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The Effect of Oryzalin on Growth and Gravitropism in Arabidopsis Roots

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Oryzalin is a dinitroaniline herbicide that has been known to disrupt microtubules. Microtubules and microfilaments are components of cytoskeletons that are implicated in plant cell growth, which requires the synthesis of cellulose when cell walls elongate. In addition, microtubules are also involved in the sedimentation of statoliths, which regulate the perception of gravity in the columella cells of root tips. In this study, we investigated the effect of oryzalin on the growth and gravitropic response of Arabidopsis roots. The role of ethylene in oryzalin's effect was also examined using these roots. Treatment of oryzalin at a concentration of 10^{-4} M completely inhibited the roots' growth and gravitropic response. At a concentration of 10^{-6} M oryzalin, root growth was inhibited by 47% at 8 hr when compared to control. Gravitropic response was inhibited by about 38% compared to control in roots treated with 10^{-6} M oryzalin for 4 hr. To understand the role of oryzalin in the regulation of root growth and gravitropic response, we measured ethylene production in root segments treated with oryzalin. It was found that the addition of oryzalin stimulated ethylene production through the activation of ACC oxidase and ACC synthase genes, which are key components in the synthesis of ethylene. From these findings, it can be inferred that oryzalin inhibits the growth and gravitropic response of Arabidopsis roots by stimulating ethylene production. The increased ethylene alters the arrangement of the microtubules, which eventually interferes with the growth of the cell wall.

Key words : Arabidopsis root, ethylene, gravitropic response, growth, oryzalin

Introduction

The pattern of growth of plants is different from that of animals since the way cell divides is different each other. The growth of plant cell is mediated via elongation of cells rather than the increase of cell numbers that animal does. However, unlike animal cells, plants have cell walls, composed of cellulose microfiber, which limits the elongation of cells. When plant cells grow, relaxation of cellulose is a prerequisite and the turgor pressure induced by the intake of water allows the cell wall to be elongated through the addition of newly synthesized cellulose [14]. Microtubules play a key role in plant morphogenesis, which usually determines the sites where cellulose synthase complexes are inserted into the plasma membrane and guides the synthase complexes during cellulose deposition in the cell wall [8].

There are some studies about the role of microtubules to regulate the growth and/or cell shape in plants. In Arabidopsis hypocotyls, the alignment of the microtubule in outer epidermal cell wall regulated growth rate, whereas inner epidermal cell wall controlled growth direction [5]. Microtubule also plays a role in the development of plant such as cell wall formation, and the sedimentation of amyloplast in columella cell of root tip [9]. The sedimentation of amyloplast in columella cell of root is related to the sensing of gravity, resulting in the downward growth into the soil in order to absorb water and minerals [3].

Oryzalin is an herbicide of the dinitroaniline class. It acts through the disruption (depolymerization) of microtubules of plants by binding to α-tubulin, thus blocks anisotropic growth of plant cells. It can also be used to induce polyploidy in plants as an alternative to colchicine [13, 16]. Dinitroanilines, such as oryzalin, are potent inhibitors of plant microtubule formation, while not effective in fungi and animals [7]. It has been known that the orientation of microtubule is changed depending on the level of ethylene in plant cells [18]. Wang et al. reported that ethylene inhibits root elongation via reorientation of microtubule in the cell wall in Arabidopsis [19].

In this study, we found that the treatment of oryzalin interrupted the root growth and gravitropism and increased

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ethylene synthesis. So, it is considered that the increased ethylene induced by oryzalin affected the assembly and re-orientation of microtubules, which consequently inhibited the normal growth and gravitropic response of roots.

Material and Methods

Plant material

The sterilized seeds of *Landsberg erecta* (Ler), *Arabidopsis thaliana* were planted on the agar medium with half-strength of MS salts, 1% sucrose and 1 mM MES (pH 5.8). The seeds were incubated in vertical position at 4℃ for 1 day and then were incubated for another 6 days at 22℃.

Measurement of ethylene production

Ethylene production was measured in 100 root segments (10 mm). The root segments were placed in vials containing 1 ml of MES buffer (100 mM, pH 6.8, 50 μg/ml chloramphenicol) with the test compounds. The vials were shaken in the dark at 27℃ in an incubator. To measure the ethylene production, 1-ml of gas sample was withdrawn from the vial using a syringe and injected to the gas chromatograph (HP5890 Series II; Hewlett-Packard, USA) equipped with an alumina column (80/100 Porapak-Q; 1.8-m x 2.1-mm).

