the light phase (ZT4-12) in the roots of As-treated plants, compared to the control (Fig. 4D, E). By contrast, there were significant increases in the enzymatic activities of APx (Fig. 4D) and GPx (Fig. 4E) at ZT16 and ZT20 in roots treated with AsIII. GPx activity was similarly induced during the dark phase (from ZT16 to ZT24) in leaves under the same treatment (Fig. 3E). APx activity also increased at the end of the dark phase (ZT24) in these leaves (Fig. 3D). The activity of both enzymes depends on GSH content, because it is used by GPx as an electron donor and facilitates the recycling of ascorbate used by APx (Yadav, 2010). Our results showed a significant increase in the enzymatic activity of APx (Fig. 3D and 4D) and GPx (Fig. 3E and 4E) during the dark phase in AsIII-treated plants, which was coincident with the low GSH content, mainly in roots (Fig. 3B, 4B). An increase in antioxidant enzyme activity at night has also been observed in other stress-exposed plant species such as *Crocus sativus* and *Medicago sativa* exposed to low temperatures, and *M. crystallinum* and *Sedum sp.* under saline and water stress, suggesting that these adverse environmental conditions might increase oxidative stress at night (Poór et al., 2018 and

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references therein). Moreover, the low activity by GPx and APx detected in roots during the light phase (Fig. 4D, E) was also coincident with the lowest GSH levels, which means that during this phase the GSH pool could be used for the synthesis of PCs. These compounds are key in the As detoxification pathway and their maximum content was observed at ZT8 (Fig. 4A).

The findings of the present work, which are summarized in Figure 5, demonstrate the importance of considering diurnal variations in the study of stress responses. For example, previous research by our laboratory found that APx and GPx activities did not change in soybean leaves and decreased in roots under AsV and AsIII treatments, which might have indicated that these enzymes did not play an important role in metalloid tolerance (Vezza et al., 2019). However, these assays were carried exclusively during the early light phase, which prevented the detection of the significant increase in APx and GPx activities that occurred during the dark phase, mainly under AsIII treatment (Fig. 3D-E, 4D-E). Thus, this new study carried out during a full diurnal cycle allows us to conclude that the occurrence, type and magnitude of stress responses could be different depending on the time of the day. Besides, these circadian responses could differ widely from one plant species and type of stress to another, so specific studies are required for each particular biological system (Poór et al., 2018 and references therein). Further studies are needed, moreover, to continue elucidating the relationship between circadian clock genes and the physiological processes involved in the response to As. Recent research provides evidence of cross-talk between the circadian clock and plant metabolism. Although light and temperature are the most common exogenous inputs that synchronize the clock, other non-photic and endogenous inputs have been proposed. Sugars and ROS, for instance, might entrain circadian rhythms by regulating the gene expression of clock components (Schippers et al., 2013; Stangherlin and Reddy, 2013). Changes in sugar content due to alterations in photosynthesis and oxidative stress due to ROS generation, the most important adverse effects observed in soybean under As-stress (Armendariz et al., 2016a; Vezza et al., 2019), could both provide the metabolic feedback to which the changes in clock gene expression observed here could be attributed. After synchronization, the circadian clock conveys the temporal information to several output pathways. For example, it has a direct regulatory function in the biosynthesis and signaling of phytohormones (Atamian and Harmer, 2016), many of which regulate responses to As, such as the previously discussed interaction between TOC1-PRR9 and ABA. However, since the circadian clock is a recently explored aspect in plants, plenty of interesting questions remain for a better understanding of its involvement in the tolerance to As-stress, such as how the molecular and physiological dimensions communicate with each other and how they are affected by As stress.

4. CONCLUSION

Our findings show that stress induced by AsV and AsIII affects the gene expression profile of key circadian clock components (*GmLCL1*, *GmTOC1*, *GmPRR9*, *GmELF4*, *GmGI*) in the leaves and roots of soybean plants. Interestingly, we also observed that several physiological events involved in the response to As, such as stomatal aperture, PC and GSH content, and TAA, APx and GPx activities, exhibit diurnal oscillation, which showed changes in As-treated plants depending on the time of day. For example, PC synthesis was higher during the light phase, while APx and GPx activities increased during the dark phase, indicating a possible temporary coordination. These circadian dynamics suggest a regulatory interaction between clock genes and physiological responses to As in soybean plants. Moreover, differences between circadian profiles of leaves and roots open the possibility that the regulatory mechanism is organ-specific.

The results of the present work are novel since the As effects on circadian rhythms in soybean plants were unknown until now. We believe that these results contribute to the generation of a new way of studying and understanding the stress responses. However, many further studies will be needed to know the crosstalk between the molecular and physiological clock and its role in As tolerance.

