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A mammalian steroid action inhibitor spironolactone retards plant growth by inhibition of brassinosteroid action and induces light-induced gene expression in the dark

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Abstract

We screened steroid derivatives and found that spironolactone, an inhibitor of both 17β-hydroxysteroid dehydrogenase (17β-HSD) and aldosterone receptor, is an inhibitor of phytohormone brassinosteroid (BR) action in plants. Under both dark and light growing conditions, spironolactone induced morphological changes in *Arabidopsis*, characteristic of brassinosteroid-deficient mutants. Spironolactone-treated plants were also nearly restored to the wild-type phenotype by treatment with additional BRs. In the spironolactone-treated *Arabidopsis*, the *CPD* gene in the BR biosynthesis pathway was up-regulated, probably due to feedback regulation caused by BR-deficiency. Spironolactone-treated tobacco plants grown in the dark showed expression of light-regulated genes as was observed in the deficient mutant. These data suggest that spironolactone inhibits brassinosteroid action probably due to the blockage of biosynthesis and exerts its activity against plants. Thus, spironolactone, in conjunction with brassinosteroid-deficient mutants, can be used to clarify the function of BRs in plants and characterize mutants. The spironolactone action site was also investigated by feeding BR biosynthesis intermediates to *Arabidopsis* grown in the dark, and the results are discussed.
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Keywords: Brassinosteroid biosynthesis; Receptor; Inhibitor; Photomorphogenesis

1. Introduction

The application of biologically active brassinosteroid (BR) homologues causes remarkable growth responses in plants, including stem elongation, pollen tube growth, leaf bending, leaf unrolling, root inhibition, proton pump activation [1], promotion of ethylene production [2], tracheary element differentiation [3,4], and cell elongation [5]. The functions of endogenous BRs have been revealed by analysis of several BR-deficient mutants in *Arabidopsis*, pea, tomato [6] and rice [7]. These mutants have been invaluable in determining the essential roles BRs have in plant growth and development; consequently, BRs have recently been recognized as a new class of phytohormones [6,8].

The use of specific inhibitors is an alternative way to determine the physiological functions of BRs. KM-01 is the first reported selective BR inhibitor, but it appears to be of limited use for investigating BR function in plants due to its very low activity when it is applied alone [9]. Recently, we reported the series of specific BR biosynthesis inhibitors and found that all of them target the conversions of 6-oxocampestanol to cathasterone catalyzed by the cytochrome P450 monooxygenases DWF4 [10–16]. Although BR biosynthesis inhibitors were proved to be very useful for investigating BR functions and gene expressions in plants [17–19] and isolating and characterizing mutants in which a component involved in BR signal transduction is altered [20–22], BR biosynthesis inhibitors, which target other enzymes catalyzing reactions in BR biosynthesis pathway, are required. If we have two types of BR biosynthesis inhibitors targeting different enzymes, then we are readily able to identify whether the mutant is altered in BR signal transduction or in inhibitor target enzymes. In this context, we started the

Abbreviations: BR, brassinosteroid; GA, gibberellin; BL, brassinolide; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase

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search for new BR biosynthesis inhibitors targeting different enzymes other than DWF4.

Mammals have steroid biosynthesis pathways that have steps similar to those of plants. Inhibitors of these steps have been identified and used to investigate the function of steroids in mammalian cells. Some of these inhibitors might be used successfully in plants, since they have similar enzymes in their BR biosynthesis pathways, such as 5α-reductase (DET2 in Fig. 1) [23] and 3β-hydroxysteroid dehydrogenase (3β-HSD in Fig. 1) [24]. We started to screen animal BR biosynthesis inhibitors and eventually identified spironolactone, an inhibitor of both aldosterone receptor and 17β-HSD [25,26], as an inhibitor of BR action. Here, we report the characterization of spironolactone as an inhibitor of BR action and examine its potential targets.

