

Review

Importance of Daily Rhythms on *Brassicaceae* Phytochemicals

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Abstract: The circadian clock serves to coordinate metabolism and physiology with the diurnal cycles derived from the daily rotation of the earth. In *Brassicaceae*, circadian rhythms contribute to the temporal daily variation in diverse phytochemicals and, hence, to both resistance to biotic stress and edible crop health value. Understanding the temporal variation in the specialized metabolites present in *Brassica* crops can improve selection and future breeding strategies. In this review, we focus on the importance of daily rhythms in the phytochemical biochemistry of the main bioactive compounds present in *Brassicaceae* plants. We provide a general overview of the mechanisms that can drive the daily variation in phytochemical levels and then provide specific examples of compounds that show daily variation. Finally, we discuss how these rhythmic patterns in plant phytochemicals may impact plant protection against biotic stress, the content of nutraceuticals, and the longevity of post-harvest crops.

Keywords: circadian clock; glucosinolates; antioxidants; secondary metabolites; *Brassica* crops; clock-regulated metabolism



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1. Introduction

During each 24 h day, there are large fluctuations in light intensity (photocycles) and temperature (thermocycles), which also lead to daily fluctuations in other aspects of the environment, such as humidity and predator behavior. The requirement to coordinate biological processes with the environmental cyclic rhythms has led to the evolution of circadian clocks. Circadian clocks are defined as an endogenous biological oscillation with a cycle of 24 h (although natural variation exists among individuals in terms of period length) which persist in the absence of external signals [1]. Roughly 1/3 of the transcripts in *Arabidopsis* show circadian rhythms [2,3], although the proportion of the transcriptome that cycles over a day increases substantially under a variety of environmental changes [4,5]. Similar broad circadian control of the transcriptome has been observed in crop plants, including *Brassica rapa* L. [6–8]. This circadian regulation of the transcriptome leads to time-of-day changes in a wide variety of downstream events in plants, including cotyledon movement, flowering time, metabolism, photosynthesis, nutrient homeostasis, and immunity, among others [9].

Vegetables from *Brassica* are characterized by a relatively high content of vitamins (A, E, C, and K), minerals (Ca, Fe, K, Cu, Zn, P, Mn, and Mg, among others), fiber, folate, and plant secondary metabolites, mainly phenolic compounds and glucosinolates (GSLs). These essential nutrients and phytochemicals may act synergistically in the food matrix to modulate the cell metabolism and help with the prevention and treatment of certain types of cancer, cardiovascular health problems, and neurodegenerative conditions of the aging human being [10,11]. In addition, these phytochemicals are involved in a variety of functions in plants. They can modulate essential physiological processes, such as growth, development, adaptation, symbiosis, diseases, and responses to pathogen attack [12]. Therefore, it would be expected that biosynthesis and the accumulation of

Brassica phytochemicals is precisely regulated in coordination with diverse endogenous signals and environmental conditions [13].

During the last ten years, evidence that levels of specialized metabolites present in *Brassica* crops can oscillate rhythmically during a day has begun to accumulate [14,15]. Consistently, circadian clock also seems to play an essential role in the immunity timing coordination by ensuring appropriate chemical defenses in the right tissue and at the right time, controlling their biosynthesis, transport, and storage [16]. In addition, it has been shown that circadian periodicity during the post-harvest period can also improve the longevity of tissue integrity and phytochemical content in diverse *Brassica* vegetables [17]. Thus, temporal variation in metabolite concentrations can alter the accumulation of diverse phytochemicals and thereby the overall edible crop health value. In this review, we focus on the importance of daily rhythms in the phytochemical biochemistry of the main bioactive compounds present in *Brassicaceae*. We provide a general overview of the mechanisms that can drive daily variation in metabolite levels and then provide specific examples of compounds that show daily variation. Finally, we discuss how these rhythmic patterns in plant phytochemicals may affect plant protection against biotic stress and the longevity of post-harvest crops.

