The Root Growth-Regulating Brevicompanine Natural Products Modulate the Plant Circadian Clock

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Supporting Information

ABSTRACT: Plant growth regulating properties of brevicompanines (Brvs), natural products of the fungus Penicillium brevicompactum, have been known for several years, but further investigations into the molecular mechanism of their bioactivity have not been performed. Following chemical synthesis of brevicompanine derivatives, we studied their activity in the model plant Arabidopsis thaliana by a combination of plant growth assays, transcriptional profiling, and numerous additional bioassays. These studies demonstrated that brevicompanines cause transcriptional misregulation of core components of the circadian clock, whereas other biological read-outs were not affected. Brevicompanines thus represent promising chemical tools for investigating the regulation of the plant circadian clock. In addition, our study also illustrates the potential of an unbiased -omics-based characterization of bioactive compounds for identifying the often cryptic modes of action of small molecules.

Natural products have a long-standing history as starting points for the development of chemical probes. In the plant sciences, this approach has been less frequently used, although the need for chemical probes with interesting biological activities has recently been recognized. Accordingly, the number of examples of natural product-derived chemical probes in plant systems is still limited. The natural product class of brevicompanines (consisting of BrvA=BrvC, Figure 1a) were isolated from the culture filtrate of the fungus Penicillium brevicompactum and found to display plant growth regulating properties. Chemically, brevicompanines are diketopiperazine derivatives of the larger hexahydro pyrrolindolo[2,3-b] (HPI) natural product family, with the particular feature of containing a (d)-configured amino acid residue as part of their diketopiperazine moiety and a reverse prenyl residue at the HPI core. Intriguingly, they displayed differential biological activities in different plant species. In lettuce (Lactuca sativa) seedlings, they promoted root growth in a dose-dependent manner, whereas in rice (Oryza sativa), at the same concentrations, no such effect was observed. The molecular mechanism underlying the root growth modulating activity however remains unknown so far. Moreover, the biological activities of brevicompanines in other plants, in particular the model plant Arabidopsis (Arabidopsis thaliana), have not yet been determined, thereby limiting a potential application of brevicompanines in plant chemical biology research. Accordingly, we here describe a molecular analysis of the bioactivity of brevicompanines in Arabidopsis. These investigations revealed that brevicompanines selectively attenuate the oscillation of the plant circadian clock.

RESULTS AND DISCUSSION

We chemically synthesized the three major naturally occurring brevicompanine derivatives (BrvA=BrvC, Figure 1a), following essentially established synthesis protocols (Supporting Information). We then first investigated their impact on...
In order to gain an unbiased insight into the molecular responses caused by brevicompanine treatment, we next performed a microarray-based gene expression study. For this analysis, we focused on BrvC because of its selective bioactivity. We analyzed gene expression after a short (6 h) and long (48 h) exposure to 50 μM BrvC to monitor rapid and continuous responses (Figure 2a, both time intervals refer to the same starting time, which was 10 am, 2 h after lights-on). The short-term treatment (6 h) resulted in significant reprogramming of gene transcription in comparison to DMSO-treated plants, yielding 1317 and 867 signature sequences in annotated genes that were up- and downregulated with log₂ FC ≥ 1, respectively (for P ≤ 0.001, Supporting Table 1). The changes of the expression profile after long-term treatment (48 h) with BrvC were less severe (Supporting Table 1), but most of the significantly altered genes were unidirectionally up- or downregulated at both time points (Figure 2a and b).

Subsequently, we focused our analysis on those genes that were significantly up- or downregulated at both time points because these genes may best reflect a potential “chemical gene knockout” underlying the bioactivity of BrvC. Accordingly, we ranked all genes that were upregulated after 6 and 48 h with log₂ FC ≥ 2.0 (for P ≤ 0.001, Supporting Table 2) or that were downregulated after 6 and 48 h with log₂ FC ≤ −2.0 (for P ≤ 0.001, Supporting Table 3). Thorough analysis of these data revealed that the BrvC treatment affected the transcription of core component genes of the plant circadian clock. These genes are responsible for generating and maintaining the approximately 24-h rhythm and display characteristic oscillating expression patterns peaking at a distinct time of the day. BrvC application downregulated the expression of the morning clock component genes CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), REV-ELILLE8 (RVE8), and REVEILLE4 (RVE4) and upregulated the expression of the evening genes PSEUDO-RESPONSE REGULATOR 3 (PRR3) and TIMING OF CAB EXPRESSION 1 (TOC1; Figure 2c).