2. Materials and methods

2.1. Chemicals

Brassinolide and castasterone were purchased from CIDtech Research Inc. (Cambridge, Ont., Canada). Other intermediates in the BR biosynthesis pathway used in this report were synthesized as described previously [27–30]. Murashige and Skoog salt and vitamin mixture was purchased from GIBCO-BRL (Grand Island, NY, USA). Spironolactone was purchased from Kanto Kagaku Co. Ltd. (Tokyo, Japan).

2.2. Plant materials and growth conditions

Wild-type *Arabidopsis* seeds (ecotype Columbia) were purchased from LEHLE Seeds (Round Rock, TX, USA). Cress seeds were purchased locally. Wild-type and *det2* seeds were cold treated (4 °C) for 2 days, and then surface sterilized in a 1% NaOCl solution for 20 min and washed five times with sterile distilled water. Seeds were sown on 1% agar-solidified medium containing 0.5x Murashige and Skoog salts and 1.5% sucrose (w/v) in plastic plates with or without chemicals. Wild-type and *det2* plants were grown in 16-h light (240 μ $Em^{-2}s^{-1}$) and 8-h dark conditions in a growth chamber (22 °C). The plates were sealed with Parafilm (American National Can Co., Chicago, IL, USA) for the screening experiment. For the rescue

