Involvement of phytohormones in root elongation activity of isoprothiolane in Arabidopsis

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Isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidenemalonate, IPT), an active ingredient of “Fuji-one,” has been used as a plant growth regulating agent to control non-parasitic damping-off (MURENAE disease) of rice seedlings. To understand plant growth regulating activity of IPT, its effect on root development was investigated in Arabidopsis. IPT enhanced root elongation at a lower concentration (12.5 µg/mL) but suppressed it at a higher concentration (75 µg/mL). Analysis using phytohormone-related mutants and chemical inhibitors revealed that the enhancement of root elongation by IPT required auxin, jasmonic acid, and ethylene signal transduction. Activation of the signal transduction mediated by these three phytohormones was confirmed by gene expression analysis. More detailed mechanisms of IPT’s effect on root development were demonstrated via investigation using Arabidopsis and chemical inhibitors. © Pesticide Science Society of Japan

Keywords: isoprothiolane, root elongation, ethylene, jasmonic acid, Arabidopsis.

Introduction

Isoprothiolane (diisopropyl 1,3-dithiolan-2-ylidenemalonate, IPT) has been developed by Nihon Nohyaku Co. and used as an agent, “Fuji-one,” to control rice blast disease (Fig. 1).1,2) IPT also has a plant growth regulating activity and has been used to protect rice seedlings cultured in nursery flats from non-parasitic damping-off, MURENAE disease.3) One of the plausible mechanisms for this activity is the acceleration of rice root development by IPT, which was observed at IPT concentrations as low as 6 µg/mL.4,5) Extensive studies have revealed that IPT promotes the activity of auxin in a lamina inclination assay, induces the ethylene (ET) production in seedlings and callus, and enhances the acid phosphatase activity in root.6,7) The up-regulated methionine sulfoxide reduction leading to ET production8) and the increase in water permeability of the root cell membrane9) have also been reported as effects of IPT; however, the contribution of these physiological changes to plant growth regulation has not been determined. These studies suggested the unique plant growth regulating activity of IPT as a kind of biostimulant and the existence of unidentified physiological mechanisms in the development of seedlings, especially of root system development. To gain a better understanding of IPT’s activity, we investigated its effects on root elongation in relation to phytohormones using Arabidopsis.

Materials and Methods

1. Root elongation assay

Seeds of Arabidopsis wild-type (Col-0) and phytohormone-related mutants were placed on 1/2 Murashige and Skoog (MS) (Duchefa Biochemie) agar plates and incubated in a vertical position under a constitutive light condition with a light intensity of 150 µEinsteins and a temperature of 22°C for 2 days. To test the effect of chemicals, seedlings with a root of ca. 2 mm were transferred to 1/2MS agar plates containing chemicals (13 seedlings in a row) and incubated in vertical position under the same condition. Root elongation was measured at several time points to evaluate the effects of chemicals.

2. Real-time polymerase chain reaction analysis

Plant samples consisting of 3 Arabidopsis wild-type seedlings were collected and immediately frozen in liquid nitrogen. RNA was extracted using the Quick RNA Plant Mini Kit (Zymo Research, CA, USA). After purification of RNA, the concentration of RNA was measured using a NanoDrop (Thermo Scientific, Waltham, MA, USA). The cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The expression level of each gene was measured using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The expression levels were calculated using the comparative CT method (2−ΔΔCt).

Fig. 1. Structure of isoprothiolane (IPT).
lungs were used for total RNA extraction using Sepasol-RNA I Super Reagent (Nacalai Tesque). Total RNA (0.4 µg) was converted into cDNA with a PrimeScript RT Reagent with gDNA Eraser Kit (Takara Bio) according to the manufacturer’s instructions, yielding 10 µL of cDNA solution. Real-time polymerase chain reaction (PCR) was performed using the LightCycler 96 system (Roche) with SYBR Premix Ex Taq (Takara Bio). The PCR reaction was performed with 0.4 µM of each primer, 1 × SYBR Premix Ex Taq, and the appropriate dilution of cDNA in a final volume of 20 µL under the following condition: initial denaturation at 95°C for 30 sec, then 40 cycles of 95°C for 5 sec and 60°C for 20 sec. The expression level of each sample was normalized to ubiquitin (UBQ). The gene-specific primer pairs used are as follows: for ARF19, forward 5'-AAG CTC CTT CTT GTT CAA CCT CAC-3', reverse 5'-GCT TGT CCT TGC TGT TGA TTT CT-3'; for IAA5, forward 5'-TCC GCT CTG CAA ATT CTG TTC-3', reverse 5'-ACA TCT CCA GCA AGC ATC CA-3'; for PDF1.2, forward 5'-TCA TCA TGG CTA AGT TTG CTT CC-3', reverse 5'-ATT GCC GGT GCG TCG AA-3'; for ERF1, forward 5'-GAG CCG ATA CTC AGT GAG TCG A-3', reverse 5'-GCT CTC GGT GAA GCA AGG ATA-3'.