Additionally, our analysis also revealed the downregulation of NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE 2 and 1 (LNK2 and LNK1), which are also implicated in the regulation of the circadian clock, although the underlying molecular mechanism is only partially understood. As the circadian clock is known to act as a major regulator of gene transcription, affecting the expression of ~30% of all plant genes, many additional genes that we identified as significantly changed in our microarray analysis are regulated by the circadian clock (e.g., NIGHT LIGHT-INDUCIBLE AND CLOCK-REGULATED GENE 3 (LNK3) or the CYCLING DOF FACTOR 1, 2 and 3 (CDF1, CDF2, and CDF3, Supporting Table 2 and Supporting Table 3)).

Next, we tested whether the exposure of Arabidopsis seedlings to brevicompanines indeed has an effect on circadian rhythms by monitoring the expression of the well-characterized circadian marker GIGANTEA::LUCIFERASE (Gi::LUC), which is known to show robust and synchronized rhythms under a variety of conditions and was previously used in a chemical screen for compounds that alter plant circadian rhythms. Initially, we assessed the effect of brevicompanines on Gi::LUC expression in constant darkness (DD), when the circadian rhythm is uncoupled from the modifying effects of light. Under these conditions, application of BrvC significantly reduced the circadian amplitude (half of the difference between peak and trough, hereafter called “amplitude”) of Gi::LUC.
expression in a dose-dependent manner (Figure 3a). In order to assess whether this effect was caused by a specific molecular interaction of BrvC with a target, we chemically synthesized D-BrvC, the (D)-isomer of BrvC (for chemical structure, see Supporting Information Figure 1; chemical synthesis is described in the Supporting Information). In contrast to BrvC, D-BrvC did not significantly affect the amplitude of GI::LUC rhythms, thus indicating a specific effect of BrvC (Figure 3b).

We next evaluated the impact of BrvB on the circadian rhythm as BrvB is commercially available (thereby facilitating future application as a chemical research tool) and overall displayed the highest inhibition of plant root growth. Exposure of Arabidopsis seedlings to BrvB (100 μM) reduced the amplitude of rhythmic GI::LUC expression in DD but, in contrast to BrvC, also increased the circadian period length (duration of the circadian cycle, hereafter called “period”; Figure 3c and Supporting Figure 2a). More detailed analyses revealed that BrvB exerted its effects on the amplitude and period length of rhythmic GI::LUC expression in a dose-dependent manner (Figure 3d). In a similar fashion, BrvB also affected the amplitude and period length of the rhythmic expression of CCA1::LUC, another well-established circadian marker that is also a core component of the circadian oscillator (Supporting Figure 2b). Notably, the rhythm of CCA1 expression in DD dampened faster than that of GI, which may explain why the effects of BrvB on this marker were less consistent across experiments.11

Finally, we extended the analysis of brevicompanines on circadian rhythms to include constant light (LL) conditions and essentially confirmed the bioactivities of BrvB and BrvC and the inactivity of D-BrvC. As previously seen under DD conditions, BrvB also reduced the amplitude and lengthened the period of the oscillating GI::LUC and CCA1::LUC expression, whereas BrvC only affected the amplitude and D-BrvC was inactive (Figure 3e and f and Supporting Figure 3). Taken together, these results indicate that brevicompanines alter the rhythmic amplitude of two core components of the circadian oscillator under DD and LL conditions, thus demonstrating that these chemicals are modulators of the circadian clock. The selective bioactivity of BrvC on the amplitude over the period of the oscillation contrasted with the more general effect of BrvB, an...
observation that parallels the results obtained with the root growth assays. Amplitude and period length are regulated by different molecular mechanisms in animals, and BrvC might therefore be used in future studies as a tool to study specific physiological effects of amplitude variation in plants.