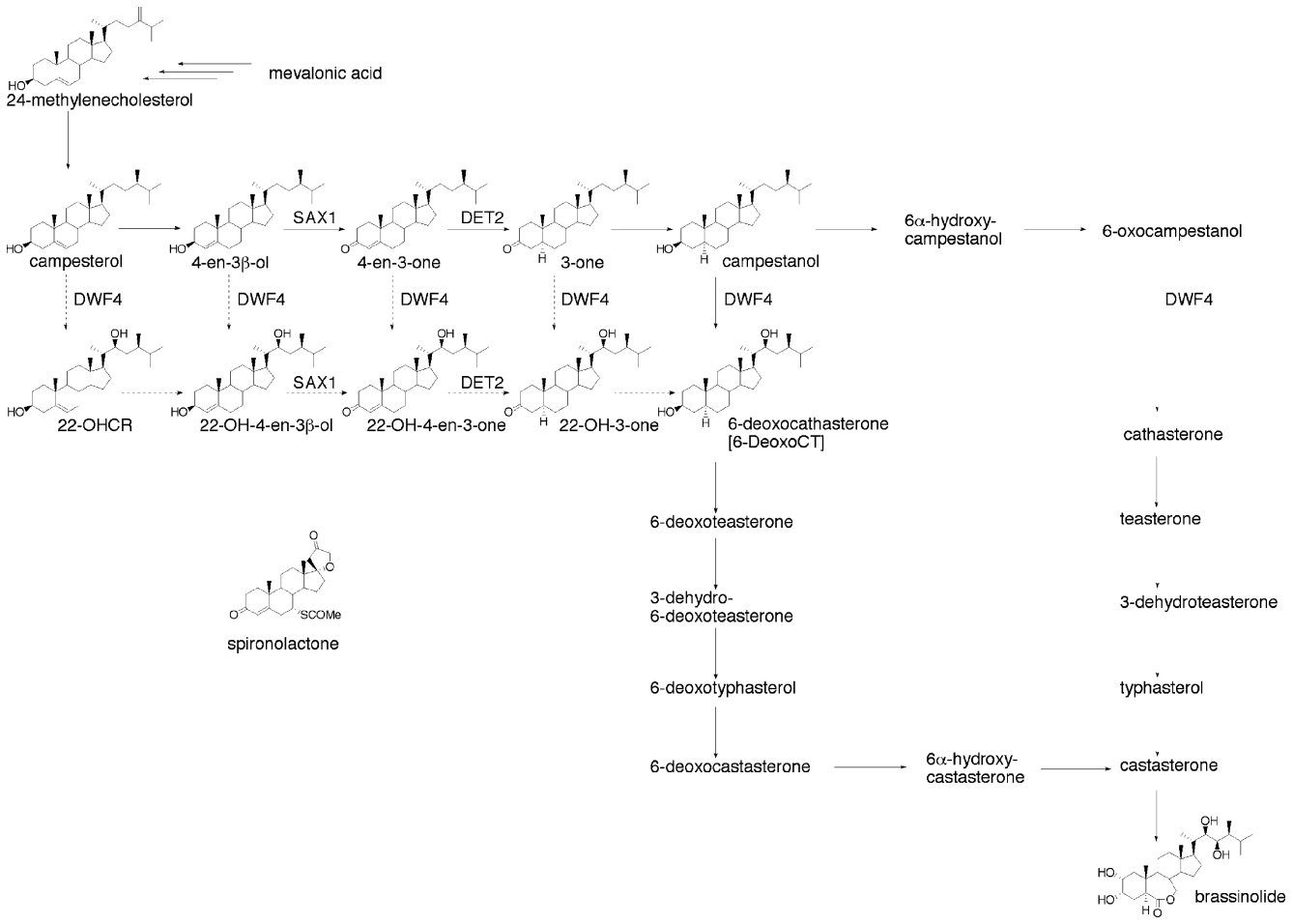

Fig. 1. Structure of spironolactone and the proposed biosynthetic pathway for brassinolide. Campesterol is converted to campestanol by four reactions. Campestanol is then metabolized to castasterone through two different pathways, the early C6 oxidation (right downward pathway) and the late C6 oxidation pathways (left downward pathway). Steps mediated by DET2 [9] and SAX1 [12] are indicated.

experiment, which required a longer experimental period, seeds were sown on 1% agar-solidified medium containing 0.5x Murashige and Skoog salts and 1.5% sucrose (w/v) in Agripots (Kirin Brew. Co., Tokyo, Japan). Plants were grown in 16-h light (240 μ Em^{-2}s^{-1}) and 8-h dark conditions in a growth chamber (25 °C). Cress seeds were sown on 1% agar-solidified medium containing 0.5x Murashige and Skoog salts and 1.5% sucrose (w/v) in Agripots (Kirin Brew. Co., Tokyo, Japan) with or without chemicals. Plants were grown in 16-h light (240 μ Em^{-2}s^{-1}) and 8-h dark conditions in a growth chamber (25 °C).

2.3. Northern hybridization

For Northern analysis, RNA (2 μg) was electrophoresed on 1.5% formaldehyde denaturing agarose gels, blotted onto Hybond-N+ membranes (Amersham), and hybridized with DNA and RNA probes according to the method of Sambrook et al. [31]. Full-length cDNA fragments of tobacco chloroplast genes and ribosomal RNA were cloned by the polymerase chain reaction (PCR) using the Expand High Fidelity PCR system (Boehringer Mannheim) and a cloning kit (Invitrogen). The sequences of the clones were determined to be known tobacco genes: chlorophyll *a/b*-binding protein (*cab21*, GenBank (GB) accession number X52743), ribulose bisphosphate carboxylase small subunit (*rbcS-8B*, GB: M36685), and 25S ribosomal RNA (25S rRNA, GB: X76056). Oligonucleotide PCR primers located at the 5′ and 3′ ends of these genes were designed: 5′-ATGGTACGGCCCAGACCGTGTTAAGTAC-3′ and 5′-TCACTTTCCGGGGACAAAGTTTGTGGCG-3′ for *cab*; 5′-ATGGCTTCCTCAGTTCTTTCCTCTGCAG-3′ and 5′-TTAGTAGCCTTCTGGCTTGTAGGCAATGAA-3′ for *rbcS*; 5′-GAATTCACCAAGTGTTGGATTGT-3′ and 5′-ACGAATCGGAGCGACAAAGGG-3′ for 25S rRNA. The fragments were cloned into pCR2.1 (Invitrogen) and sequenced with a DNA sequencer (model PRISM310, ABI) using a Dye Terminator Cycle Sequencing Kit (ABI).