Author statement

M.E.V., L.G.S.A. and M.A.T. planned the experiments. M.E.V. did all the experiments. L.G.S.A. contributed with the analysis of circadian clock gene expression. M.E.V., L.G.S.A., E.A. and M.A.T. contributed to the interpretation of the results. M.E.V. wrote the manuscript with input from all authors.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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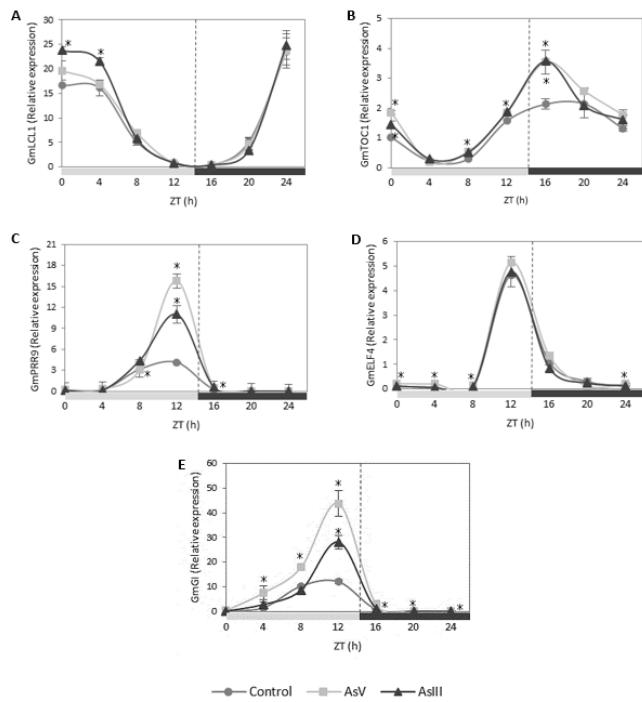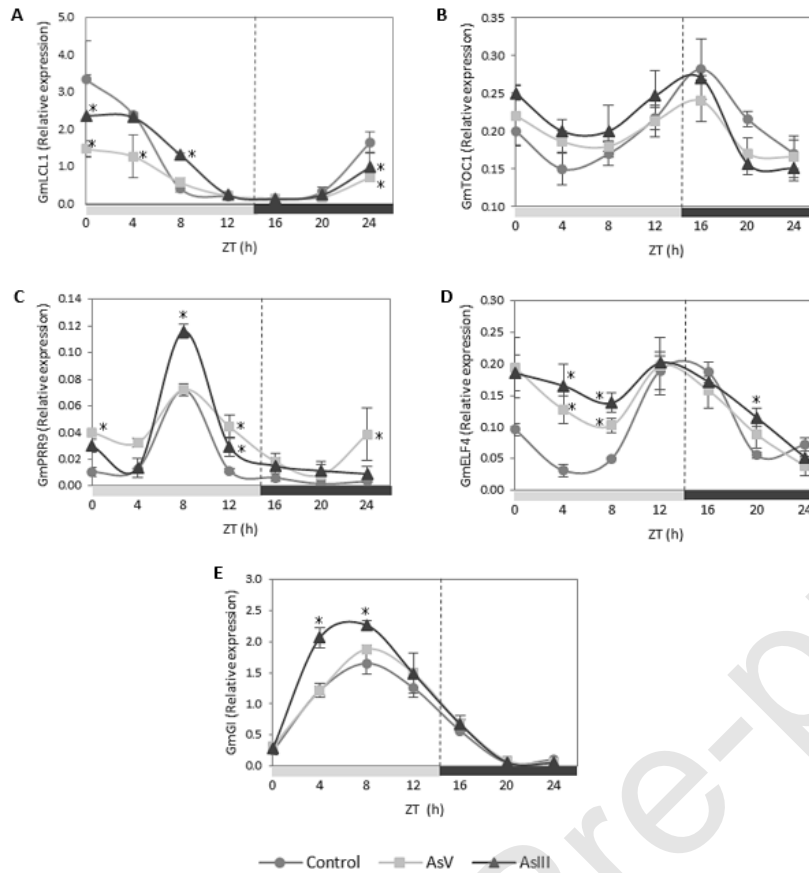
Figure 1: Expression of clock genes: *GmLCL1* (A), *GmTOC1* (B), *GmPRR9* (C), *GmELF4* (D) and *GmGI* (E) in leaves of soybean plants under control conditions and treated with 25 µM AsV or AsIII. Light and dark gray bars indicate light and dark phases, respectively. The results represent the mean ± SE (n=3). * indicates significant difference between control and treated plants in each time point (Duncan test, p ≤ 0.05). More information about statistical analysis results is presented in the Tables S2 and S3.

