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Brief Report

Fenoxycarb, a carbamate insect growth regulator, inhibits brassinosteroid action

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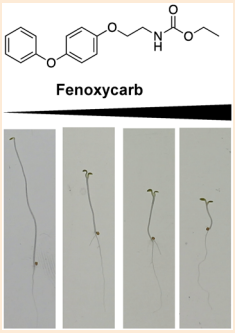
Supplementary material

Brassinosteroids (BRs) are steroid hormones that regulate plant growth, development, and stress resistance. In this study, we evaluated the effect of agrochemicals on dark-induced hypocotyl elongation, which is regulated by BRs, to identify novel chemicals that regulate BR action. We found that the juvenile hormone agonist fenoxycarb inhibited dark-induced hypocotyl elongation in *Arabidopsis*. Treatment with the same class of juvenile hormone agonist, pyriproxyfen, did not affect hypocotyl elongation. Co-treatment with fenoxycarb and BR partly canceled the fenoxycarb-induced hypocotyl suppression. In addition, gene expression analysis revealed that fenoxycarb altered the BR-responsive gene expression. These results indicate that fenoxycarb is a BR action inhibitor.



Keywords: brassinosteroid, fenoxycarb, insect growth regulator, *Arabidopsis*.

Introduction

Plant growth and development are controlled through the regulation of plant hormone biosynthesis and signaling in response to environmental changes. Brassinosteroids (BRs) are a group of polyhydroxylated steroid hormones involved in dark-induced hypocotyl elongation, xylem development, reproductive growth, and seed germination. In addition, BRs protect plants from various abiotic and biotic stresses by regulating the mechanisms by which plants respond to stress. Hence, regulating BR functions in plant tissues using chemicals is a useful approach for enhancing plant growth and crop yields. Endogenous BR functions have been demonstrated in BR-deficient mutants. These mutants have been useful for determining the fundamental roles of BRs in plant growth and development. Small molecules have also proven to be valuable tools for determining the physiological roles, biosynthesis, and signaling of BRs. For example, the BR biosynthesis inhibitor brassinazole has greatly contributed to identifying BR signaling genes.[1)] *BIL1*/*BZR1* was the first gene identified from mutant screening to cancel the brassinazole-induced phenotype.[2,3)] BIL1/BZR1 and its homolog, BES1, are key transcriptional regulators of BR signaling and control the expression of numerous genes.[4,5)]

Juvenile hormones (JHs) play critical roles in regulating various physiological processes such as metamorphosis, reproduction, diapause, and behavior in most insects. When larvae grow to the appropriate size, JH synthesis ceases, permitting the formation of an adult.[6)] Because JH-like compounds prevent metamorphosis, an important event for insects, many biologically active JH-like compounds have been synthesized as novel insect growth regulators. Fenoxycarb and pyriproxyfen act as agonists of JH receptor and are commercially registered as potent insecticides against various insects.

Some pharmaceutical and agrochemical compounds function as plant growth regulators and several plant growth

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regulators used in basic research have been developed using these compounds as molecular scaffolds. For example, brassinazole was discovered as a BR biosynthesis inhibitor because uniconazole-P, which is used as a plant growth retardant, exhibits the inhibitory activities of BR biosynthesis.[7] In addition, TIS108, a strigolactone (SL) biosynthesis inhibitor, was found in a screening and structure–activity relationship study from a chemical library constructed during brassinazole development.[8–10] Recently, our group reported that insect growth regulators, chromafenozide and methoxyfenozide, were SL biosynthesis inhibitors.[11]

In this study, we screened commercial agrochemicals for compounds that inhibit dark-induced hypocotyl elongation in *Arabidopsis* to find the novel BR action regulators and investigated the inhibitory activities of BRs by fenoxycarb.

Materials and methods

1. Plant material, chemicals, and growth conditions

The *Arabidopsis* ecotype Columbia (Col-0) was used as the wild-type plant. The *Arabidopsis bil1-1D/bzr1-1D* mutant showing a BR-insensitive long hypocotyl phenotype was selected. After surface sterilization of seeds and incubation at 4°C, seeds were germinated on 1/2 Murashige and Skoog (MS) medium containing 0.8% phytoagar with 1.5% sucrose. The MS medium was supplemented with 0.1% (v/v) DMSO (control) or test chemicals dissolved in DMSO. The fenoxycarb, pyriproxyfen, insecticides, and insect growth regulators used in Fig. 1 were purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Juvenile hormone III was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Plants were grown at 22°C in the dark for 7 days, and the hypocotyl length was measured using the ImageJ software.