2. The Plant Circadian Clock

In plants, the primary rhythmic input is sunlight, which acts through photoreceptive proteins to reset the phase of the clock to local time. Circadian oscillations originate at the cellular level from the interactions of at least a dozen clock genes that interact through a series of transcriptional and post-transcriptional feedback loops to create further rhythmic gene expression [1,18]. Up to this point, the majority of work on the plant circadian oscillator has been conducted with the model plant *Arabidopsis*. Although several homologs of clock-related genes have been found to be conserved in *Brassica* crop species, not all the components have yet been identified, and the mechanistic details of signaling are only incompletely understood. Thus, in this section we first provide a general overview of the molecular mechanisms and clock network organization in the model plant *Arabidopsis*; then, we summarize the current knowledge of circadian oscillator in *Brassica* species.

2.1. The *Arabidopsis* Circadian Oscillator

In *Arabidopsis*, the circadian clock genes include over 20 transcription factors connected by an intricate network of feedback loops, including both activating and repressive components [19]. Core circadian clock genes are expressed throughout the day, but distinct morning, day, and evening transcriptional phases exist, and each phase represents the activity of multiple core circadian clock proteins (Figure 1). The core loop is comprised of two morning-expressed MYB transcription factors that have partially overlapping functions—*CIRCADIANCLOCK-ASSOCIATED1* (*CCA1*), *LATE ELONGATED HYPOCOTYL* (*LHY*); an evening-expressed gene, *TIMING OF CAB EXPRESSION1* (*TOC1*); as well as a PSEUDO RESPONSE REGULATOR (*PRR1*) protein [20]. The expression of *CCA1* and *LHY* peaks just after dawn, whereas the expression of *TOC1* peaks in the early evening. *CCA1* and *LHY* are members of the larger *REVEILLE* (*RVE*) gene family, which also contains the principal clock activators *RVE8*, *RVE6*, and *RVE4* [21,22]. Furthermore, several other genes, including those codifying for additional *PRR*s proteins (*PRR5*, *PRR7*, and *PRR9*), *GIGANTEA* (*GI*), *LUX ARRHYTHMO* (*LUX*), *BROTHER OF LUX ARRHYTHMO* (*NOX*), as well as *EARLY FLOWERING 3* (*ELF3*) and *ELF4*, are responsible for the generation of the morning and evening complex loops [21,23–25].

During the morning, *CCA1* and *LHY* repress the expression of evening-expressed genes, including *TOC1*, *LUX*, *ELF3*, and *ELF4* [26,27], and, at the same time, promote the expression of *PRR* gene family members *PRR9* and *PRR7* [28]. The sequential expression of *PRR* genes over the course of the day imposes transcriptional repression on other *PRR* family members and additional core clock genes (for a review, see [29]). During the evening,

the trimeric protein assembly composed of *LUX*, *ELF3*, and *ELF4* suppresses the expression of *PRR9*, *PRR7*, *GI*, and *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED1 (LNK1)* in the late evening [25]. The evening complex represses itself near dawn through the inhibition of *LUX*, which allows the clock regulatory cycle to repeat the next day [30].