Figure 2: Expression of clock genes: *GmLCL1* (A), *GmTOC1* (B), *GmPRR9* (C), *GmELF4* (D) and *GmGI* (E) in roots of soybean plants under control conditions and treated with 25 µM AsV or AsIII. Light and dark gray bars indicate light and dark phases, respectively. The results represent the mean ± SE (n=3). * indicates significant difference between control and treated plants in each time point (Duncan test, p ≤ 0.05). More information about statistical analysis results is presented in the Tables S4 and S5.
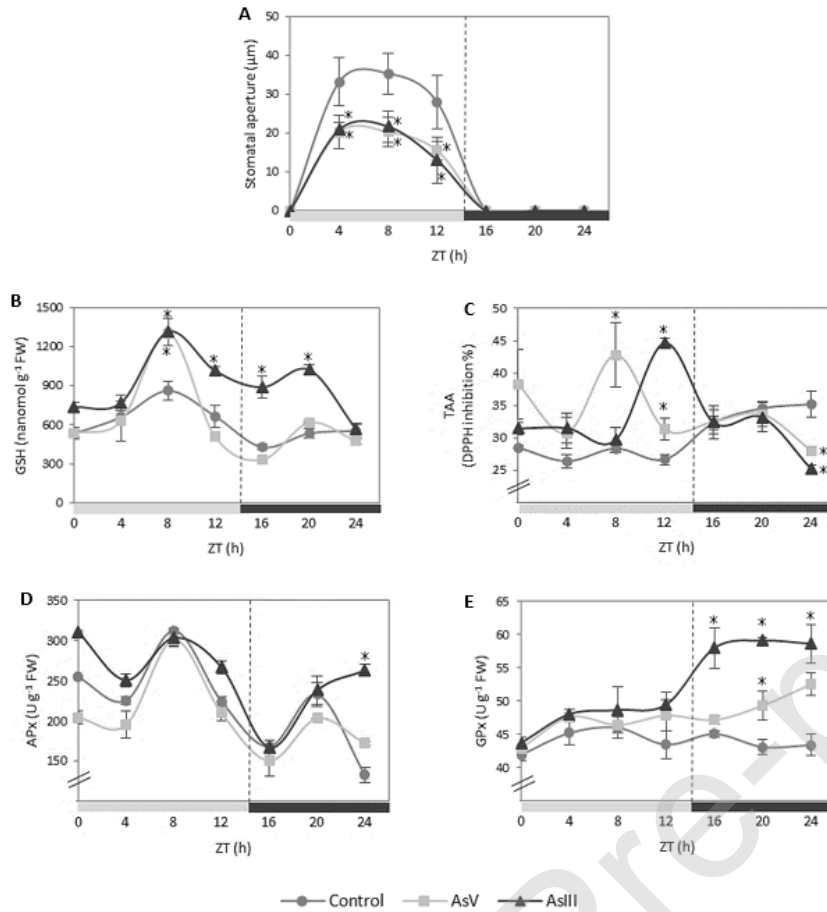
Figure 3: Diurnal oscillation of stomatal aperture (A), GSH content (B), TAA (C), APx (D) and GPx (E) activities in leaves of soybean plants under control conditions and treated with 25 µM AsV or AsIII. Light and dark gray bars indicate light and dark phases, respectively. The results represent the mean ± SE (n=3). * indicates significant difference between control and treated plants in each time point (Duncan test, p ≤ 0.05). More information about statistical analysis results is presented in the Tables S6 and S7.
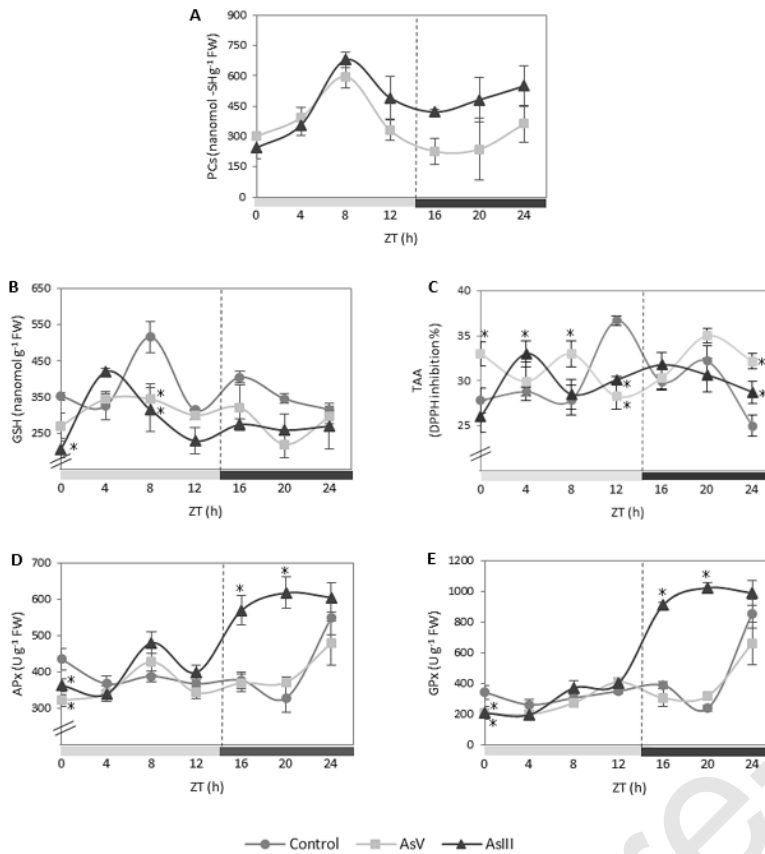
