β-cyclocitral is a natural root growth regulator

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Abstract

Natural compounds capable of increasing root depth and branching are desirable tools for enhancing stress tolerance in crops. We devised a sensitized screen to identify natural metabolites capable of regulating root traits in Arabidopsis. Application of nanomolar concentrations of β-cyclocitral were found to promote cell divisions in root meristems, stimulating branching and growth. β-cyclocitral had a conserved effect on root growth in tomato and rice. β-cyclocitral fundamentally altered root system architecture in rice, generating deeper and more compact root systems without affecting shoot growth. Importantly, β-cyclocitral treatment enhanced root growth under abiotic stress conditions in rice. These results indicate that β-cyclocitral is a conserved regulator of root growth across monocots and dicots and could be a valuable tool to shield crops from environmental stress.

One Sentence Summary: β-cyclocitral, a metabolite of β-carotene, was identified as a conserved enhancer of root growth and branching using a sensitized chemical screen.

Introduction

A rapidly increasing world population, coinciding with changes in climate, creates a need for new methods to stabilize and improve crop productivity under harsh environmental conditions. Exogenously applied phytohormones, such as auxin, cytokinin, and ethylene, have had profound impacts on agriculture by selectively killing weeds, promoting shoot growth, and optimizing fruit ripening (*1-4*). Root traits such as growth and branching are also appealing targets for enhancing plant performance, due to their essential role in nutrient and water uptake. In Arabidopsis, root development begins with the formation of a primary root during embryogenesis. Root branching is initiated by oscillations in gene expression at the tip of the primary root (*5*). These oscillations establish the future position of *de novo* roots called lateral roots (LRs) and are referred to as the "LR clock." Stereotyped cell divisions promote LR primordia development by increasing cell number and generating all root cell types (*6*). In both primary and LRs, growth is maintained through cell divisions in the meristem and the subsequent elongation of daughter cells. Previously, inhibition of the carotenoid pathway was shown to reduce root branching in Arabidopsis, suggesting that this pathway is important for LR development (*7*).

The carotenoid pathway is a rich source of metabolites, called apocarotenoids, several of which are known regulators of root development (Figure S1) (*8, 9*). Strigolactones and abscisic

acid (ABA), apocarotenoid phytohormones, reduce root growth and LR branching, respectively (*10-12*). Previously, an inhibitor of carotenoid cleavage dioxygenases (CCDs) called D15 was found to decrease LR branching through an ABA and strigolactone independent-mechanism in Arabidopsi*s* (*7*). This suggested one or more unidentified apocarotenoids could be positive regulators of LR development. Isolating these compounds through genetic means is difficult because each CCD has multiple substrates and produces a variety of different compounds, making it impossible to selectively knock out a single apocarotenoid. Therefore, to identify new natural compounds capable of promoting root growth and development, we leveraged D15 to characterize the effects of exogenously applied apocarotenoids on root traits in a sensitized background.

Results

To identify apocarotenoids involved in LR development we utilized a targeted chemical genetic approach to screen endogenous apocarotenoids for their ability to enhance root branching in the presence of D15. The maximum concentration of D15 tested (100 μM) decreased primary root length and completely inhibited LR capacity, the number of LRs that emerge after excision of the primary root apical meristem. By titrating D15 to 30 μM, the concentration at which it decreases LR branching by 50% (IC_{50}), we could measure changes in LR capacity with enhanced sensitivity (Figure 1A). Most apocarotenoids tested, including ABA and GR24, a synthetic strigolactone analogue, further decreased LR capacity when combined with D15 (Figure S2). Two apocarotenoids, dihydroactinidiolide (DHAD) and β-cyclocitral, were found to increase LR branching in the presence of D15. These compounds were previously found to trigger the reactive oxygen species response and increase leaf tolerance to high light stress (*13-15*).