2. Gene expression analysis

Seven-day-old seedlings grown on 1/2 MS medium containing 0.8% phytoagar and 1.5% sucrose were treated with 30 μM fenoxycarb for one day. Seedlings were frozen in liquid nitrogen and crushed using a mortar and pestle. RNA was extracted using the PureLink Plant RNA Reagent (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's protocol. cDNA synthesis was performed using ReverTraAce qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan), according to the manufacturer's protocol. qRT-PCR and data analysis were performed using the QuantStudio RealtimePCR System (Thermo Fisher). The PCR primers used for qRT-PCR are listed in Table S1.[12]

Results and discussion

To identify novel plant growth regulators, we focused on the regulation of BR action because BRs affect plant growth and crop yield. First, we screened for chemicals that regulate dark-induced hypocotyl elongation, which is positively regulated by BRs, among the insecticides and insect growth regulators (Fig. S1). Seedlings were grown on 1/2 MS agar plates with

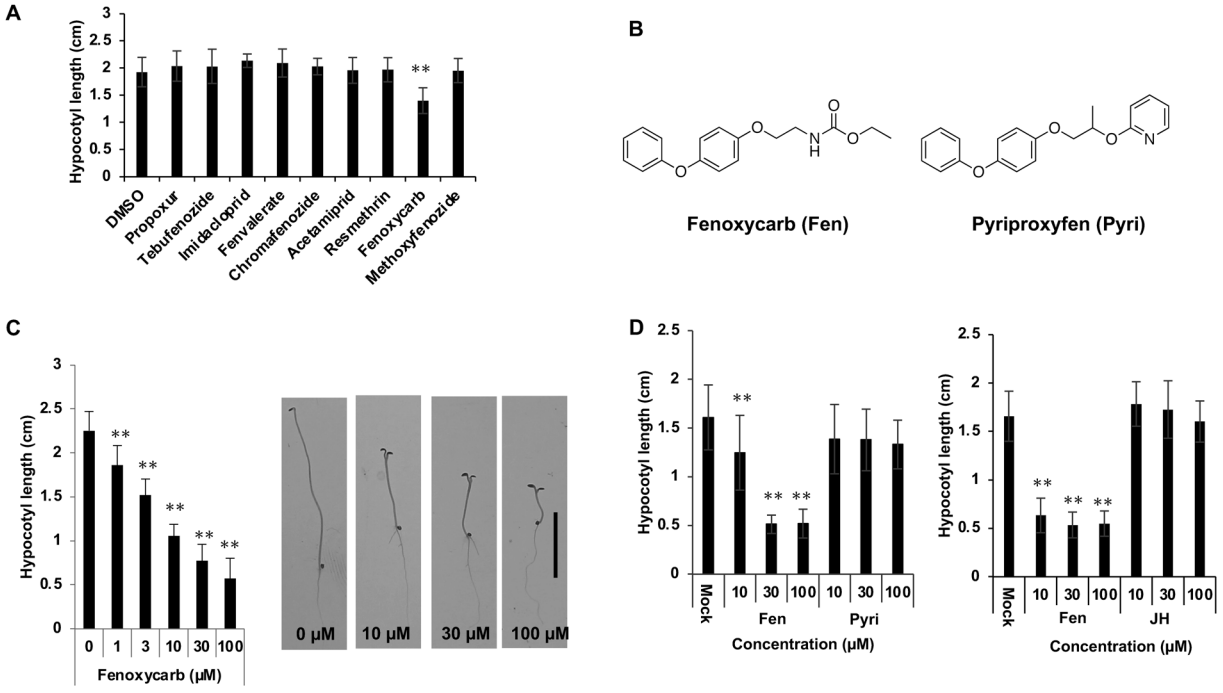

Fig. 1. Effect of fenoxycarb on dark-induced hypocotyl elongation. (A) Effect of insecticide and insect growth regulators (3 μM) on dark-induced hypocotyl elongation. The data are the mean±S.D. (n=13–52). (B) Structures of fenoxycarb and pyriproxyfen. (C) Dose-response of fenoxycarb in dark-induced hypocotyl elongation. Scale bar: 1 cm. The data are the mean±S.D. (n=16–20). (D) Effect of fenoxycarb, pyriproxyfen, and natural juvenile hormone on dark-induced hypocotyl elongation. Fen: fenoxycarb, Pyri: pyriproxyfen, JH: juvenile hormone III. The data are the mean±S.D. (n=18–45). ** denotes a statistically significant difference from hypocotyl length in mock-treated (DMSO or Mock) plants (Dunnett's test; $p<0.01$).

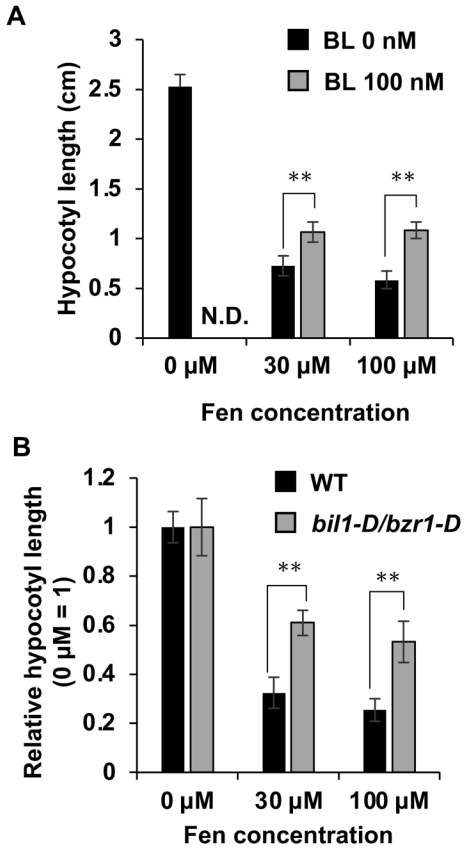