Measurement of root growth and gravitropic curvature

The seedlings were placed in vertical or horizontal position in petri dishes, depending on the experiments. Growth and gravitropic curvature were measured using a camera (Rexsa, DS-400 PC-camera) with the time-interval software (SupervisionCam ver. 3.2.2.4; http://supervisioncam.com). Images were recorded every 15 min and analyzed using UTHSCSA Image Tool Program (ver. 3.0; http://comdent.uthscsa.edu/dig/itdes.html).

Assay of *in vitro* ACC oxidase (ACO) activity

One hundred of root segments were treated with various concentrations of oryzalin for 4 hr, and the sample was frozen in liquid nitrogen. The frozen samples were ground with liquid nitrogen, and added an extraction buffer (100 mM MES, pH 7.5, 10% glycerol, 30 mM ascorbate and 2 mM DTT). The resuspended samples were centrifuged at 15,000 rpm for 10 min at 4℃. The supernatant was transferred to a new vial containing incubation buffer (50 mM MES, pH 7.5, 10% glycerol, 30 mm ascorbate, 2 mM DTT, 30 mM $NaHCO_3$, 50 μM FeSO4, 1 mM ACC) and incubated on a shaker for 1 hr at 22℃ in dark. After incubation, 1-ml of gas sample was withdrawn from the vial using a syringe and injected to the gas chromatograph. The amount of ethylene production is regarded as *in vitro* ACO activity.

Assay of *in vitro* ACC Synthase (ACS) activity

One hundred of root segments were treated with various concentrations of oryzalin for 4 hr, and the sample was frozen in liquid nitrogen. The frozen samples were ground with liquid nitrogen, and combined with 250 mM potassium phosphate buffer (pH 8.0) containing 10 μM pyridoxal phosphate, 1 mM EDTA, 2 mM PMSF and 5 mM DTT. Samples were centrifuged at 15,000 rpm for 15 min at 4℃. The supernatant was incubated with 5 mM AdoMet (0.1 ml) for 1 hr at 22℃. Then ACS activity was measured by adding a mixture of 0.1 ml of 20 mm $HgCl_2$ and 0.1 ml of NaOH/NaOCl (saturated NaOH : 5% NaOCl = 1:1 [v/v]) to the supernatant to produce ethylene, and the reaction was stopped by incubating the solution on ice for 10 min. The ethylene production was measured as described above. The amount of ethylene production is regarded as *in vitro* ACS activity.

Extraction of total RNA and RT-PCR

Tissue samples were ground into a powder with a mortar and pestle under liquid nitrogen. The powder was suspended with an RNA extraction buffer [0.1 M Tris-HCl (pH 9.0), 0.1 M NaCl, and 1% SDS]. A volume of phenol: chloroform: isoamylalcohol (25:24:1; v:v:v) equal to the volume of the buffer was added and the mixture incubated for 10 min at 50℃. The reaction mixture was centrifuged at 13,000 rpm for 15 min at 4℃; the supernatant was transferred into new tubes and incubated with 2.5 M LiCl for 30 min at -20℃. The mixture was centrifuged for 15 min at 13,000 rpm (4℃); the pellet was washed with 70% ethanol and dissolved in DEPC-treated water. Total RNA was quantified at 260/280 using a microplate reader (Microplate Reader Infinite® 200, Tecan Group Ltd, Morrisville, NC, USA).

The purified total RNA was used for first-strand complementary DNA synthesis in a AccuPower® RT Premix (Bioneer, Korea). Polymerase chain reaction (PCR) conditions included 30 cycles of denaturing at 95℃ for 5 min, annealing at 55℃ for 20 sec, and extension at 72℃ for 5 min; and, a final elongation step at 72℃ for 10 min. The gene-specific primers were used as listed in Table 1. *ACO2* and *ACS2*

Table 1. Gene-specific primers used for RT-PCR experiments. *ACO2* and *ACS* are primers for the ACC oxidase and ACC synthase gene from *Arabidopsis thaliana* root, respectively

Gene	Forward (5′ to 3′)	Reverse (5′ to 3′)
ACO2	AGGATGTCGGTTGCATCGTT	CTTCATTGCTGCGAACCGTG
ACS2	TACCACGGGGATCAAGAAAG	AGGAAGAGCCAGGAGACACA
GAPDH	TGAAGGACTGGAGAGGTGGA	GGTTGGGACACGGAAAGACA

are primers for ACC oxidase and ACC synthase gene in Arabidopsis, respectively. The gels were analyzed using Gel Image Analysis System (Core, i-MAX-D500, Korea). The band density was measured and normalized with that of the GAPDH gene expression.