To further explore the effects of DHAD and β-cyclocitral on LR branching, we first confirmed that these compounds are endogenously present in Arabidopsis using GC/MS (Figure S3). Next, we varied their concentrations in the presence of D15 at its IC_{50} concentration. We found that even the most effective concentration of DHAD only increased root branching by 14%. However, application of volatile β-cyclocitral had a much more dramatic effect, promoting root branching by nearly 40% (Figure 1B-C). Therefore, we focused on β-cyclocitral's mechanism of action. β-cyclocitral has been identified endogenously in dozens of plant species, including tomato (*16*), rice (*17*), parsley (*18*), tea (*19, 20*), grape (*21*), various trees (*22, 23*), and moss (*24*), indicating that its presence in plants is evolutionarily conserved. A gene ontology (GO) term enrichment analysis of previously published data in Arabidopsis revealed that genes upregulated by β-cyclocitral are important for the immune system, metabolite catabolism, and abiotic stress responses, indicating that β-cyclocitral may play a number of roles in growth and development (Table S1) *(13)*. In the absence of D15, β-cyclocitral increased root branching at nanomolar concentrations, which is comparable to the levels at which ABA and strigolactones are active and is approximately the same concentration of β-cyclocitral endogenously present in leaves (Figure S4) *(13)*. Importantly, apocarotenoids with nearly identical chemical structures, such as dimethyl-β-cyclocitral and β-ionone, did not have the ability to increase root branching (Figure S1-2). These results suggest that β-cyclocitral, and not an impurity or structurally-similar compound, is the active molecule regulating LR branching.

To understand how β-cyclocitral promotes LR formation, we characterized its effect on root development in the presence of D15. β-cyclocitral's effect suggested that it enhanced LR

branching either by increasing initiation or inducing LR outgrowth. We first tested how D15 and β-cyclocitral affect the initiation of LR primordia by examining their effect on the LR clock. To test this, we utilized *pDR5:LUC*, a marker line that gives a readout of LR clock oscillations. The region of the root tip that experiences the peak luminescence oscillation intensity becomes competent to form LR primordia (Figure 1D) (*5*). In D15-treated roots, the peak oscillation intensity was significantly lower as compared to control roots (Figure 1D-E). This suggests that D15 prevents root branching by inhibiting the initiation of LR primordia. Next, we examined the effect of adding β-cyclocitral to D15 treated roots. β-cyclocitral did not affect the peak oscillation intensity, indicating that it does not promote LR initiation (Figure 1D-E). To further test this, we examined the effect of β-cyclocitral on the formation of the first cell division in LR primordia. As expected, the IC_{50} concentration of D15 decreased the number of initiated LR primordia by approximately 50% compared to untreated plants, as determined using the *pWOX5:GFP* marker line (Figure S5A). Consistent with its inability to restore LR clock amplitude, β-cyclocitral did not restore the number of $WOX5^+$ primordia in D15-treated plants. Additionally, it did not increase $WOX5^+$ sites in the absence of D15. These results suggest that β-cyclocitral does not have a role in LR founder cell patterning or primordium initiation, and instead promotes lateral root branching by stimulating later stages of LR primordia development. To test this hypothesis, we examined its effect on the formation of the primordia using EN7 as a marker for the endodermis (*pEN7:GAL4; UAS:H2A-GFP*). β-cyclocitral doubled the number of $EN7^+$ primordia in D15-treated plants and increased $EN7^+$ primordia by 16% compared to untreated plants (Figure S5B). This suggested that β-cyclocitral does not affect LR initiation, but instead enhances the outgrowth and subsequent emergence of LR primordia.

To determine if β-cyclocitral has an effect on root traits other than LR outgrowth, we quantified its effect on primary root growth (Figure 2A-B). Treatment with β-cyclocitral enhanced primary root length by over 30% compared to control treatment. Because β-cyclocitral stimulates endodermal formation in LR primordia prior to cell elongation, we hypothesized that it induces root growth by increasing cell divisions in the root meristem. To test this hypothesis, we examined meristematic cell divisions and cell elongation in primary roots treated with β-cyclocitral. The number of meristematic cells increased more than 20% upon treatment with β-cyclocitral, while cell elongation remained unchanged (Figure 2C-D, Figure S6). In contrast, D15, which inhibits primary root growth by 35%, does not affect meristem cell number, and instead reduces cell elongation by 40% (Figure S7). Taken together, these results indicate that β-cyclocitral compensates for the inhibitory effects of D15 treatment by enhancing divisions in undifferentiated cells and highlights the utility of the D15-sensitized screen.