**Results**

1. **Effect of IPT on root growth in Arabidopsis**

In rice, treatment with IPT at a concentration of 6 µg/mL enhanced root elongation; however, the higher concentration (50 µg/mL) reduced root elongation. Biochemical analyses suggested that auxin and ET are involved in this effect, but the necessity of these hormones remained to be clarified. To know the enhancement mechanism of root elongation by IPT, we examined the effect of IPT treatment on the root growth of Arabidopsis. First, we examined the root elongation activity of IPT in wild-type Arabidopsis (Col-0) plants by culturing the plants on agar plates containing various concentrations of IPT. Measurement of the root length indicated that root elongation was enhanced by 12.5 µg/mL IPT but strongly suppressed by 75 µg/mL IPT (Fig. 2). Thus IPT has both positive and negative effects on root growth in Arabidopsis as well as in rice. The effect was not observed at IPT concentration of 37.5 µg/mL, suggesting that the positive and negative effects were independently regulated and neutralized each other at this concentration (Fig. 2).

2. **Roles of growth-related phytohormones in IPT’s effect on root elongation**

To clarify the mechanism of the positive effect of IPT (12.5 µg/mL) on root elongation in Arabidopsis, the involvement of growth-related phytohormones in IPT’s effect was examined. The involvement of gibberellin (GA) in IPT’s effect was examined using paclobutrazol (PBZ), a GA biosynthetic inhibitor. Root elongation was drastically inhibited by 0.1 µM PBZ; however, IPT enhanced root elongation even in the media containing PBZ, similar to those without PBZ (Fig. 3A). This result indicated that GA was not required for IPT’s effect.

The spatial distribution of auxin caused by directional transport plays important roles in root elongation, gravitropism, and lateral development, which are arrested by auxin polar transport inhibitors. The involvement of auxin in the positive effect of IPT on root elongation was examined using N-(1-naphthyl)-

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**Fig. 2.** Effect of IPT on root elongation of Arabidopsis wild-type plants. Seedlings were placed on 1/2MS medium containing various concentrations of IPT as indicated. The averages of root elongation for 5 days are presented. Error bars indicate SD. Asterisks indicate significant difference between non-treated control and IPT-treated plants (unpaired t test, p<0.01). The experiment was repeated three times with similar results.

**Fig. 3.** Involvement of growth-related phytohormones in IPT’s effect on root elongation. Wild-type (A, B) or det2 mutant (C) seedlings were placed on 1/2MS medium or 1/2MS medium supplemented with 12.5 µg/mL IPT or a combination of 12.5 µg/mL IPT and 0.1 µM PBZ (A) or 0.1 µM NPA (B). The averages of root elongation for 5 days are presented. Error bars indicate SD. Different letters indicate statistically significant differences between treatments (ANOVA, p<0.05). Asterisks indicate significant difference between control and IPT-treated plants in each mutant (unpaired t test, p<0.01). The experiment was repeated three times with similar results.
phthalamic acid (NPA), one of the auxin polar transport inhibitors. Root elongation was suppressed in the media containing 0.1 µM NPA; surprisingly, however, the combination of 12.5 µg/mL IPT and NPA enhanced the suppression of root elongation by NPA (Fig. 3B). This result indicated that auxin was at least involved in the effect of IPT on the enhancement of root elongation, though the reason for the synergistic suppressive effect on root elongation was unknown.

The promotion of lamina inclination by auxin suggested the possibility that IPT can activate the brassinosteroid (BR)-mediated signaling pathway. To determine the involvement of BR in IPT's effect, the root elongation was examined using a BR biosynthesis-deficient mutant det2. The root elongation of the det2 mutant was greater in the media containing 12.5 µg/mL IPT than in the control, indicating that BR was not involved in the enhancement of root elongation by IPT (Fig. 3C).

