# ISOLATION AND IDENTIFICATION OF PHOTOTROPISM-REGULATING SUBSTANCES BENZOXAZINOIDS FROM MAIZE COLEOPTILES

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**Abstract** – As candidate(s) for phototropism-regulating substance(s), three substances were isolated from blue light-illuminated maize coleoptiles and identified as 2-*O*-ß-D-glucopyranosyl-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA glucoside), 2,4-dihydroxy-7-methoxy-(2*H*)-l,4-benzoxazin-3(4*H*)-one (DIMBOA) and 6-methoxy-2-benzoxazolinone (MBOA) from their $^1$H NMR spectra. Phototropic stimulation rapidly decreased the inactive DIMBOA glucoside, and abruptly increased the most active DIMBOA and then the active MBOA in the illuminated halves of maize coleoptiles. These results suggest that DIMBOA plays the essential role in the phototropism of maize coleoptiles.

The phenomenon of plant seedlings bending toward light to optimize the exposure of their photosynthetic organs (e.g., a leaf blade is oriented perpendicularly to the direction of light) is called phototropism, and is a typical example of an environmental response found in plants. 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 hypocotyls of radish, sunflower and *Arabidopsis thaliana*, oat coleoptiles, and pea epicotyls.[1-5] As candidates for growth inhibitory substances involved in phototropism,

4-methylthio-3-butenyl isothiocyanate and raphanusanins from radish hypocotyls,[1] uridine from oat coleoptiles,[2] 8-epixanthatin from sunflower hypocotyls [3] and indole-3-acetonitrile from *Arabidopsis thaliana* [4] have been isolated and identified. We therefore concluded that different growth inhibitors are involved in plant response to phototropic stimulation. In maize coleoptiles commonly used for phototropic studies as well as above plant species, Togo and Hasegawa reported that growth inhibitor(s) in the neutral fraction plays an important role in the phototropic curvature of maize coleoptiles.[6] However, the true character of these substances is still unknown. In this study, we report the structures and a possible formation of phototropism-regulating substance(s) from maize coleoptiles.

Five-day-old etiolated maize (*Zea mays* L. cv. Canadian Rocky 85, Kaneko Seed Co., Gunma, Japan) coleoptiles were cultured under blue light ($\lambda_{max}$ 448 nm, half band width 43 nm) or in the dark at 25°C for 60 min. The blue light was obtained from a blue fluorescent lamp (National High Light S, National Corp., Japan) through a blue acrylic plate (Kyowalite PG, Kyowa Gas Chemical Industry Corp., Japan). Incident energy was 0.3 $\mu$mol m$^{-2}$ s$^{-1}$ at the plant level. Tips of 2 cm length were excised with a razor blade under dim green light (0.03 $\mu$mol m$^{-2}$ s$^{-1}$). Excised coleoptiles each were immediately frozen in liquid $N_2$ and stored at −40°C until use. The frozen samples of 10 g FW were homogenized in 50 vol. of 80% acetone using homogenizer. The filtered extracts were evaporated to dryness at 40°C *in vacuo*. The samples were each dissolved in methanol and applied to a HPLC (Handy-ODS, Wako, Japan, 4.6 × 250 mm, 0-5 min; 5% $CH_3CN$ in $H_2O$, 5-25 min; linear gradient from 5% to 100% $CH_3CN$, 0.8 mL/min, detector at 205 nm). In the HPLC chromatograms, differences in at least three major peaks were detected between the blue light-illuminated coleoptiles and the dark control: two peaks (B and C) increased in the coleoptiles with blue illumination, whereas a peak (A) decreased.

