HETEROCYCLES, Vol. 71, No. 3, 2007, pp. 523 - 529. © The Japan Institute of Heterocyclic Chemistry
Received, 7th November, 2006, Accepted, 12th January, 2007, Published online, 15th January, 2007. COM-06-10940

DIRECT INVOLVEMENT OF BENZOXAZINOIDS IN THE GROWTH SUPPRESSION INDUCED BY PHOTOTROPIC STIMULATION IN MAIZE COLEOPTILES

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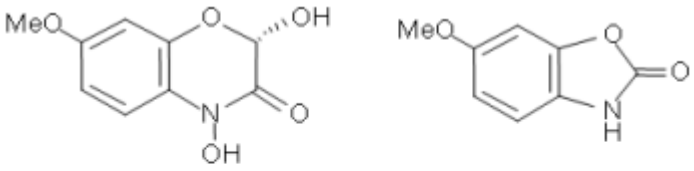
Abstract – The process of growth suppression observed in the illuminated sides in response to phototropic stimulation was investigated in maize coleoptiles. In our previous studies, the increased level of benzoxazinoids, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 6-methoxybenzoxazolinone (MBOA), and the up-regulation of β-glucosidase (DIMBOA-glucosidase) activity have been observed in the illuminated halves. In this report, significant accumulation of hydrogen peroxide (H_2O_2) was observed in the phototropically stimulated sides. Moreover, exogenously applied DIMBOA and MBOA induced a significant up-regulation of H_2O_2 in the applied sides, respectively. In addition, lignin level was also slightly increased in the illuminated sides within 60 min. These results suggest that phototropic stimulation induced the production of benzoxazinoids, resulting to the up-regulation of H_2O_2 in the illuminated halves. Consequently, the up-regulated H_2O_2 promoted the cell-wall stiffness, leading to the growth suppression in the illuminated sides. This is the first indication that blue-light induced benzoxazinoids play the role of directional effecter in the growth suppression in the illuminated sides in maize coleoptiles.

INTRODUCTION

Evidence that phototropism is caused by light induced local accumulation of growth inhibitor(s) in the presence of an unchanged, even distribution of auxin, has been obtained for coleoptiles of maize and oat,

hypocotyls of radish, sunflower and *Arabidopsis* and pea epicotyls.[1-6] As candidates for blue light-induced growth inhibitory substances (defined as phototropism-regulating substances), two benzoxazinoids, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 6-methoxy-2-benzoxazolinone (MBOA) have been isolated and identified from maize coleoptiles (Figure 1).[1,7]



DIMBOA MBOA

Figure 1. Phototropism-regulating substances in maize coleoptiles.

In our previous report, DIMBOA and MBOA levels in the illuminated halves were transiently up-regulated by phototropic stimulation and the activation of DIMBOA glucoside (DIMBOA-Glc) specific β-glucosidase (DIMBOA-glucosidase), which acts as key enzyme for the hydrolysis of inactive DIMBOA-Glc into bioactive aglycone DIMBOA in maize, has also been up-regulated by blue light.[7,8] In addition, pre-treatment of β-glucosidase inhibitor significantly decreased the growth suppression in the illuminated sides induced by phototropic stimulation.[8] Recently, an inhibitory effect of DIMBOA on the coleoptile segments has been reported in oat segments.[9] The effect of DIMBOA on cell-wall PODs would contribute to its growth inhibitory activity by promoting H_2O_2 synthesis and lignification, which could increase the stiffness at the primary cell-wall level. On the other hand, a recent publication showed the induction of H_2O_2 accumulation by blue light and the bending towards the side of H_2O_2 application in wheat coleoptiles, suggesting a close linkage between an oxidative burst and phototropic curvature.[10] However, the directional evidence for the correlation between DIMBOA and the growth suppression induced by phototropic stimulation has remained obscure.

The objective of present study was to elucidate the mechanism of growth suppression by blue light with the action of benzoxazinoids in maize coleoptiles. Our hypothesis is that the up-regulated DIMBOA and its metabolic compound, MBOA, from their glucoside by DIMBOA-glucosidase in response to phototropic stimulation induces the accumulation of H_2O_2 in the illuminated halves, leading to the phototropism.