These fragments were labeled with ^{32}P-dCTP (6000 Ci/mmole) using Ready-To-Go DNA Labeling Beads (Pharmacia) for use as DNA probes. The radioactivity of the hybridized bands was determined using the BAS2000 system (Fuji Film).

3. Results

Arabidopsis mutants such as *det2* and *cpd* show strong dwarfism with curly, dark green leaves in the light, and a de-etiolated phenotype with short hypocotyls and open cotyledons in the dark, which are characteristics of light-grown plants [32,33]. This phenotype was rescued by application of BL, but other plant hormones, such as auxin and GA, had no effect. Based on these results and using an *Arabidopsis* seedling assay, we started screening a variety of steroid derivatives including progesterone [34] and trilostane [35], which have been reported to be 3β-HSD inhibitors in mammalian systems, and found spironolactone to be an inhibitor of BR action. Spironolactone was the only active inhibitor identified among steroidal compounds tested, and it caused marked morphological seedling malformations similar to those of BR-deficient mutants. At a concentration of 100 μM the phenotype became very much similar to that of BR-deficient mutants (Fig. 2A and B). These spironolactone-induced phenotypes were rescued by co-application of 10 nM brassinolide (Fig. 2C). In the dark, spironolactone induced a de-etiolated phenotype with a short hypocotyl and open cotyledons (Fig. 2D), again similar to BR-deficient mutants. These phenotypes were also rescued by application of 10 nM BL.

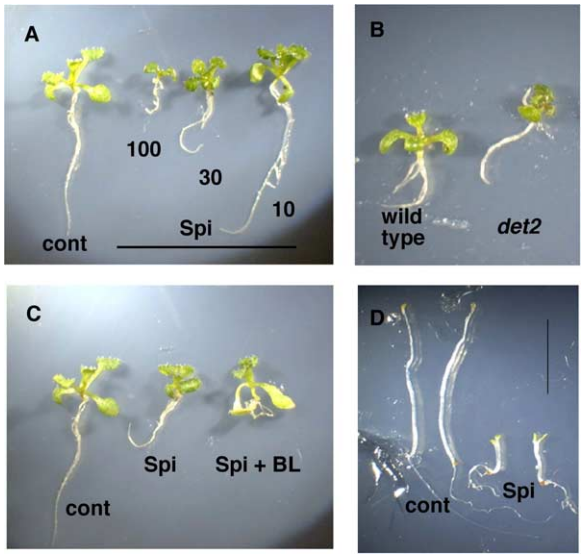

Fig. 2. Effect of spironolactone on *Arabidopsis* seedlings grown in both light and dark conditions. (A) Spironolactone (100, 30, 10 μM)-treated *Arabidopsis* (14-day-old grown in the light) show dwarfism in a concentration-dependent manner. (B) Wild-type and brassinosteroid-deficient mutant (*det2*) (14 days old, grown in the light). (C) Spironolactone (100 μM) treated *Arabidopsis* (14 days old, grown in the light) shows a brassinosteroid-deficient mutant-like phenotype, which is rescued by the application of brassinolide (10 nM). (D) Spironolactone (100 μM)-treated *Arabidopsis* (7 days old, grown in the dark) shows a brassinosteroid-deficient-mutant-like phenotype, that is, a short hypocotyl and open cotyledon. Bar in (D) = 10 mm. Cont, control; Spi, spironolactone; BL, brassinolide.