For the purpose of identifying the chemical substances in these peaks, a large amount of 5-day-old etiolated maize coleoptiles were exposed to blue light (0.3 $\mu$mol m$^{-2}$ s$^{-1}$) for 60 min. The blue light-illuminated maize coleoptiles (70 gFW) were homogenized in cold acetone. The filtered extract was evaporated to dryness *in vacuo* at 40°C to give 957 mg of crude material. The crude material was applied to a silica Sep-pak cartridge column (Waters), and eluted with 30%, 60% and 100% ethyl acetate in *n*-hexane, and finally 100% methanol (40 mL per step). The peaks A, B and C were detected in the 100% methanol eluate. The methanol fraction (681 mg) was further chromatographed on a $C_{18}$ Sep-pak cartridge column (Waters) eluted with 30%, 60% and 100% methanol in $H_2O$ (40 mL per step). The 60% methanol fraction (223 mg), which contained the peaks A, B and C, was finally purified by HPLC (ODS-120A, Tosoh, Japan, 7.8 × 300 mm, 0-5 min; 5% $CH_3CN$ in $H_2O$, 5-25 min; linear gradient from 5% to 100% $CH_3CN$, 2 mL/min, detector at 205 nm). The retention time of peak A was 15.5-15.9 min, that of peak B 18.2-18.8 min, and that of peak C 20.4-20.8 min. Each eluate was evaporated to dryness *in vacuo* at 40°C to give 2.8 mg, 2.4 mg and 1.2 mg, respectively.

These three substances were identified by their ^{1}H NMR spectra (Brucker AVANCE 500 spectrometer, in CD$_3$OD) and Platform LC (Waters), respectively. Peak A was elucidated to be 2-*O*-ß-D-glucopyranosyl-4-hydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one (DIMBOA glucoside) by the ^{1}H NMR spectrum as follows: three aromatic protons at δ 7.36 (1H, d, *J*= 8.8 Hz), 6.78 (1H, d, *J*=2.7 Hz), 6.73 (1H, dd, *J*=8.8 and 2.7 Hz), an acetal proton at δ 5.93 (1H, s), a methoxy group at δ 3.83 (3H, s), an anomeric proton at δ 4.71 (1H, d, *J*= 7.9 Hz), and oxymethine and oxymethylene protons at δ 3.2-3.8. Peak B was
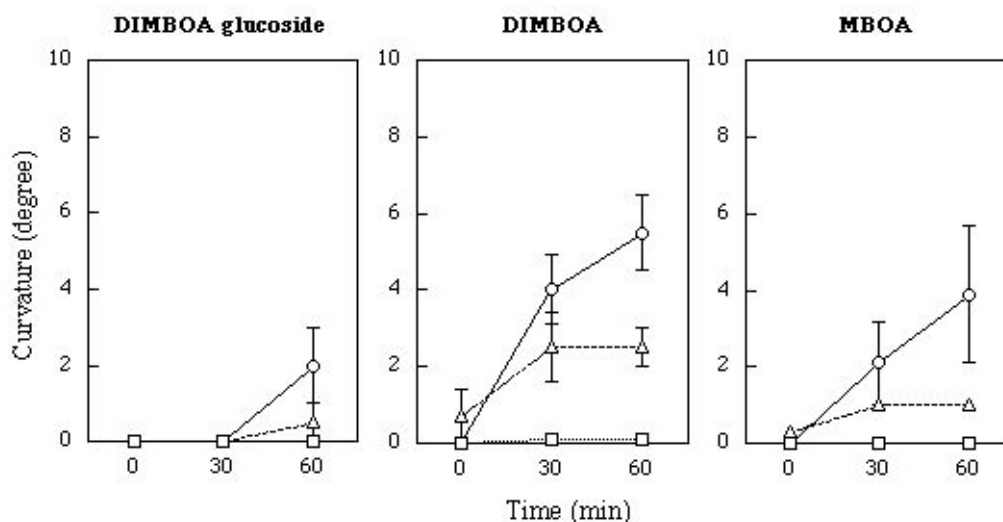