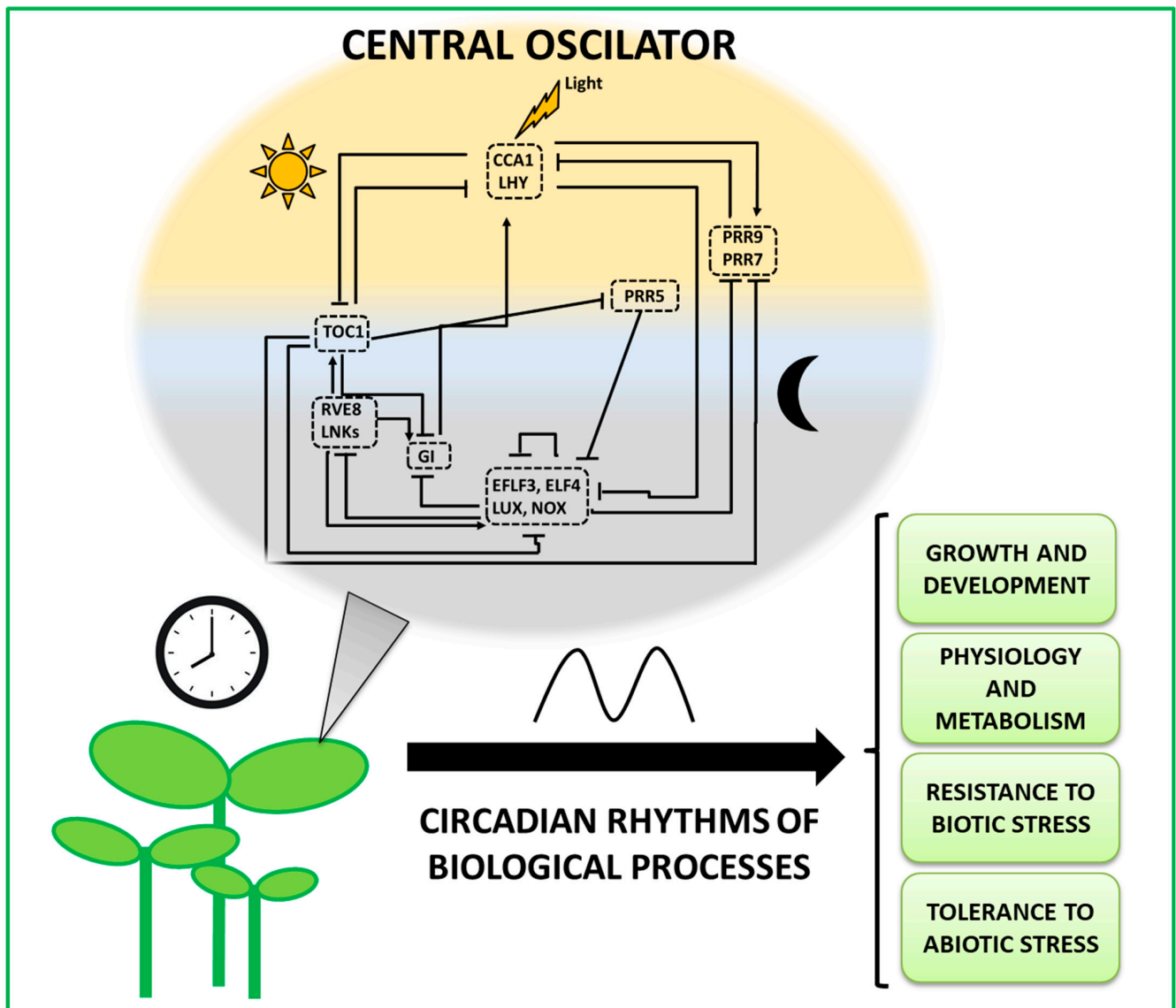


Figure 1. The plant circadian oscillator contributes to the regulation of multiple biological processes. The left area represents a simplified model of the regulatory interactions within the core circadian clock described in *Arabidopsis*. The clock components are represented from up to down according to the time of day of their peak expression. Yellow and gray backgrounds depict day and night, respectively. Rectangles denote functional groups, either because the components are members of the same gene family or because they act as a complex. Arrowheads and perpendicular lines illustrate the induction and repression of transcriptional activity, respectively. The right area region shows biological processes directly controlled by circadian clock genes or the rhythmic output of the circadian clock. For references and a complete description, please refer to the main text. CCA1, *CIRCADIANCLOCK-ASSOCIATED1*; LHY, *LATE ELONGATED HYPOCOTYL*; PRR9, *PSEUDO RESPONSE REGULATOR9*; PRR7, *PSEUDO RESPONSE REGULATOR7*; PRR5, *PSEUDO RESPONSE REGULATOR5*; EFLF3, *EARLY FLOWERING3*; ELF4, *EARLY FLOWERING4*; LUX, *LUX ARRHYTHMO*; NOX, *BROTHER OF LUX ARRHYTHMO*; GI, *GIGANTEA*; RVE8, *REVEILLE8*; LNKs, *NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED*; TOC1, *TIMING OF CAB EXPRESSION1*.