Statistical analysis

All experiments were conducted at least three times, with no fewer than 30 primary roots each. To test for significance at p values of <0.05, the data mean values were calculated according to one-way ANOVA and Tukey test.

Results and Discussion

Oryzalin inhibits the root growth

The root growth was inhibited by the treatment of oryzalin (Fig. 1). The inhibition was started at 2 hr after the addition of 10^{-4} M oryzalin, and inhibited 94% of control at 8 hr. When treated with 10^{-6} M oryzalin, root growth was inhibited 30% at 4 hr and 47% at 8 hr compared to control. However, 10^{-8} M oryzalin did not inhibit the root growth for 8 hr at all. Root growth was more severely inhibited as the concentration of oryzalin increased.

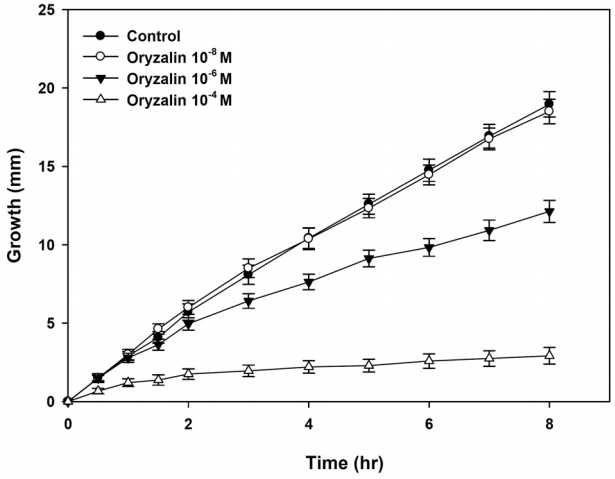

Fig. 1. Effect of oryzalin on root growth in Arabidopsis seedlings for 8 hr. After a vernalization for 1 day, seeds were grown for 6 days under diurnal rhythm of day and night cycle with 16 hr and 8 hr, respectively. These seedlings were transferred the agar plate containing several concentrations of oryzalin, and placed in vertical position. The growth was measured for 8 hr using a camera as described in Material and Methods. Symbols are mean values ± SE from 20 independent experiments.

Baskin et al. [2] reported that cortical microtubules regulate the expansion of root in Arabidopsis. Oryzalin is a microtubule disrupting agent, and plays a role in regulating cell morphogenesis by determining the orientation and location of cellulose microfibrils deposition, which is related to the arrangement of microtubule [10]. Therefore, this result suggested that impaired microtubule assembly caused by oryzalin resulted in root growth inhibition. To examine the possibility of the role of oryzalin in the gravitropic response, we applied the oryzalin to the root which were perceived the gravity.

Oryzalin inhibits the root gravitropism

Application of oryzalin to roots positioned horizontally caused the inhibition of the gravitropic curvature (Fig. 2). No inhibition of gravitropic response was observed at 10^{-8} M oryzalin, however, gravitropic response was inhibited 38% compared to controls treated with 10^{-6} M oryzalin at 4 hr. And then the gravitropic response was disappeared in the course of the treatment of oryzalin at 10^{-4} M for 8 hr.

Gravitropic response in roots induced by differential growth between upper and lower side of roots in horizontal position. Gravitropism takes place in 3 steps such as the perception of gravity in root tip, signal transduction form the root tip to the elongation zone and response of the elongation zone with resultant curvature [6]. Microtubule is involved in the sedimentation of amyloplast in columella cell of root tip [9]. It is suggested that the perception of gravity is associated with the movement of amyloplast in columella cell. The movement of amyloplast might initiate physiological changes in the root tip cells via the interaction between the amyloplast and other cellular component such as endoplasmic reticulum, or cytoskeletal elements [3].

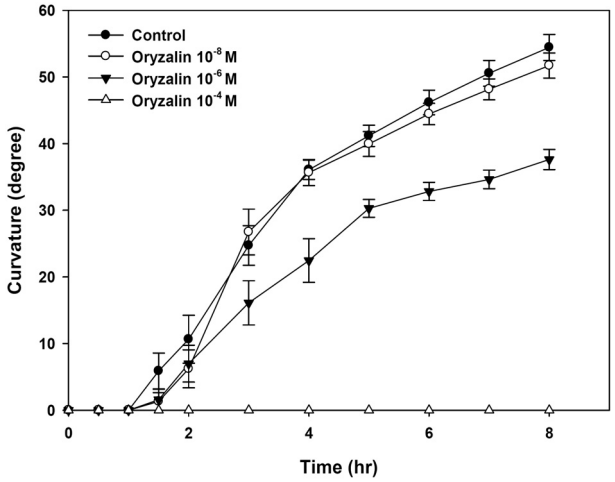

Fig. 2. Effect of oryzalin on root gravitropic response in Arabidopsis seedlings for 8 hr. After a vernalization for 1 day, seeds were grown for 6 days under diurnal rhythm diurnal rhythm of day and night cycle with 16 hr and 8 hr, respectively. These seedlings were transferred the agar plate containing several concentrations of oryzalin, and placed in horizontal position to give a gravity. The gravitropic curvature was measured for 8 hr using a camera as described in Material and Methods. Symbols are mean values ± SE from 20 independent experiments.