Brassinazole, a specific BR biosynthesis inhibitor, induces BR deficiency in a variety of plant species [6]. To examine whether spironolactone can cause a BR-deficient-mutant-like phenotype in plants other than *Arabidopsis*, spironolactone was also applied to cress (*Lepidium sativum*), a plant that has been used previously to investigate the effects of brassinolide [36,37]. The hypocotyl length of cress seedlings treated with 100 μM spironolactone is about 30% of that of the control (Fig. 3). This effect is reversed by application of 0.01 μM BL, but not by 1 μM GA. The hypocotyl length of cress seedlings treated with the potent GA biosynthesis inhibitor uniconazole [38] at

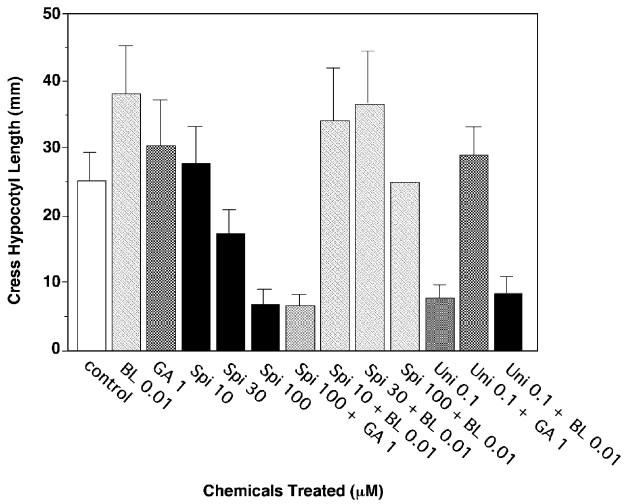

Fig. 3. Retardation of cress seedling growth by spironolactone or uniconazole and rescue by BL or GA_3.Data are the mean ± S.E. obtained from 20 seedlings. BL, brassinolide; Spi, spironolactone; Uni, uniconazole; GA, GA_3. BL added to cress rescues plants treated with spironolactone but not those treated with uniconazole. Addition of GA_3 rescues plants treated with uniconazole but not those treated with spironolactone.

concentrations 1 μM and higher is also about 30% of that of the control, but in this case the effect of uniconazole is negated by application of GA, but not by BL.

One of the characteristics of BR biosynthesis-deficient mutants under dark conditions is the accumulation of transcripts of light-regulated genes [32,39]. Hybridization of steady-state RNAs prepared from spironolactone-treated tobacco seedlings grown in the dark clearly showed that photomorphogenesis in the spironolactone-treated tobacco was accompanied by an increase in the expression of light-regulated genes coding for the small subunit of ribulose 1,5-diphosphate carboxylase (*rbcS*) and chlorophyll *a/b* binding protein (*cab*) (Fig. 4). These levels were significantly higher than those in dark-grown, non-treated seedlings.

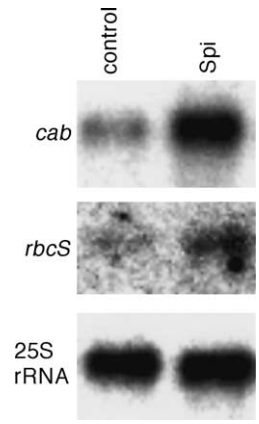

Fig. 4. Accumulation of *rbcS* and *cab* mRNA in dark-grown spironolactone-treated and non-treated plants. Lane 1, non-treated plants; lane 2, spironolactone-treated plants. Two micrograms of total RNA was loaded per lane.

To investigate whether the biosynthetic step(s) was affected by spironolactone, we examined the effects of two sets of biosynthetic intermediates on hypocotyl elongation with both spironolactone-treated and non-treated etiolated *Arabidopsis* seedlings grown in the dark. The first set was designed to analyze potential spironolactone targets lying downstream of cathasterone; the second was intended to analyze potential targets upstream of 6-deoxocathasterone (as performed by Ephritikhine et al [30]). Application of 1 μM cathasterone, teasterone and typhasterol, 100 nM castasterone, and 10 nM BL had no effect on either the hypocotyl or root length of non-treated seedlings, and all of these compounds were effective in rescuing the hypocotyl and root growth of spironolactone-treated *Arabidopsis* seedlings. In other words, spironolactone exerts its effect by reducing the supply of BL signal in the plant, and the spironolactone targets in the BR biosynthesis pathway are not downstream of cathasterone if the biosynthesis steps could be the target site of spironolactone.