2.2. Circadian Clock Network in Brassica Crops

During the last few decades, much effort has been put into studying whether information of the Arabidopsis circadian clock can be extended to elucidate the molecular elements of the circadian core clock in *Brassica* crop species. The first studies began by identifying the natural allelic variation in clock function based on quantitative trait locus (QTL) analysis [8,31]. These studies investigated cotyledon movement from *B. oleracea* and *B. rapa* to detect genetic loci affecting the circadian period. Comparative genomic analysis based on collinearity between *Brassica* and Arabidopsis also allowed the identification of candidate genes known to regulate the period in Arabidopsis that may account for the additive circadian effects of specific QTL [32–34]. This includes gene families encoding *PRRs*, *TOC1*, *CCA1*, and *LHY*. *Brassica* species experienced an extra whole-genome triplication event compared with Arabidopsis. Therefore, as was expected, genes contributing to circadian clock function were retained in multiple copies in *Brassica* species. Song et al. [35] cloned and analyzed *CCA1* genes from seven inbred lines and one cultivar of cabbage (*Brassica oleracea*). Two types of *CCA1* alleles were detected and related to freezing-tolerant cabbage traits. In another study, the natural variation in *CCA1* was associated with the flowering time in *B. rapa* and a high level of sequence variation was identified [36]. Genetic mapping and analysis of families of heterogeneous inbred lines showed that the natural variation in *GI* is responsible for a major quantitative trait locus in the circadian period in *B. rapa*. Loss-of-function mutations of *GI* from *B. rapa* and *B. oleracea* cultivars confer delayed flowering, perturbed circadian rhythms in leaf movement, caused leaf senescence, and increased freezing and salt tolerance, consistent with the effects of similar mutations in Arabidopsis [37,38].

More recently, Greenham et al. [6] and Kim et al. [39] performed high-resolution circadian transcriptome experiments to elucidate the *B. rapa* circadian network. They found that genes related to the clock displayed distinct phases, increasing or decreasing in regular patterns. In addition, the different copies of duplicated and triplicated genes did not necessarily all behave in the same way. Many of the copies had different rhythms, and some increased and decreased in patterns totally opposite to their counterparts. Not only did the daily patterns differ, but responses to stressors were also altered. Comparing these patterns to the patterns seen in Arabidopsis revealed that often, one *B. rapa* gene behaved just like its Arabidopsis equivalent, while its copies had evolved new behaviors. The authors conclude that different behaviors of the copies of each gene in *B. rapa*, relative to its biological clock, allow this plant to grow in different environments with varying temperatures and day lengths.

The clock-regulated genes identified in *Brassica* are required for primary and secondary metabolism, photosynthesis, cold stress, and response to biotic stimulus [6,34,40,41], which strongly evidences the role of the circadian clock in vegetative growth and plant physiological processes. In line with that, recent works are starting to show that the circadian core clock genes in *Brassica* crops underlie QTLs that have been related to beneficial influences on key agricultural traits, especially flowering time but also yield, biomass, and growth [42–45]. In addition, new insights into the circadian clock regulation of other fundamental plant processes, such as responses to abiotic and biotic stresses, could help to guide future work in targeting genes to improve crop growth and stress resilience.

3. Clock-Regulated Phytochemicals

Phytochemicals are specialized compounds that play critical roles in plant adaptation under stressful environmental events [46,47]. Recent advances in the plant metabolome have given unambiguous proof supporting the association of the circadian clock with the daily accumulation of a broad range of plant defense metabolites [48]. Once entrained to cyclic environmental conditions, the accumulation of some phytochemicals, including GSLs and phenolic compounds, shows a rhythmic pattern in the absence of light, temperature, or humidity cues, indicating that this pattern of accumulation is under the control of the circadian clock [14,49]. The variation in phytochemicals in *Brassica* crops may affect shelf

life, food safety, and health benefits that determine the value and drive the consumer perception of quality [50].