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Figure 4: Diurnal oscillation of PC (A) and GSH (B) content, TAA (C), APx (D) and GPx (E) activities in roots of soybean plants under control conditions and treated with 25 µM AsV or AsIII. Light and dark gray bars indicate light and dark phases, respectively. The results represent the mean ± SE (n=3). * indicates significant difference between control and treated plants in each time point (Duncan test, p ≤ 0.05). More information about statistical analysis results is presented in the Tables S8 and S9.
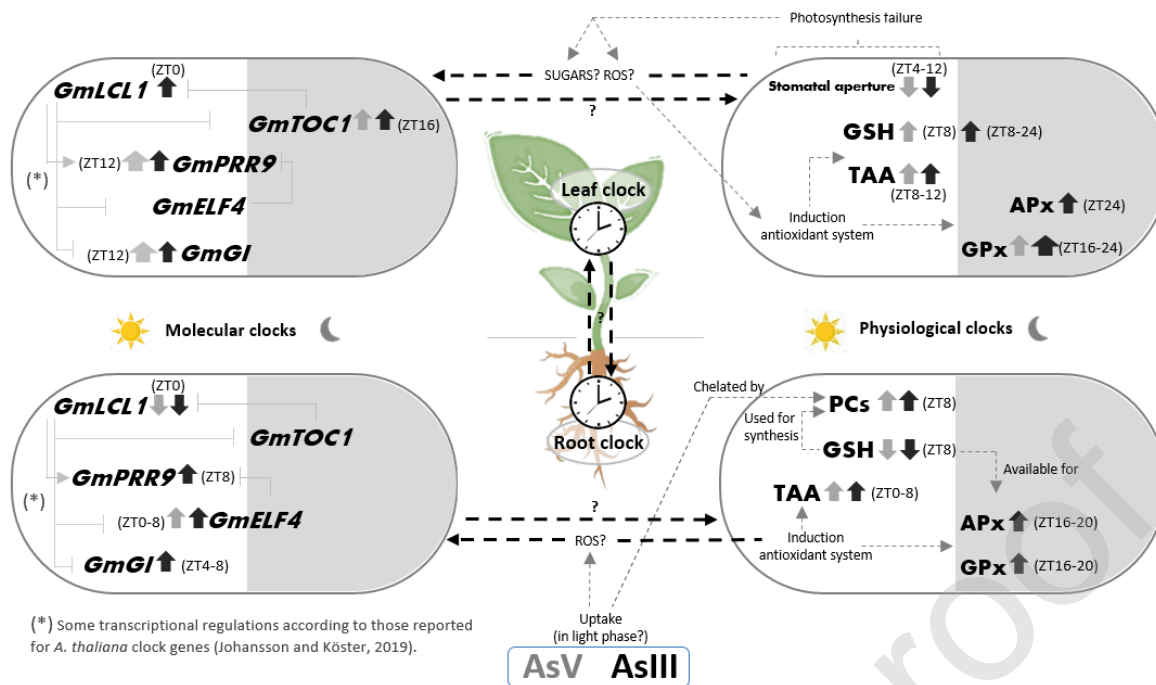
Figure 5: Representative scheme of the main effects of AsV and AsIII treatments on the clock gene expression and physiological events (stomata aperture, PCs: phytochelatins synthesis, GSH: glutathione content, TAA: total antioxidant activity, APx: ascorbate peroxidase activity, GPx: glutathione peroxidase activity) in leaves and roots of soybean plants. The relative position within the squares is according to the time of the day that variable changes occurs (white color for day, light grey for night). The arrows indicate increase (up arrow) or decrease (down arrow) for the analyzed variables under As treatments compared to control condition. Light grey arrows refer to AsV treatment while black arrows refer to AsIII treatment. Putative links between clock components and physiological events were suggested as dash lines.