Fig. 2. Effects of fenoxycarb on BL-treated WT and *bil1-1D/bzr1-1D* mutant. N.D.: not determined, Fen: fenoxycarb. The data are the mean±S.D. (n=14–28). ** denotes a statistically significant difference from hypocotyl length in 0 nM BL-treated (A) and WT (B) plants (t-test; $p<0.01$).

insecticides or insect growth regulators under dark conditions, and the hypocotyl length of seven-day-old *Arabidopsis* seedlings was measured. Most chemicals did not affect hypocotyl elongation, whereas treatment with 3 μM fenoxycarb significantly inhibited dark-induced hypocotyl elongation (Fig. 1A and B). Fenoxycarb treatment reduced the hypocotyl length in seven-day-old *Arabidopsis* seedlings in a dose-dependent manner within a concentration range of 1–100 μM (Fig. 1C). In addition, fenoxycarb treatment induced a de-etiolated phenotype with open cotyledons, which is characteristic of BR-deficient mutants. Because fenoxycarb is commercially used as an insect JH agonist, we estimated the effect of a JH agonist, pyriproxyfen, and natural juvenile hormone on dark-induced hypocotyl elongation under the same conditions (Fig. 1B and D). While fenoxycarb reduced the hypocotyl length by one-third at 30 μM and 100 μM, pyriproxyfen or juvenile hormone III treatment showed only a slight inhibition of dark-induced hypocotyl elongation, even at a concentration of 100 μM. These results suggest that the chemical structure of fenoxycarb, rather than its JH agonist activity, is important for inhibiting dark-induced hypocotyl elongation (Fig. 1D). Both fenoxycarb and pyriproxyfen have 4-phenoxyphenoxy structures. However, fenoxycarb has a carbamate structure, whereas pyriproxyfen has a pyridine ring (Fig. 1B). This structural difference may have caused a significant difference in the inhibitory activity of dark-induced hypocotyl elongation.