3.1. Cyclic Accumulation of Phytochemicals and Nutritional Value

Among all the phytochemicals presents in *Brassica* vegetables, GSL are the most studied compounds in terms of variation under the control of circadian clock inputs. Rosa et al. [51] showed that GSLs in the leaves of young *B. oleracea* plants grown under field conditions can vary within a single day. A later study demonstrated that under a constant photoperiod, temperature, light, and relative humidity, individual and total GSLs varied within a 24 h period in kale and cabbage (*B. oleracea*). This variation was more evident when the temperature was optimum for growth and development [52,53]. It has been shown that, in general, the lowest total GSL levels were observed during the first half of the light cycle with a single recovery in the next hours and reached maximum levels during the dark period. Further research carried out [54] with broccoli (*B. oleracea*) under semi-field conditions demonstrated that circadian clock rhythms influenced GSL, flavonols, and, in consequence, the sensory quality of broccoli.

More recently, Soengas et al. [14] investigated for species-specific patterns in circadian rhythmicity of GSLs under controlled environmental conditions. Plants from different GSL-containing cultivars belonging to three *Brassica* species (*B. oleracea*, *B. rapa*, and *B. napus*) were entrained to light–dark cycles prior to release into continuous light. Then, the GSL levels were monitored at four time points of the day during two consecutive days. These experiments corroborated the circadian rhythmicity of total and individual aliphatic GSL levels. However, each cultivar showed a different phase output of maximal GSL accumulation in a day. Even cultivars with a similar aliphatic GSL profile and/or belonging to the same species differ in their shape of GSL fluctuation through time. This suggests that differences could be more related with the plant internal clock rather than the GSL pathway by itself.

Diurnal changes in antioxidant phytochemicals were also observed in *Brassica* plants. Antioxidant capacity measured using ABTS and DPPH assays found that broccoli heads harvested at the end of the day had higher antioxidants and phenolic compounds than samples harvested in the morning and evening [55]. Soengas et al. [49] reported that the antioxidant activity (measured as ABTS and FRAP) of extracts from different *Brassica* cultivars belonging to *B. oleracea* and *B. rapa* fluctuates rhythmically though the day. In this work, plants were entrained to light–dark cycles prior to release into continuous light. The antioxidant activity and phenolic content was monitored at four time points of the day during four consecutive days (two days under light–dark conditions followed by two days under continuous light). Variations under constant light conditions were related with endogenous circadian rhythms. In general, all the analyzed cultivars accumulated higher levels of individual phenolics correlated with the antioxidant properties at the end of the night period and/or at the beginning of the light period. Since the principal role of plant polyphenols is protection against excess light damages these results evidenced that that *Brassica* plants anticipate the dawn and adjust their biology accordingly.

3.2. Post-harvest Life of Brassica Vegetables and Circadian Clock

Traditionally, research focused on *Brassica* food products to delay deterioration during storage has focused on the application of diverse post-harvest treatments such as refrigeration storage, heat treatments, controlled and modified atmospheres, and UV-C light, among others [56]. However, recent advances are starting to show that circadian rhythms [17] and the time of day at which vegetables are harvested [57] could also influence their post-harvest life. In addition to the discussed daily fluctuations in GSLs and phenolics, changes can be also detected in the metabolism of chlorophyll precursors, sugar metabolism, and ascorbic acid, affecting several aspects of post-harvest performance, which will be discussed in this section.

Liu et al. [17] found that keeping the rhythms of light-dark cycles during post-harvest storage preserved the tissue integrity and nutritional content of leafy plants including *Brassica* vegetables. The tissue integrity, green coloration, and chlorophyll content from kale and cabbage were generally enhanced by the cycling of light and darkness compared to constant light or darkness during storage. These results were comparable to storage under refrigeration. Similarly, a reduction in senescence was noted for post-harvest broccoli stored under natural light-dark cycles [58]. Therefore, the cycling of light treatment with darkness periods may not only maintain clock function but may also provide the additional benefit of keeping the rhythms of biological processes, such as photosynthesis, during post-harvest storage avoiding physiological damage.