**Figure 1**. Time course of the curvature in etiolated maize coleoptiles after unilateral application of DIMBOA glucoside, DIMBOA and MBOA. O, 50 nM; △, 17 nM; □, 0 nM. Each value is the mean of 10 seedlings ± SE. These experiments were repeated three times and the results were similar.
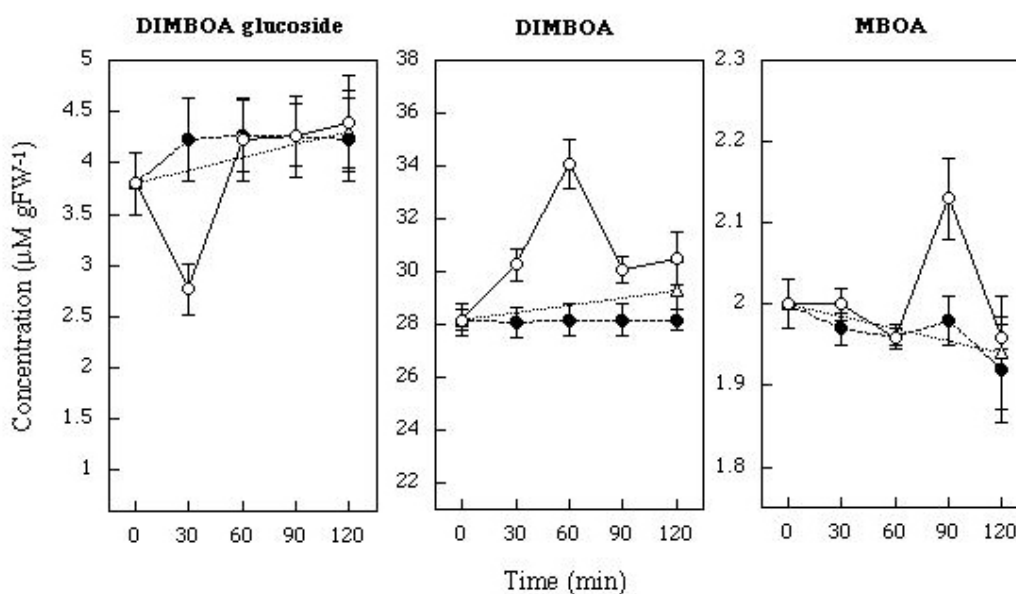


**Figure 2**. Lateral distribution of DIMBOA glucoside, DIMBOA and MBOA in etiolated maize coleoptiles in darkness and in the illuminated and shaded halves of maize coleoptiles until 120 min after the onset of 10 s unilateral blue illumination. O, Illuminated halves; ●, shaded halves; △, halves of dark control coleoptiles. Each point is the average of four independent experiment ± SE.

elucidated to be 2,4-dihydroxy-7-methoxy-(2*H*)-l,4-benzoxazin-3(4*H*)-one (DIMBOA) by proton signals at δ 7.30 (1H, d, *J*= 8.9 Hz), 6.73 (1H, dd, *J*= 8.9 and 2.6 Hz), 6.67 (1H, d, *J*= 2.6 Hz), 5.71 (1H, s), and 3.81 (3H, s). Peak C was determined to be 6-methoxy-2-benzoxazolinone (MBOA) by proton resonances at δ 7.01 (1H, d, *J*=8.6 Hz), 6.94 (1H, d, *J*=2.4 Hz), 6.79 (1H, dd, *J*= 8.6 and 2.4 Hz), and 3.83 (3H, s). DIMBOA and MBOA were already isolated and identified from maize as well as from rye and wheat as antifungally active substances.[7, 8] DIMBOA glucoside, of which the antifungal activity was negligible, was also isolated and identified as a precursor of DIMBOA from maize and wheat.[8] The spectrum of ^{1}H NMR of each compound isolated in this study was in full agreement with that in the paper scited above. Moreover, MBOA and DIMBOA were already shown to possess not only antifungal but also plant growth-inhibitory activity.[9-12] However, their role in phototropism of maize coleoptiles was hitherto not clear. Whether or not unilateral application of DIMBOA glucoside, DIMBOA and MBOA on the etiolated maize coleoptiles, resulting in unequal distribution, causes bending towards the site of application, was determined. As shown in Figure 1, the substance with the highest activity appeared to be DIMBOA, followed by MBOA. DIMBOA and MBOA in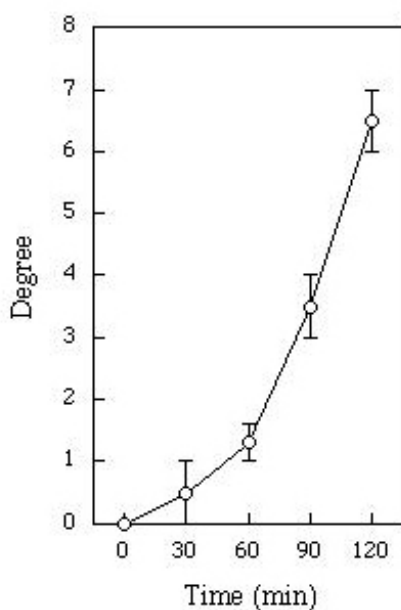duced maize coleoptiles to bend within 30 min after application. The growth inhibitory activity of DIMBOA glucoside was low compared to the other two substances.