β-cyclocitral is a natural compound that is inexpensive, active in low concentrations, and can be applied exogenously, making it is a promising candidate for agricultural applications. To determine if β-cyclocitral has a conserved regulatory effect on agriculturally important plant species, we first assayed root growth in β-cyclocitral-treated tomato seedlings. We found that β-cyclocitral did indeed significantly increase primary and lateral root length in *Solanum lycopersicum* (Figure 2E-G). Interestingly, D15 did not have a negative effect on LR branching in tomato ($p = .99$), suggesting that its inhibition of LR initiation is not conserved in this species. The most parsimonious explanation for this is either that D15 may target different enzymes or that the patterning mechanism for LR founder cells is not conserved in tomato. This provides

further evidence that β-cyclocitral has a D15-independent effect on root development. Overall, these results demonstrate that β-cyclocitral regulates primary and LR growth in both tomato and Arabidopsis, suggesting that it is a conserved root growth promoter in eudicots.

Because many agriculturally important crops are derived from the evolutionarily divergent monocot family, we next applied β-cyclocitral to rice seedlings. We discovered that β-cyclocitral has a striking ability to modify root growth and architecture in 9311, a traditional *indica* cultivar (Figure 3). β-cyclocitral-treated root systems grew twice as deep compared to control plants (Figure 3A-B) and were significantly narrower (Figure 3C). β-cyclocitral enhanced rice root system architecture depth mainly by increasing primary root growth (Figure 3D). Crown roots also grew about 80% deeper when exposed to β-cyclocitral (Figure 3E), due to an increase in crown root length (Figure 3F) as well as a steeper angle of growth (Figure 3G). β-cyclocitral treatment also significantly decreased the total number of crown roots per plant by approximately 50% (Figure 3H). The combination of these factors generated deeper, more compact root systems. Importantly, the overall enhanced root growth caused by β-cyclocitral did not have an obvious effect on shoot mass, which indicates that β-cyclocitral does not have deleterious effects on shoot growth. (Figure S8). The added complexity of monocot root systems leads to additional emergent phenotypes that would not have been predicted based on the eudicot studies and reveals new potential roles of β-cyclocitral in root development.

Deeper, more compact root systems have been shown to increase tolerance to osmotic stress in monocots, indicating that β-cyclocitral's effects could benefit rice grown in harsh environments (*25-27*). To determine if β-cyclocitral can promote root growth under abiotic stress, we applied it to salt-stressed roots (Figure 4). Treatment of seedlings with 50 mM and 100 mM sodium chloride (NaCl) significantly decreased root depth compared to control treatment. Root depth could be completely recovered upon co-treatment with β-cyclocitral (Figure 4A-B). In fact, β-cyclocitral had a significantly larger effect on salt-treated plants compared to unstressed plants (p value $\leq$ 0.0001). Salt stress also significantly increased the solidity, of the root network (Figure 4C), measured by calculating the total area of each root divided by the convex area of the root system (*28*). Increased solidity during salt stress indicates that the plants are producing denser and smaller root systems. Although β-cyclocitral does not affect the solidity of the root system in the absence of salt, it rescues solidity during salt treatment. Overall, these results indicate that β-cyclocitral not only enhances root growth during normal conditions but could also enable roots of crop plants to adjust to salt stress.

Conclusion

Through a sensitized chemical genetic screen, we identified β-cyclocitral as a naturally occurring β-carotene derived apocarotenoid, which regulates root architecture in monocots and dicots. In *Arabidopsis*, we found that β-cyclocitral increases primary and LR growth by inducing cell divisions in root meristems. β-cyclocitral additionally promotes root growth in tomato and rice, with a particularly potent effect in rice. Along with its conserved role as a root growth promoter in rice, β-cyclocitral also affects other aspects of root architecture, including the numbers and angles of roots. In salt-stressed rice roots we found that β-cyclocitral significantly promotes root growth, potentially shielding the root systems from the effects of this abiotic stress. These results indicate that β-cyclocitral is a natural compound that could be a valuable

tool to improve crop fitness, especially in harsh environmental conditions. Intriguingly, β-cyclocitral does not directly rescue LR initiation in the presence of D15, suggesting that there remains at least one unidentified apocarotenoid crucial for root development. By refining the D15-sensitized screen, we plan to identify this elusive compound in future studies.