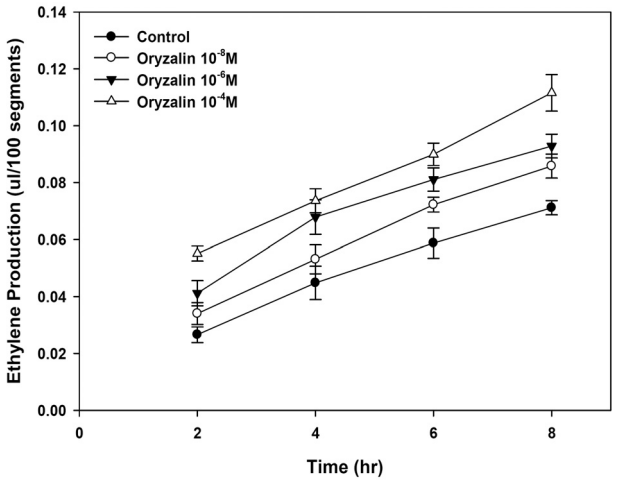

Fig. 3. Effect of oryzalin on the ethylene production in root segment for 8 hr. Root segments (10 mm) were incubated in solution containing 10^{-8} M, 10^{-6} M and 10^{-4} M oryzalin. At every 2 hr, 1 ml of gas sample was withdrawn from the vials for measuring the production of ethylene. Symbols are mean values ± SE from 15 independent experiments.

Therefore, it is highly like that the delayed gravitropic response by the treatment of oryzalin caused by the interruption of amyloplast sedimentation, which was due to the disruption of microtubule alignment in root tip or in root elongation zone.

Oryzalin stimulates the ethylene production in the root

Addition of 10^{-6} M and 10^{-4} M oryzalin resulted in stimulating the ethylene production for 8 hr (Fig. 3). The treatment of 10^{-4} M oryzalin for 8 hr increased 57% compared to the control in ethylene production. Ethylene production from roots treated with 10^{-6} M and 10^{-8} M oryzalin was increased by 37% compared to the control. From these results, we observed that the ethylene production was increased in proportion to the oryzalin concentrations.

Ethylene affects the growth pattern of plant cells by reducing the rate of elongation and increasing lateral expansion, leading to swelling of the tissue and modifying the gravitropic response [4]. Thus, the inhibition of growth and curvature by oryzalin might be due to the disruption of microtubule in the root. Ruzicka et al. [15] reported that ethylene stimulated auxin biosynthesis and increased the capacity of auxin transport by regulating the transcription of auxin transport components such as AUX1 and PIN2. The increase in auxin production by ethylene could interfere with distribution of auxin from root cap to the elongation zone via outer cell layer, and auxin could inhibit growth in the elongation zone. Recent study by Ma and Ren showed that the gravitropic response of the flax root during the germination become weaker due to the decrease in auxin sensitivity and transport, which was caused by increased ethylene production [12]. Therefore, possible increase of ethylene production by oryzalin could affect auxin movement and/or lateral auxin transport in horizontal roots. Ma et al. [11] reported that ethylene regulates root elongation and its gravitropic responses via the alignment of microtubule.

Ethylene synthesis begins from methionine and processed via two major intermediates, S-adenosylmethionine (Ado Met) and 1-aminocycopropane-1-carboxylic acid (ACC), in sequence [1]. The enzyme of ACC synthase (ACS) and ACC oxidase (ACO) regulate the steps from AdoMet to ACC and from ACC to ethylene, respectively. Several factors regulate these two enzymes, especially auxin which stimulates the ethylene production through increasing the expression level of the ACS gene [18]. To confirm the effect of oryzalin on ethylene production, we measured *in vitro* activities of ACC synthase (ACS) and ACC oxidase (ACO).