RESULTS AND DISCUSSION

Previously, the transient increase of DIMBOA and MBOA levels in the illuminated halves in response to phototropic stimulation have been reported.[8] In addition, β-glucosidase inhibitor, (D-gluconic acid δ-lactone, GL) treatment significantly decreased growth suppression in the illuminated sides. It has recently been reported that H_2O_2 accumulation occurs in response to phototropic stimulation within a few minutes.[10]

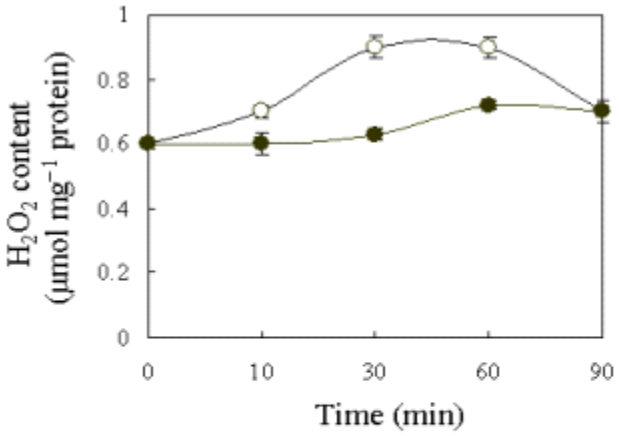


Figure 2. Time course of H_2O_2 levels in response to phototropic stimulation. The endogenous level of H_2O_2 in the illuminated (○) and shaded (●) halves was determined at the indicated time periods. The data represents the mean of three independent experiments. Error bars indicate ± SE (n = 6).

Therefore, we have investigated the kinetics of H_2O_2 content in the illuminated and shaded halves upon phototropic stimulation. Continuous unilateral blue light illumination elevated the endogenous H_2O_2 level in the illuminated halves at least 10 min after the onset of illumination, and peaked between 30 and 60 min (Figure 2). In contrast, the peaking of the DIMBOA and MBOA accumulation due to phototropic stimulation occurred 30 min later.[8] The kinetics of H_2O_2 accumulation in the illuminated halves seemed to correlate with those of DIMBOA and MBOA levels, suggesting the early oxidative burst in response to blue light stimulation could be the trigger for DIMBOA and MBOA production. The tissue printing assay for H_2O_2 localization also strongly confirmed the accumulation of H_2O_2 in the illuminated halves upon phototropic stimulation, supporting the results described above (data not shown).

Although the inhibitory activity of DIMBOA on the growth of oat segments through the generation of H_2O_2 has been reported,[9] it is not clear whether blue light can also induce H_2O_2 accumulation through the up-regulation of benzoxazinoids. To clarify our working hypothesis that the H_2O_2 accumulation generated within 30 min might be due to the increased level of benzoxazinoids caused by blue light, we measured the endogenous level of H_2O_2 with DIMBOA and MBOA application in maize coleoptiles at several time periods. As expected, DIMBOA induced a significant up-regulation of H_2O_2 within 10 min after the onset of application in the applied coleoptiles (Figure 3A). Moreover, its level was up-regulated in the applied halves compared with the opposite ones (Figure 3B). In addition, MBOA also caused significant up-regulation of H_2O_2 in the applied sides, after 30

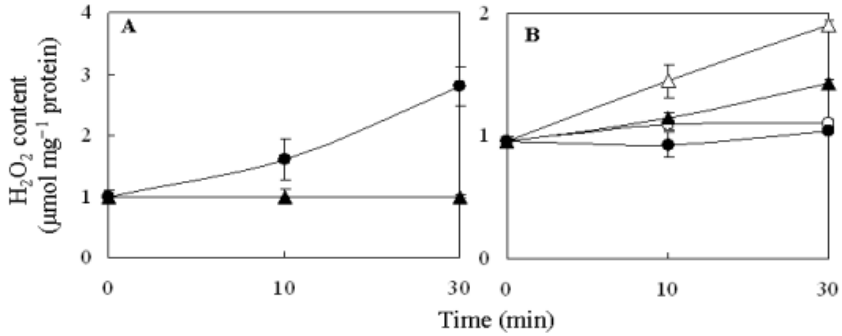


Figure 3. The effect of DIMBOA application on the endogenous level of H_2O_2 in the dark. DIMBOA (94 μM) was applied unilaterally from 0.5 to 2.5 cm below the tip of seedlings. (A) Endogenous level of H_2O_2 with DIMBOA (●) and without DIMBOA (▲); (B) H_2O_2 level in the applied halves with DIMBOA (△) and without DIMBOA (○, lanolin alone); opposite halves with DIMBOA (▲) and without DIMBOA (●). The data represents the mean of three separate experiments. Error bars indicate ± SE (n = 6).

min of MBOA application (Figure 4). The coleoptiles with unilateral DIMBOA application exhibited a little larger bending towards the applied sides compared with MBOA application, after 90 min which was in good agreement with the former report.[7] The present result suggests the involvement of both DIMBOA and MBOA in the growth suppression through H_2O_2 up-regulation.