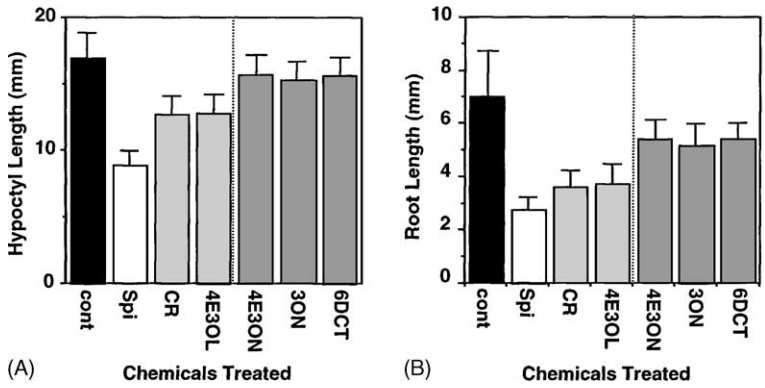

Fig. 5. Spironolactone-treated *Arabidopsis* hypocotyl elongation (A) and root growth (B) in the dark in response to applied 22-hydroxylated biosynthesis intermediates lying upstream of 6-deoxocathasterone. Data are the mean ± S.E. obtained from 30 seedlings. Concentration of spironolactone applied was 30 μM. Concentration of biosynthesis intermediates applied was 1 μM. Cont, control; Spi, spironolactone; CR, 22-OHCR; 4E3OL, 22-OH-4-en-3β-ol; 4E3ON, 22-OH-4-en-3-one; 3ON, 22-OH-3-one; 6DCT, 6-deoxocathasterone. Each broken line indicates a biosynthesis step that spironolactone may block.

We therefore next tested whether BRs lying upstream of 6-deoxocastasterone could negate the effect of spironolactone. Fig. 5 shows that BR biosynthetic intermediates, 22-OH-4-en-3-one and its products, 22-OH-3-one and 6-DeoxoCT, induced both hypocotyl and root growth in spironolactone-treated *Arabidopsis* seedlings to the levels of non-treated *Arabidopsis*. On the other hand, the effects of 22-OH-4-en-3-ol and 22-OHCR, both precursors of 22-OH-4-en-3-one, on the growth of both the hypocotyl and root were less than those of 22-OH-3-one, 22-OH-4-en-3-one, and 6-DeoxoCT. There was a clear difference between the 22-OH-4-en-3-one and 22-OH-4-en-3β-ol in promoting activities. This result suggests that one of the target sites of spironolactone could lie between 22-OH-4-en-3-one and 22-OH-4-en-3β-ol, a step converting a 3β-hydroxyl group into a carbonyl group. The conversion test used 22-OH derivatives of BRs because no activity of biosynthetic intermediates without 22-OH group was detected upstream of campesterol in bioassays and these derivates existed and were converted to BL in plants [40]. In the case of reversion tests, plants tend to be sensitive to growth conditions, perhaps because of the slow uptake and transport of BL within cress and/or the non-specific effect(s) of spironolactone on other aspects of plant metabolism. This may be one of the reasons that BRs did not completely reverse spironolactone inhibition in our assays.

4. Discussion

Under both light and dark growing conditions, spironolactone-induced morphological changes were reversed by the addition of BL. In the dark, spironolactone-treated *Arabidopsis* plants develop similar to light-grown plants, and express light-regulated genes, as BR-deficient mutants do. The *cpd* and *det2* mutations induce de-etiolation and expression of light-induced genes in the dark, phenotypes also seen in spironolactone-treated *Arabidopsis*. These results indicate that spironolactone treatment causes BL deficiency in *Arabidopsis*.