Casajus et al. [57] studied the effect of harvesting at different times of the day on the post-harvest senescence of kale leaves stored at 20 °C in darkness for nine days. They found that leaves harvested in the morning presented earlier symptoms of yellowing, higher rates of chlorophyll degradation, and a lower sugar content compared with leaves harvested at evening. Thus, harvesting in the evening contributes to delaying kale leaves yellowing and senescence metabolism during post-harvest storage. These changes in shelf life are attributed to diurnal changes in leaf status. However, much research is needed to determine whether this effect on post-harvest quality is due to endogenous core clock or is a mere consequence of light.

The effect of light intensity and/or light spectral quality on shelf life, quality, and phytochemical content in post-harvest *Brassica* vegetables has been investigated [58–63]. In this regard, low-intensity light treatment during storage improves the appearance and quality of kale, broccoli, and pak choi by maintaining the chlorophyll content and activating photosynthesis [58,59,63]. In the same way, antioxidants, monosaccharides, and starch were retained in Chinese kale leaves after a treatment with low-intensity continuous light during post-harvest storage [60]. Exposure to white-blue light delayed the senescence of harvested broccoli [61], while the content of total phenols and GSLs was markedly increased by green light [62]. These findings indicate that detached plant organs and post-harvest vegetables maintain responsiveness to light after harvest and more interestingly, if the light intensity is adequate, leaves could continue light-dependent biological process, including photosynthesis and phytochemical retention.

4. The Clock Role on Plant Resistance to Biotic Stress

In the last decade, given the role of biological clocks as master integrators of external information, several authors have focused on the study of the circadian system as a key regulator of plant defense responses against biotic stress. Supporting this notion, several genetic studies demonstrated that *Arabidopsis* clock mutants show impaired defense responses [64–66]. In addition, daily oscillations in susceptibility correlate with circadian regulation of expression of many defense-related genes, including those related with plant secondary metabolites [15,66–68]. However, there are only a handful of studies addressing the role of the circadian clock in biotic stress responses. In this section, we will discuss the results obtained in different studies performed with diverse pathogens and insects to elucidate the clock role in plant defense response to biotic threats.

4.1. Pathogen Infection and the Clock

Plant defense mechanisms against pathogens have been reported to vary daily in *Arabidopsis*. Plants inoculated with bacteria (*Pseudomonas syringae*) at dusk displayed higher bacterial growth after infection than plants inoculated at dawn did [69]. However, the authors attributed this to a direct effect of light rather than endogenous circadian effects on the induction of plant defense responses. Similarly, the inoculation of *Arabidopsis* with oomycete (*Hyaloperonospora arabidopsidis*) at dawn and dusk resulted in significantly higher levels of susceptibility, as measured by sporangiophore counts, at dusk [70]. Nevertheless, the bacterial and sporangiophore counts, which represent the outcome of the plant-pathogen interaction, were not determined under constant conditions in these exper-

iments to confirm endogenous circadian clock regulation of plant defenses. Further work carried out under constant light conditions revealed that the central core clock components *CCA1* and *LHY* act as a positive integrator between the clock and defense pathways in resistance against oomycete and bacterial pathogen infections in *Arabidopsis* [64,65]. These studies also found that *CCA1* and *LHY* act independently of SA mediated defense but through downstream target genes, such as regulating stomata pathways.

It is well known that stomatal opening is influenced by light intensity, the concentration of atmospheric CO₂, endogenous plant hormonal stimuli control, membrane ion transport, and the metabolic activity of guard cells. In addition, current data suggest a link between the circadian system and stomatal opening [71]. In consequence, circadian sensitivity of stomatal responses to pathogens is likely to vary over a day. In the field, the bacterial and sporangiophore counts are higher during the day when stomata are open. This could partially explain why plants accumulate the highest basal defenses during the light period compared with night.

Results from Ingle et al. [68] showed that plant responses to necrotrophic fungus (*Botrytis cinerea*) are also temporally regulated and play a key role in determining the outcome of infection in *Arabidopsis*. They profiled the transcriptome of *Arabidopsis* leaves inoculated with *B. cinerea* at different times of day and identified a subset of transcription factors that are known to be direct targets of clock proteins that respond differentially to this pathogen depending on the time at which inoculation occurs. Furthermore, they found that jasmonic acid (JA) and ethylene (ET) signaling pathways are involved in this response.