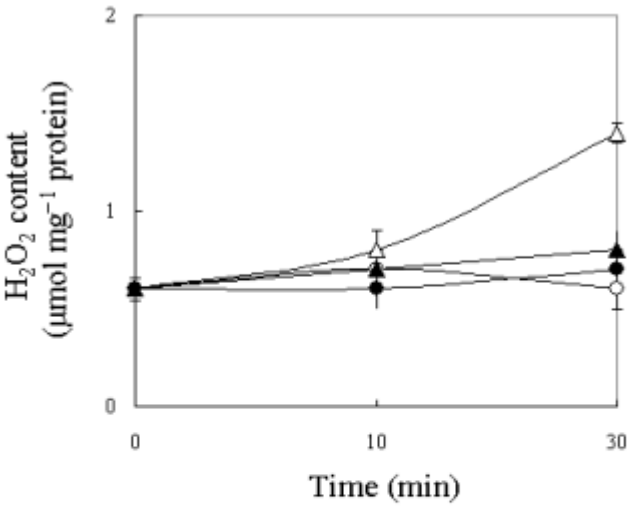


Figure 4. The effect of MBOA application on the endogenous level of H_2O_2 in the dark. MBOA (94 μM) was applied unilaterally from 0.5 to 2.5 cm below the tip of seedlings. Symbols used in the graph are same as in Figure 3B. The data represents the mean of three separate experiments. Error bars indicate ± SE (n = 6).

In the literature, light mediated inhibition of the shoot growth through H_2O_2 up-regulation, followed by the lignification.[11,12] It is probably that the growth suppression in the illuminated side due to phototropic stimulation observed here will be accompanied with cell-wall stiffness. To investigate the possible mechanism of cell-wall stiffness in the blue-light stimulated coleoptiles, the endogenous level of lignin was determined. As shown in Table 1, the lignin content was elevated in the illuminated halves compared with the shaded one, 60 min after the onset of blue light illumination. This result indicates the involvement of lignification in the cell-wall stiffness in the illuminated sides. However, the clarification of the involvement of oxidative burst that leads to the cross-linking of glycoproteins and the microtubule (MT) reorientation in the cell-wall stiffness during phototropic curvature needs further investigation.[13,14]

Finally, together with the results obtained here, it is concluded that phototropic stimulation can up-regulate the level of benzoxazinoids in the illuminated halves. The increased level of DIMBOA and MBOA may enhance the accumulation of H_2O_2, resulting the growth suppression in the illuminated sides due to cell-wall stiffness (Figure 5). This differential growth caused by the suppression in the illuminated side may lead to the phototropic curvature. This is the first indication that blue-light induced benzoxazinoids play the role of directional effecter in the growth suppression in maize coleoptiles.

Table 1. Effect of phototropic stimulation on the lignin accumulation. Lignin content (A_{280} g^{-1} FW) was determined 60 min after the onset of phototropic stimulation. The data represents the mean of three independent experiments ± SE (n = 6).

Initial (0 min)	Dark (60 min)	Illuminated halves	Shaded halves
4.7 ± 0.1 (100%)	4.5 ± 0.2 (95.7%)	5.2 ± 0.3 (110.6%)	4.3 ± 0.1 (91.5%)