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Supplementary Materials:

Materials and Methods

Figures S1-S8

Table S1

References (*29-32)*

Figures

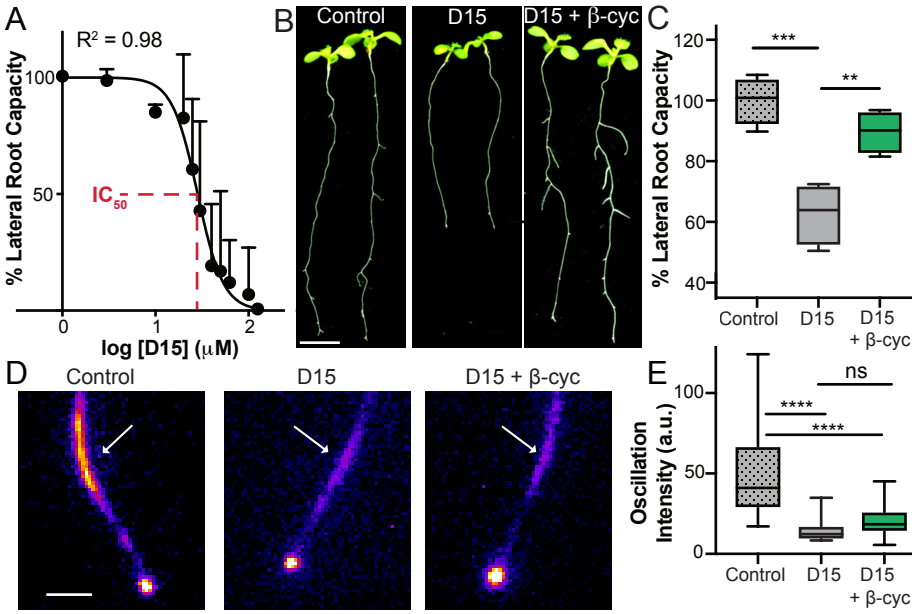

Figure 1. Identification of β-cyclocitral in a sensitized LR screen. A) The LR capacity of D15-treated plants, normalized to control plants. The IC_{50} is highlighted in red. B) Seedlings after treatment with D15 and β-cyclocitral (β-cyc) (scale bar = 5 mm) C) LR capacity in plants treated with D15 and β-cyclocitral. D) Luminescence images of *pDR5:LUC* primary roots taken at the peak of the LR clock oscillation intensity (scale bar = 0.5 mm). The arrows indicate the center of the oscillation zone. E) The maximum oscillation zone intensity in treated roots. The abbreviation "ns" stands for non-significant ($p = 0.6$). The symbols **, ***, and **** indicate p values $\leq$ 0.01, 0.001, and 0.0001, respectively.

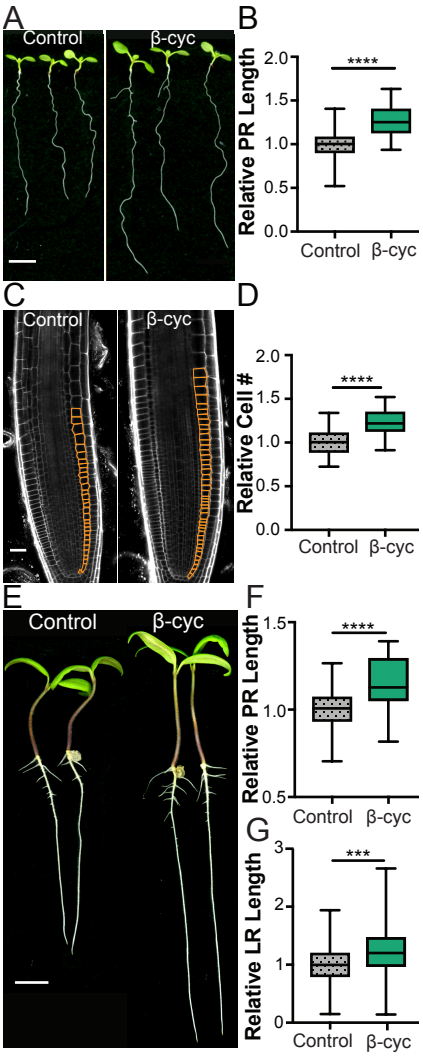

Figure 2. β-cyclocitral is a conserved promoter of root growth. A) Arabidopsis seedlings treated with β-cyclocitral (scale bar = 5 mm). B) Quantification of primary root length in β-cyclocitral-treated plants. C) Confocal images of primary root meristems (scale bar = 50 microns). Meristematic cortex cells are highlighted in orange. D) Relative number of cortex cells in the primary root meristems of treated and control plants. E) Tomato seedlings treated with β-cyclocitral (scale bar = 1 cm). F) Primary root (PR) length in tomato seedlings treated with β-cyclocitral (normalized to control roots). G) LR length in tomato seedlings treated with β-cyclocitral (normalized to control roots), calculated using the longest three LRs per plant. The symbols *** and **** indicate p values ≤ 0.001 and 0.0001, respectively.