These results indicated that IPT's effect on root elongation requires auxin signaling but not GA or BR, which is reasonable because auxin functions in root elongation by promoting cell division.12)

3. Roles of stress-related phytohormones in IPT's effect on root elongation

Next, to understand the involvement of stress-related phytohormones, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and ET, in the positive effect of IPT on root elongation, we analyzed the root growth of Arabidopsis mutants defective in the biosynthesis or signal transduction of these phytohormones. The enhancement of root elongation by 12.5 µg/mL IPT was observed in the ABA biosynthesis-deficient mutant aao3, indicating that ABA was not involved in IPT's effect (Fig. 4). The enhancement of root elongation by IPT in the SA biosynthesis-deficient mutant sid2 and the NahG transgenic plant expressing an SA-degrading enzyme indicated that SA was not involved in IPT's effect on root elongation (Fig. 4). In contrast, in the jar1 mutant, defective in JA signal transduction due to the lack of conversion of JA to JA-isoleucine conjugate, IPT was not able to enhance root elongation (Fig. 4). Then enhancement of root elongation by IPT was not observed also in ein2 mutant, defective in ET signal transduction (Fig. 4). These results indicated that JA and ET, but not ABA and SA, are involved in IPT's effect on enhancement of root elongation in Arabidopsis.

The involvement of JA and ET in IPT’s effect on root elongation was also examined in wild-type plants using biosynthetic inhibitors for these phytohormones, that is, ibuprofen (IBP) for JA and 2-aminoethoxyvinyl glycine (AVG) for ET. Seedlings treated with IPT in the presence of 10 or 20 µM IBP exhibited lower root elongation than those treated with only IBP, although IBP itself reduced the root elongation in a dose-dependent manner (Fig. 5A). Taken together with the result obtained with the jar1 mutant, it is suggested that JA is required for IPT’s effect on the enhancement of root elongation. Next, the involvement of ET was examined using an ET biosynthetic inhibitor AVG. Although AVG reduced the root elongation in a dose-dependent...
The analyses using Arabidopsis mutants and phytohormone-related inhibitors indicated that auxin, JA and ET are involved in the enhancement of root elongation by 12.5 μg/mL IPT. To determine whether 12.5 μg/mL IPT activates the signal transduction of these phytohormones, we analyzed the expression of phytohormone-responsive genes. In the wild-type plant, treatment with IPT induced the expression of auxin-responsive genes ARF19 and IAA5, the JA-responsive gene PDF1.2, and the ET-responsive gene ERF1 (Fig. 6A, B). These results suggest that treatment with IPT activates the auxin-, JA-, and ET-related signal transductions, which is consistent with the fact that these phytohormones are necessary for the enhancement of root elongation by IPT.

**Discussion**

In this study, we demonstrated that treatment with IPT has positive and negative effects on root elongation in Arabidopsis. Root elongation was enhanced by 12.5 μg/mL IPT, whereas it was reduced by 75 μg/mL IPT, and the intermediate concentration of 37.5 μg/mL had no effect. Similar activities were reported in rice, namely 6 μg/mL for enhancement, 50 μg/mL for reduction, and no effect at the intermediate concentration, suggesting that IPT’s effect with dual activities on root elongation is common to both monocotyledonous and dicotyledonous plants. The intensities of IPT’s effect on root elongation in rice were considerably greater than in Arabidopsis. This may be explained by differences in the sensitivities and crosstalk of phytohormones and the root systems between these plants in addition to experimental conditions; however, this remains to be clarified.

Analyses using mutants and chemical inhibitors revealed that auxin, JA and ET are involved in IPT’s enhancement of root elongation, which was confirmed by gene expression analysis after IPT treatment. Endogenous auxin is known to function in root elongation by activating cell division in the root apical meristem, whereas JA and ET are known to suppress root elongation. However, it was recently reported that JA and ET play positive roles in root by promoting cell division in the quiescent center of the root apical meristem. The root apical meristem, consisting of many types of cells, is intricately regulated by internal and external conditions for cell division in the root growth process; however, the detailed regulation mechanisms remain to be clarified. Although only the involvement of auxin and ET was indicated in rice, the necessity of JA in addition to auxin and ET for IPT’s effect was demonstrated by using Arabidopsis mutants and chemical inhibitors, suggesting that the molecular mechanism is not so simple. Thus, the analysis of crosstalk among auxin, JA, and ET signaling pathways will be important to clarify the mechanisms of IPT’s effect. Further analysis of IPT’s mode of action would be helpful for a better understanding of the physiological mechanism of root growth regulation.

The positive effect of IPT on root elongation is important for an improved crop cultivation system. From practical points of view such as faster growth and avoiding drought stress by efficient use of soil water, further analysis would reveal more about effectiveness of IPT as a biostimulant.
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