The circadian clock controls root growth, and brevicompanines control both the clock and root elongation (Figures 1−3). The regulation of circadian rhythms and of root elongation could be two independent functions of brevicompanines, but there is also the possibility that brevicompanines control root growth by modulating the regulation of central components of the oscillator (Figures 2 and 3). To this end, we first confirmed that the inactive enantiomer of BrvC, D-BrvC, had no effect on primary root elongation in the Col-0 WT (Figure 4a). We then performed experiments to exclude that brevicompanines were altering root growth by acting on hormone signaling pathways. Root growth and development is controlled by several plant hormones (auxin, cytokinins, abscisic acid, and others) and particularly auxin functions as a critical regulator of lateral root formation. Accordingly, we monitored the effect of BrvB on various reporter genes that selectively respond to different phytohormones. To this end, we used transgenic Arabidopsis lines expressing (i) the DR5p::GUS reporter, which is frequently used to monitor alterations in auxin levels, (ii) the ARR5p::GUS reporter as a proxy of altered cytokinin levels, (iii) the DC3p::GUS reporter responsive to abscisic acid (ABA), and (iv) the jasmonate-responsive VSP1::GUS reporter. None of these reporters were strongly affected in their expression upon treatment with BrvB, i.e., neither induced in the absence of the corresponding phytohormones nor impaired by its presence, suggesting that endogenous hormone levels (auxin, cytokinin, ABA, or jasmonate signaling) are not affected and therefore do not cause the observed root phenotype (Supporting Figure 4). Instead, the modulation of root growth by brevicompanines might be mediated by changes in the expression of circadian clock genes induced by the application of these chemicals.

Figure 3. Modulation of plant circadian rhythms by brevicompanines. (a) Amplitude of GI::LUC rhythms in response to increasing concentrations of BrvC under constant darkness (DD) conditions. Data are the means of two biological replicates, with 12 individuals per replicate. The p value (p) was determined with a two-way ANOVA using concentration and experiment as factors and indicates a significant effect of BrvC on amplitude (α = 0.05). (b) Amplitude of GI::LUC rhythms in response to increasing concentrations of D-BrvC under constant darkness (DD) conditions. Statistics was performed as described in a. (c) Waveform of GI::LUC activity in constant darkness (DD) after exposure to 100 μM BrvB (orange curve) or DMSO (blue curve); n = 12. (d) Period (left panel) and amplitude (right panel) of GI::LUC rhythms in response to increasing concentrations of BrvB in constant darkness (DD). Statistics was performed as described in a. (e) Waveform of GI::LUC activity in constant light (LL) after exposure to 100 μM BrvB (orange curve) or DMSO (blue curve; left panel) and corresponding amplitude of GI::LUC rhythms in constant light (LL; right panel); n = 12. Statistical difference from the DMSO control was determined with a Student’s t test (α = 0.05; *P < 0.05, **P < 0.001). (f) Waveform of GI::LUC activity in constant light (LL) after exposure to 100 μM BrvB (orange curve) or 100 μM D-BrvC (blue curve; left panel) and corresponding amplitude of GI::LUC rhythms in LL (right panel); n = 12. Statistics were performed as described in e. In all experiments, seedlings bearing the GI::LUC reporter were entrained for 7 days in day/night regimes of 16 h light/8 h dark. Seedlings were then transferred on day 7 to 96-well plates containing solid medium supplied with chemicals at the indicated concentrations. The resulting luminescence signals were measured over 5 days. In all panels, amplitude and raw luminescence are expressed in counts per second (cps), and period length is expressed in hours (h).
The functionally redundant genes LHY and CCA1 are central components of the oscillator and are the two circadian clock genes most strongly misregulated by the application of BrvC (Figure 2).\textsuperscript{20} LHY and CCA1 are expressed in roots,\textsuperscript{21} and their activity is essential for the oscillator to generate sustained rhythms not only in the shoots but also in the roots.\textsuperscript{22} Importantly, the analysis of loss of function mutants demonstrated that LHY and CCA1 promote root growth by acting at certain times of the diel cycle.\textsuperscript{13} Loss of function of other central circadian clock genes such as \textsc{early flowering} 3 and 4 (ELF3 and ELF4) also affects root growth. But, the rhythmic pattern of root growth differs between clock mutants,\textsuperscript{23} and ELF3 and ELF4 expression levels are not strongly affected by the application of BrvC (Supporting Table 1). These observations suggested that brevicompanines might modulate root growth by altering the expression of LHY and CCA1. Along these lines, we determined primary root length after application of BrvB in the \textit{lhy11 cca1−1} double mutant as well as in the \textit{elf3−1} and \textit{elf4−101} loss of function mutants.\textsuperscript{20} These experiments revealed the general tendency of a reduced effect of BrvB in the \textit{lhy11 cca1−1} mutant (Figure 4b) but not in the \textit{elf3−1} and \textit{elf4−101} mutants (Supporting Figure S). In fact, statistical analyses (ANOVA) supported the \textit{lhy11 cca1−1} genotype limiting the effect of BrvB on root growth (significant genotype × treatment interaction with \(P = 0.013\) for the 30 \(\mu M\) treatment and \(P < 0.001\) for the 100 \(\mu M\) treatment). On the basis of these results, we propose that brevicompanines repress root growth at least partly by repressing the expression of \textit{LHY} and \textit{CCA1}, two central components of the circadian clock.