Given the effect of the phytohormones SA, JA, and ET on the contents of GSLs; individual and total phenolic compounds; and carotenoid, chlorophyll and, anthocyanin content by affecting their gene regulation of metabolic biosynthesis, it would be expected that phytochemical accumulation is also involved in the response of the plant clock under pathogen infection. Supporting this notion, a recent study seems to indicate the importance of diurnal rhythms in the accumulation of sulfur-containing defense compounds in *B. napus*, including GSLs, against the fungal pathogen *Verticillium longisporum* [72]. Therefore, time of the day and circadian rhythms seem to play a role in the functionality of the plant immune system, which may affect the virulence of pathogens and the overall outcome of host–pathogen relationship. Further work is needed to corroborate this hypothesis.

4.2. Pest Attack and the Clock

The hypothesized interactions between circadian plant defense regulation and pest attack states that tissue defense should be correlated with the probably of biotic stress [73]. Goodspeed et al. [67] carried out the first work reporting advances on the plant clock and defense against herbivory. They show that *Arabidopsis* resistance against a generalist herbivore insect, *Trichoplusia ni*, is highly dependent on clock-regulated hormone accumulation. The feeding of *T. ni* is rhythmic and occurs predominantly around midday. This was consistent with the diurnal accumulation of endogenous JA. Entrainment of circadian rhythms to opposite light–dark cycles in plants and insects results in higher susceptibility of plants to *T. ni*. In agreement, arrhythmic plants display increased susceptibility to *T. ni*. To gain insight into this circadian behavior and test whether it can be extended to other plant species, Goodspeed et al. [15] performed a complementary experiment studying the herbivore behavior and plant defense in post-harvest crops. They reported for the first time that post-harvest *Brassica* crops retain their capacity to entrain diurnal cycles and to enhance their defense. Accordingly, they found that GSLs accumulation of the post-harvest cabbage is dependent on circadian periodicity. These observations suggest that both circadian clock regulation and cyclic activation of defensive compounds are required for a phase-dependent resistance. In addition, mutant analysis showed that the presence of a functional clock enables plants to anticipate upcoming challenges in consonance with pest behavior.

Recently, Lei et al. [66] investigated the link between the circadian clock and plant defense against green peach aphids (*Myzus persicae* S.). They found that, under constant

light, wild-type Columbia-0 (Col-0) *Arabidopsis* plants entrained with aphids in the same light/dark cycles exhibited a greater antixenotic activity than plants entrained in the opposite cycle from the aphids. Consistently, several loss-of-function clock mutants were more susceptible to aphid infestation than Col-0. However, the arrhythmic *CCA1* overexpression line exhibited enhanced resistance toward aphids. The constitutive activation of *CCA1* seemed to positively regulate the biosynthesis of indole GSLs. Thus, rhythmic GSLs accumulation has been shown to play a critical role in this time-dependent pest resistance.

Despite the clear links between the clock and immune responses, whether the circadian clock of cultivated *Brassica* plants affects biotic stress responses remains to be determined. Future research should take into account the considerable different herbivore feeding behaviors that are expected to be occurring in natural habitats. An interesting comparison will be conducting similar experiments to those discussed in this section but with other insects, including night-feeding insects. This could extend our knowledge in the area of plant–insect interactions mediated by the circadian clock.

5. Concluding Remarks

The circadian clock is a major regulator of plant physiology and metabolism. It has been demonstrated that the accumulation of major *Brassica* phytochemicals (i.e., GSLs and phenolic compounds) is regulated by a day–night temporal oscillation of regulatory genes. Some of these genes are well characterized in the model plant *Arabidopsis*, but plants from the *Brassica* genus have a more complex genome with multiple copies of these genes. This brings a higher level of complexity to the circadian regulatory network that requires further studies to elucidate the mechanisms of circadian regulation in this genus. A deeper knowledge of these mechanisms would allow us to optimize cultural practices against pathogens and pests and post-harvesting management.

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