Feeding experiments demonstrated that spironolactone-induced retardation of hypocotyl growth was rescued by the addition of exogenous BR biosynthesis intermediates, such as 22-OH-4-en-3-one, but not by 22-OH-4-en-3β-ol, suggesting that the inhibition site of spironolactone may be the step from 4-en-3β-ol to 4-en-3-one being catalyzed by 3β-HSD. The enzyme involved in this step mediates the dehydrogenation of a hydroxyl group at C-3. The effects of 22-OH-4-en-3β-ol and 22-OHCR, both precursors of 22-OH-4-en-3-one, on both hypocotyl and root growth in the reversion test shown in Fig. 5 were significantly less than those of 22-OH-4-en-3-one, 22-OH-3-one, and 6-DeoxoCT, but those 3-ols still exerted a reversion effect on spironolactone-treated plants.

Spironolactone has multiple target sites in animal cells, such as aldosterone receptor [25], type II 17β-hydroxysteroid dehydrogenase [26], androgen receptor [41] and progesterone receptor [42]. Taking this into consideration, spironolactone may affect BR biosynthesis by blocking steps or receptors other than 3β-HSD, explaining why intermediates lying upstream of 3β-HSD were effective in the reversion test. Since spironolactone is known to be a mammalian 17β-hydroxysteroid dehydrogenase inhibitor and this enzyme has not been found in plants, the structural requirements of plant 3β-HSD for substrates and inhibitors are likely different from those of mammalian 3β-HSD. As neither aldosterone nor progesterone showed significant effect on plant (*Arabidopsis thaliana*) morphologies in our preliminary tests, spironolactone may not exert its effect by blocking the action of these steroids.

In the dark, spironolactone-treated *Arabidopsis* developed as light-grown plants and inappropriately expressed light-regulated genes, as do the BR-deficient mutants *det2* and *cpd*. BR biosynthesis inhibitor-treated *Arabidopsis* also expressed light-regulated genes even grown in the dark [11,13,43]. In dark-grown *det1* [44] plant, which is not BR-deficient mutants but show photomorphogenesis in the dark, light-regulated genes such as chlorophyll *a/b* binding-protein are expressed. It is therefore postulated that expression of light-regulated genes can be involved in photomorphogenesis in the dark. Further investigation will reveal the relationship between the expression of light-regulated genes and BR deficiency, and whether specific BR biosynthesis inhibitors can be useful tools for elucidating the expression mechanisms of light-regulated genes.

Considering that dark-grown *bri1* mutant, which has a defect in the function of BL receptor and shows photomorphogenesis in the dark, also expresses light regulated genes [45], the possibility that spironolactone antagonizes BL at BL receptors cannot be excluded. Further investigation on the mechanism of spironolactone at BRI1 receptor will reveal whether spironolactone is multi-functional inhibitor in plants or not.

In this report we have demonstrated that spironolactone induces morphological changes in plants by interfering with BR biosynthesis. At present, BR-deficient mutants are known only in *Arabidopsis*, tomato and pea. This novel BR biosynthesis inhibitor will play an important role in investigating the function of BRs, not only in other plants but also in tissue, organ and biochemical processes. Varying the concentration of BRs in plants by altering the concentration of an inhibitor may make it possible to titrate the minimum concentration of BRs required for plant growth. Moreover, the ability to select a group of new mutants using GA biosynthesis inhibitors [46,47] and BR biosynthesis inhibitor [21,22] suggests that this inhibitor will provide a way to find either a new BR pathway or other novel mutants. In fact, our preliminary test demonstrated that *bzr1* mutant, which is insensitive to BR biosynthesis inhibitors, was also insensitive to spironolactone and that spironolactone-treated *bzr1* mutant grown in the dark elongated to the same level as *bzr1* mutant not treated with spironolactone, suggesting

that spironolactone inhibits the action of BRs and possesses no side effect as long as hypocotyls elongation in the dark (data not shown). The concentration of spironolactone required to change plant morphology is still high, but further modification of the chemical composition may provide new and potent BR biosynthesis inhibitors.

Acknowledgements

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