Overall, we conclude that BrvB displays apparent selectivity by modulating only the circadian clock but induces no other tested biological responses in plants. Notwithstanding differences in activity between BrvB and BrvC, the results generally converge on brevicompanines predominantly regulating the amplitude of the rhythm rather than the period length. The molecular basis of the different bioactivity of the structurally rather similar compounds BrvB and BrvC is unknown and could be caused by different factors such as different binding affinities to their protein target(s) or different pharmacokinetic properties including differential uptake, metabolism, and distribution in distinct plant tissues. As BrvB is commercially available, we anticipate that it may represent a versatile chemical tool for studying the molecular regulation of the plant circadian clock, e.g., in combinatorial chemical genetics approaches that may complement the currently popular genetic studies in the plant field.\textsuperscript{23} Of note, brevicompanines fill a gap in the current chemical tool repertoire. In contrast to small molecule modulators of the mammalian circadian clock, e.g., the CRY stabilizers KL001 (and derivatives thereof) or the kinase inhibitor longdysin,\textsuperscript{23,25−28} only one plant circadian clock modulator, the actin stabilizer prieurian/endosidin 1,\textsuperscript{11} has been reported so far. This compound, however, is difficult to use due to its indirect mode of action. Unfortunately, the direct target(s) of the brevicompanines have remained elusive so far and require further experiments. We anticipate that a systematic screening of clock mutants or biochemical approaches such as affinity purifications with chemically modified brevicompanines might represent suitable approaches to identify their enigmatic target protein(s).

Our studies furthermore indicate that the observed root growth phenotype is at least partly mediated by alterations in the expression level of two circadian clock components, although further investigations into this direction are still required. This phenotype thus joins a growing number of various growth phenotypes or effects such as heterosis that are known to be induced by a deregulated circadian clock.\textsuperscript{5,8,29,30}

Finally, our study highlights the potential of unbiased -omics-based approaches to decipher cryptic bioactivities of natural products. We believe that with the advances in the systematic analysis of such data, this approach will become an established methodology to unravel bioactivities of small molecules.\textsuperscript{31}

### METHODS

Chemical synthesis and experimental procedures are included in the Supporting Information.

### ASSOCIATED CONTENT

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00978.

Supporting figures, supporting procedures and methods, and supporting references (PDF)

Supporting Table 1 (PDF)

Supporting Table 2 (PDF)

Supporting Table 3 (PDF)

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Notes

The authors declare no competing financial interest.

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