The ACS activity was increased by 22% and 66% in roots treated with 10^{-6} M and 10^{-4} M oryzalin for 4 hr, respectively

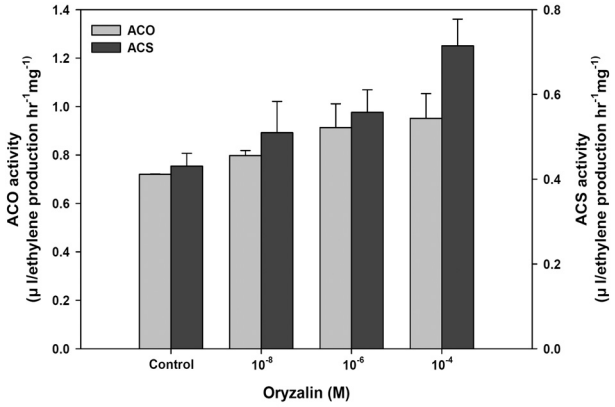

Fig. 4. Effect of oryzalin on *in vitro* ACS and *in vitro* ACO activities. To measure *in vitro* ACS activity, one hundred of roots was incubated for 4 hr in solution containing 10^{-8} M, 10^{-6} M and 10^{-4} M oryzalin. Phosphate buffer (250 mM) was added to ground root segments in liquid nitrogen, and then ethylene production was measured as described in Materials and Methods. To measure *in vitro* ACO activity, one hundred of roots were incubated with oryzalin for 4 hr. These root segments were ground in liquid nitrogen, and added to an extraction buffer. After centrifugation, the supernatant was transferred to a new vial containing incubation buffer. Ethylene production was measured as described in Material and Methods to detect ACO activity. Symbols are mean values ± SE from 10 independent experiments.

(Fig. 4). Further, in roots treated with 10^{-6} M and 10^{-4} M oryzalin, ACO activity increased by 21% and 31%, respectively compared to controls. Therefore, the increased production of ethylene by oryzalin was mediated through the activation of ACS and ACO.

To distinguish whether the increased ACO and ACS activities was due to the increase of transcript levels of the respective genes, RT-PCR was carried out with total RNAs extracted from roots treated with or without oryzalin (Fig. 5). As we expected, the gene expression level of ACO and ACS stimulated as the concentration of oryzalin increased.

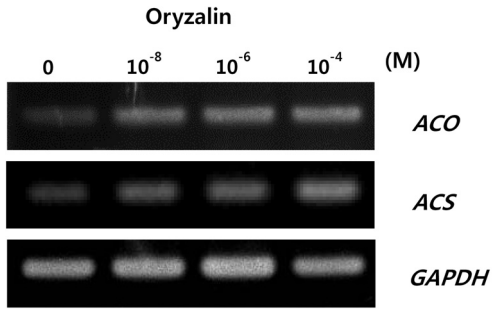

Fig. 5. Effect of oryzalin on gene expression of ACO and ACS. Root segments were incubated for 4 hr in solution containing 10^{-8} M, 10^{-6} M and 10^{-4} M oryzalin. Total RNAs were extracted and used for RT-PCR as described in Material and Methods. Gel image show result with consistent patterns from 3 independent experiments.

In conclusion, the inhibition of growth and reduction of gravitropic response in Arabidopsis root by the treatment of oryzalin was mediated via the stimulation of ethylene biosynthesis, which was attributed to the increased ACO and ACS activities. The results found in this study are well consistent with the previous findings that ethylene changes the arrangement of microtubules, leading to inhibition of root growth and reduction of gravitropic response.

Acknowledgement

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : Oryzalin이 애기장대 뿌리 생장과 굴중성 반응에 미치는 작용

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Oryzalin은 미세소관의 배열을 방해하는 dinitroaniline계 제초제이다. 미세소관과 미세섬유는 식물 세포 생장에 관여하는 세포벽을 구성하는 골격 성분이다. 또한 미세소관은 평형석의 침전에도 관여하는데, 이는 뿌리 끝에 있는 columella 세포에서 중력 인지를 조절한다. 본 연구는 애기장대 뿌리에서 에틸렌 생성을 통하여 oryzalin이 뿌리 생장과 굴중성 반응에 미치는 영향을 조사하였다. 10^{-4} M oryzalin을 뿌리에 처리하면 뿌리 생장과 굴중성 반응이 완전히 억제되었다. 뿌리 생장과 굴중성 반응을 조절하는 oryzalin의 작용을 알아보기 위해 애기장대 뿌리 절편에서 ethylene 생합성을 측정하였다. Oryzalin을 처리하면 ACC oxidase와 ACC synthase을 활성을 촉진하여 에틸렌 생성이 촉진되고, 증가된 ethylene은 미세소관의 배열을 변화시켜 뿌리 생장과 굴중성 반응을 억제한다.