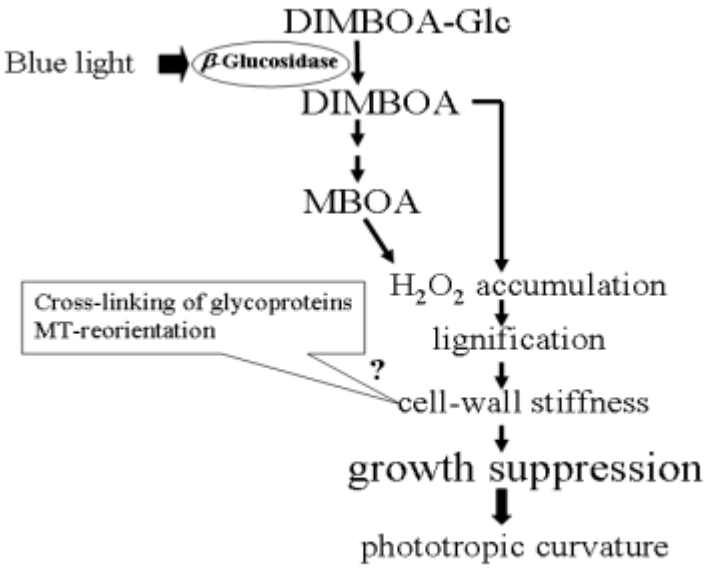


Figure 5. Phototropism in maize coleoptiles (Hypothetical model).

EXPERIMENTAL

Plant materials and growth conditions

Maize (*Zea mays* L. cv. Canadian Rocky 85, Kaneko seed Co, Japan) seeds were soaked for one day in the dark under running tap water, and were sown on moist vermiculite in a tray, under red light (0.3 μmol m^{-2} s^{-1}, λ_{max} 655 nm) at 25°C for one day. The germinated seeds were then incubated in the dark at 25°C for one more day. Uniform etiolated seedlings (coleoptiles: 1.5-2.0 cm long) were transplanted into a row in the seedling case (13.5×6.5×3.5 cm) containing moist vermiculite and were grown for a further 12 h in the dark at 25°C (each case with 10 seedlings). All the manipulation was carried out under a photomorphogenetically inactive intensity of dim green light (0.01 μmol m^{-2} s^{-1}).

Light treatments and experimental procedures

Phototropic stimulation with unilateral blue light (0.05 μmol m^{-2} s^{-1}, λ_{max} 445 nm) was done over the whole length of four-day-old maize seedlings. Six tips of 1.5-2.0 cm length were excised and bisected into the illuminated and shaded halves with a razor blade under dim green light at the indicated periods. All the coleoptiles samples were immediately frozen in liquid nitrogen until use.

Measurement of H_2O_2 content

The level of H_2O_2 in the whole and incised coleoptiles was measured following the method by Brennan and Frenkel with some modifications.[15] Frozen materials were finely ground in a mortar and homogenized in 2

mL of 100% cold acetone using a homogenizer. The homogenates were centrifuged at 3,000 rpm for 10 min at 4°C. One mL of the supernatant was then collected and mixed with 0.1 mL of 5% $TiSO_4$ in 98% H_2SO_4 and 0.2 mL of NH_4OH. After centrifugation at 3,000 rpm for 10 min at 4°C, the orange yellow-colored deposit was collected and rinsed with 100% cold acetone for 3-5 times to reduce the interference of plant pigments. After the addition of 1.0 mL of 2 M H_2SO_4 to dissolve the deposit, the absorbance was measured at 415 nm. Absorbance values were calibrated to standard curves generated with known concentrations of H_2O_2, and the H_2O_2 content was shown as μmol H_2O_2 mg^{-1} protein. The protein content was measured following the method of Bradford with BSA as a standard.[16]

Standard sample of DIMBOA was isolated from seven-day-old maize seedlings (5-6 cm long) as previously described.[17] MBOA was purchased from Wako Pure Chemical Industries, Ltd., Japan. To investigate the effect of benzoxazinoids on the endogenous level of H_2O_2, 94 μM of DIMBOA and MBOA with lanolin were applied unilaterally from 0.5 to 2.5 cm below the tip of seedlings just before the onset of the experiment, respectively.

Measurement of lignin content

Lignin analysis was carried out according to the method of Iiyama and Wallis.[18] The frozen coleoptile samples were pulverized in 95% EtOH with the mortar and pestle, and the homogenates were centrifuged at 1,000 rpm for 10 min at room temperature. The pellets were washed with 95% EtOH and 95% EtOH : *n*-hexane (1:2, v/v) solutions for three times, respectively. After drying by heating at 50°C for 5 min, the deposits were dissolved in 0.5 mL of 25% acetyl bromide in acetic acid for 30 min at 70°C. After a rapid cooling on ice, 0.9 mL of 2 M NaOH was added to stop the reaction, and then 0.1 mL of 7.5 M hydroxylamine-HCl and 1.0 mL of acetic acid were added and then was diluted with 3.0 mL acetic acid. After centrifugation at 1,000 rpm for 5 min, the supernatants were measured at 280 nm. Lignin content was expressed in $A_{280}\,g^{-1}$ FW.

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