**Figure 3**. Time course of the phototropic response of etiolated maize coleoptiles upon 10 s unilateral blue illumination. Each value is the mean of 10 seedlings ± SE. These experiments were repeated three times and the results were similar.

The lateral distribution of the endogenous DIMBOA glucoside, DIMBOA and MBOA was determined in the dark-grown coleoptiles and in the illuminated and shaded halves of etiolated coleoptiles during phototropic curvature. Figure 2 shows that the DIMBOA glucoside level in the illuminated halves fell for at least 30 min after phototropic stimulation and then returned to about its initial level. DIMBOA was increased in the illuminated halves 30 min after the phototropic stimulation started, reached a peak at 60 min, and then decreased. MBOA showed little changes within 60 min after the onset of phototropic stimulation, but the content in the illuminated halves increased rapidly after 60 to 90 min had elapsed, and then decreased again. The level of DIMBOA glucoside, DIMBOA and MBOA in the shaded halves were similar to those in the dark controls. Of these endogenous inhibitors, DIMBOA increased in the

illuminated side together or even prior to the bending response (Figure 3). This increase was sufficient to account for the curvature observed. It can therefore be concluded that a light-induced lateral DIMBOA-gradient in the tissue induces phototropic response in maize seedlings. The increase in the MBOA level was delayed. Possibly, this less active substance is rather the result than a cause of phototropic stimulation since it may well be a product of DIMBOA metabolism. Although enzymatic activities were not yet studied in the present work, these results suggest that phototropic stimulation induces conversion of the inactive DIMBOA glucoside into the active DIMBOA, which plays the essential role in the phototropism of maize coleoptiles, and then metabolizes into MBOA (Figure 4).
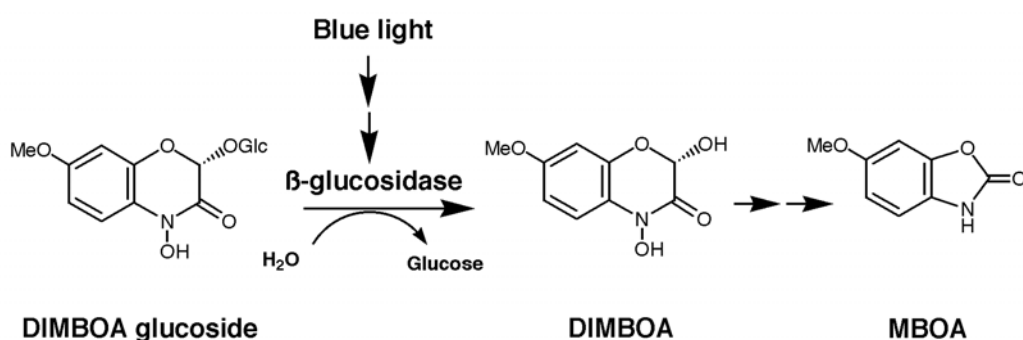
Figure 4. Metabolism of maize phototropism-regulating substances.

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