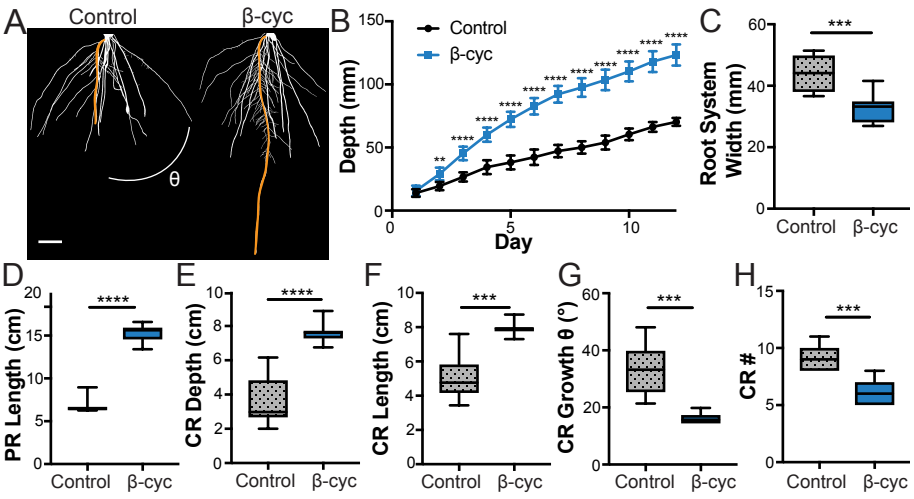

Figure 3. β-cyclocitral induces root system architecture changes in monocots. A) Root systems of 9311 rice seedlings treated with β-cyclocitral (scale bar = 10 mm). The primary roots are highlighted in orange. The growth angle (θ) measured to quantify the steepness of crown roots is shown in white. B) Depth of the 9311 root systems over the course of 12 days of treatment. C) The width of root systems at day 6. D) Quantification of primary root length (D), crown root depth (E), crown root length (F) and crown root growth angle θ (G) in treated plants. H) Quantification of the number of crown roots per seedling. The symbols **, ***, and **** indicate p values ≤ 0.01, 0.001, and 0.0001, respectively.

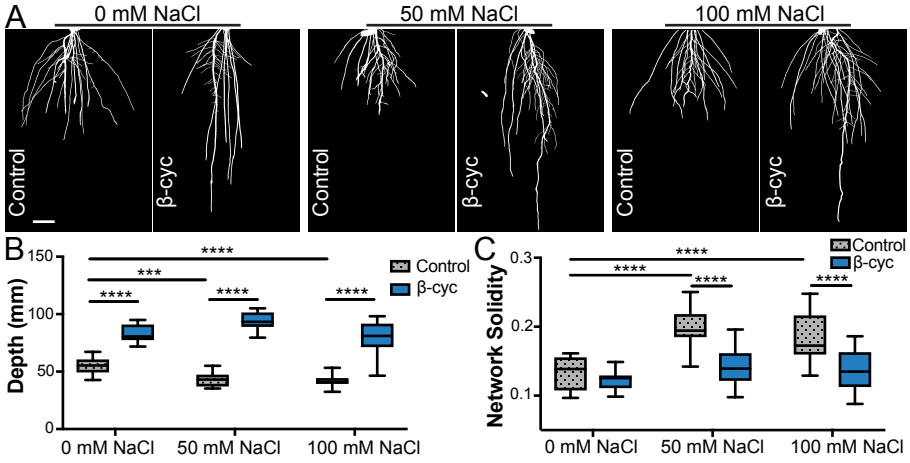

Figure 4. β-cyclocitral promotes growth under salt stress. A) Rice roots treated with β-cyclocitral and varying concentrations of NaCl (scale bar = 10 mm). B) Root system depth in seedlings treated with different concentrations of NaCl. C) The solidity of the root network (area of roots / convex area of root system) in treated seedlings. The symbols *** and **** indicate p values ≤ 0.001 and 0.0001, respectively.