Hypocotyl elongation under dark conditions is mainly regulated by BR signaling. To determine whether BR signaling inhibition caused dark-induced hypocotyl elongation by fenoxycarb, we co-treated 100 nM brassinolide (BL) with fenoxycarb. This co-treatment partly but significantly recovered dark-induced hypocotyl elongation compared to fenoxycarb treatment alone (Fig. 2A). In addition, the inhibition of dark-induced hypocotyl elongation by fenoxycarb was partly canceled in the *bil1-1D/bzr1-1D* mutant, a constitutive BR signaling mutant, compared to the wild-type, suggesting that fenoxycarb inhibits BR biosynthesis or signal transduction (Fig. 2B). The structure of fenoxycarb differs markedly from those of BR biosynthesis inhibitors (Fig. S2A).[13] For example, brassinazole, a potent BR biosynthesis inhibitor, and its derivatives have azole moiety, while fenoxycarb is a compound without a heterocyclic ring. Known BR antagonists are also structurally different from fenoxycarb, which contains a carbamate moiety (Fig. S2B).[14,15] Thus, fenoxycarb may act through a different mechanism than known BR action inhibitors.

To further investigate the mechanism of action of fenoxycarb in *Arabidopsis*, we performed a gene expression analysis of BR-responsive genes (Fig. 3). Seven-day-old wild-type seedlings were treated with 30 μM fenoxycarb for one day. BIL1/BZR1, the master regulator of BR signaling, negatively regulates BR biosynthesis genes and positively regulates auxin-related genes.[5] In this study, we selected *AtSAUR-AC1* (At4g38850) and *AtIAA19* (At3g15540), which are auxin-related genes as BR-upregulated genes, and *AtBR6ox2* (At3g30180) and *AtDWF4* (At3g50660), which are BR biosynthesis genes as BR-downregulated genes. In addition, as the inhibition of BR biosynthesis increased the

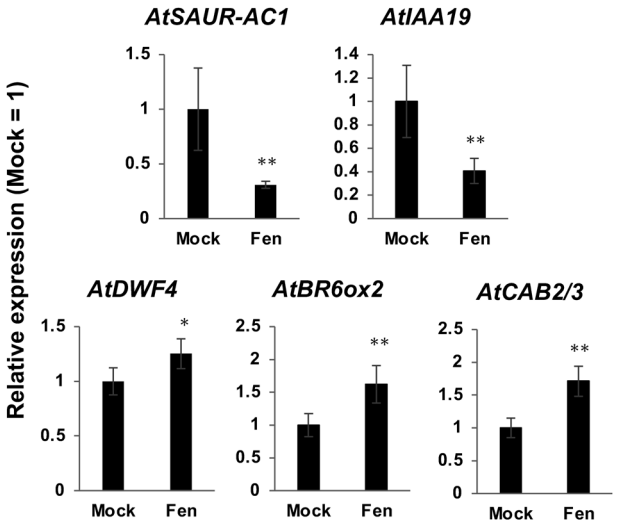

Fig. 3. Gene expression analysis of fenoxycarb treated *Arabidopsis* seedlings. The data are the mean±S.D. (n=5). Fen: 30 μM fenoxycarb. * and ** denote a statistically significant difference from hypocotyl length in mock-treated (DMSO) plants (t-test; $p<0.05$ and 0.01, respectively).

expression of photosynthesis-related genes, we also analyzed the expression level of chlorophyll A/B-binding proteins, *AtCAB2/3* (At1g29910 and At1g29920), as BR-downregulated genes.[16] Fenoxycarb treatment downregulated the expression of *AtSAUR-AC1* and *AtIAA19* and upregulated the expression of *AtBR6ox-2*, *AtDWF4*, and *AtCAB2/3*, suggesting that fenoxycarb inhibits BR action at the gene expression level.

In this study, we found that fenoxycarb, a carbamate juvenile hormone agonist, inhibited BR action in *Arabidopsis*. Although it is not clear whether fenoxycarb acts on BR biosynthesis or signal transduction, analysis of the target protein(s) and action mechanism(s) of fenoxycarb might provide new insights into BR biosynthesis and function since fenoxycarb is structurally different from previously reported inhibitors of BR biosynthesis and signal transduction.[13]

Acknowledgements

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Conflicts of interest

Declarations of interest: none

Electronic supplementary materials

The online version of this article contains supplementary materials (Supplementary Table S1, Fig. S1, and Fig. S2), which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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