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Induction of HDMBOA-Glc accumulation and DIMBOA-Glc 4-*O*-methyltransferase by jasmonic acid in poaceous plants

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Abstract

Induction of the accumulation of 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc) by jasmonic acid (JA) was investigated in wheat, Job's tears (*Coix lacryma-jobi*), and rye. An increase in HDMBOA-Glc and a corresponding decrease in 2-(2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (DIMBOA-Glc) were found in wheat and Job's tears, whereas no such changes were observed in rye. The activity of *S*-adenosyl-L-methionine:DIMBOA-Glc 4-*O*-methyltransferase which catalyzes the conversion of DIMBOA-Glc to HDMBOA-Glc was detected in wheat leaves treated with 50 μM JA. The activity started to increase 3 h after treatment with JA, reached a maximum after 9 h, and then decreased gradually. This mode of induction was well correlated with that for the accumulation of HDMBOA-Glc, indicating the induction of enzyme activity was responsible for the accumulation of HDMBOA-Glc. The enzyme was purified from JA-treated wheat leaves by three steps of chromatography, resulting in 95-fold purification. The enzyme showed strict substrate specificity for DIMBOA-Glc with a K_m value of 0.12 mM. DIBOA-Glc was also accepted as substrate, but the K_m value was 10 times larger than that for DIMBOA-Glc. The aglycones, DIMBOA and DIBOA, were not methylated by the enzyme. The K_m value for *S*-adenosyl-L-methionine was 0.06 mM. The optimum pH and temperature were 7.5 and 35 °C, respectively. The activity was slightly enhanced by the presence of 1 mM EDTA, while heavy metal ions at 5 mM completely inhibited the activity.

Keywords: *Triticum aestivum*; Poaceae; Wheat; Benzoxazinone; Jasmonic acid; Methyltransferase; Induction

1. Introduction

The benzoxazinones (Bxs), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, **1**) and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA, **2**), are the major secondary metabolites found in wheat, maize, Job's tears, and rye. They are constitutively present as glucosides **3**–**4**, and are implicated in defense responses of plants to pathogens and insects (Niemeyer, 1988). The disintegration of tissue due to infection or insect attack results in contact between glucosides and β-glucosidase stored in different cellular compartments, leading to the release of the corresponding aglycones that have antimicrobial and antifeeding activities. Bxs have also been implicted in inducible defense reactions. Changes in the total Bx content or DIMBOA **1** level following infection by pathogenic fungi, insect feeding and mechanical damage have been investigated in maize and wheat (Gutierrez et al., 1988; Niemeyer et al., 1989; Morse et al., 1991; Weibull and Niemeyer, 1995).

The content of a minor component of Bxs, 2-(2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HDMBOA-Glc, **5**), has been found to increase in wheat infected with the stem rust fungus (Bücker and Grambow, 1990). The increase in **5** was observed only in the moderately resistant interaction between wheat cultivars and rust races. Recently, we found that the accumulation of **5** is induced by treatment with chitooligosaccharides and $CuCl_2$ in maize leaves (Oikawa et al., 2001). The accumulation of **5** was also evoked by treatment with jasmonic acid (JA), suggesting involvement of JA in the signal transduction from elicitors to the biosynthesis of **5**. The increase of **5**

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was accompanied by a decrease of **3**, and the deuterium atoms of [$^2H_3$-Me]L-methionine were efficiently incorporated into **5**, indicating the conversion of **3** into **5**.

Here, we investigated the induction of accumulation of **5** by treatment with JA in Bx-accumulating poaceous plants including wheat, rye, and Job's tears to elucidate the distribution of this reaction. In addition, we identified the *S*-adenosyl-L-methionine: DIMBOA-Glc 4-*O*-methyltransferase activity, which is responsible for the conversion of **3** to **5**, in JA-treated wheat leaves. This report represents the first description of the identification and characterization of an *O*-methyltransferase that accepts hydroxamic acids as substrates.

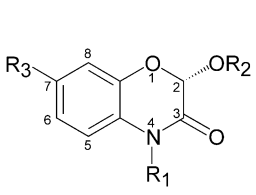
2. Results

2.1. Induction of accumulation of 5 by treatment with JA in poaceous plants

The possibility that plants respond to JA treatment by accumulating newly synthesized Bx derivatives was examined in representative Bx-accumulating poaceous plants including wheat, rye, Job's tears, and maize. The chemical structures of the Bxs **1**–**10** investigated are shown in Fig. 1. The leaf segments of these plants were floated on a solution of 50 μM JA for 48 h, and the Bx content was analyzed by HPLC. An accumulation of **5** and decrease in the amount of **3** were found in wheat and Job's tears as well as in maize (Oikawa et al., 2001). The extent of the accumulation of **5** and accompanying decrease in **3** were almost identical in each of these species, indicating the conversion of **3** to **5**. The amount of 2-(2-hydroxy-7-methoxy-1,4-benzoxazin-3-one)-β-D-glucopyranose (HMBOA-Glc, **9**) did not change significantly (Table 1). The accumulation of **5** was not detected in rye. In rye leaves, which contained little **3** but a large amount of **4**, neither **5** nor the *O*-methylation product of **4**, 4-*O*-Me-DIBOA-Glc (**6**), was detected in the JA- or water-treated leaves.

2.2. Induction of accumulation of 5 in wheat leaves

The induction of accumulation of **5** was investigated in detail in wheat leaves. Fig. 2A shows the change in the amount of **5** following treatment with 50 μM JA. The level of **5** started to increase 6–12 h after treatment, and reached a maximum after 24 h (2.4 μmol g^{-1} fr. wt). It then remained constant. In control leaves, a small increase in **5** (0.5 μmol g^{-1} fr. wt) was found, although the maximal amount was one fifth of that in JA-treated leaves. The effect of JA concentration was examined using leaves treated for 24 h (Fig. 2B). An increase in the amount of **5** was detected even in the leaves treated with 0.5 μM JA, and reached a level of saturation at 10 μM JA.



		R_1	R_2	R_3
1	DIMBOA	OH	H	OMe
2	DIBOA	OH	H	H
3	DIMBOA-Glc	OH	Glc	OMe
4	DIBOA-Glc	OH	Glc	H
5	HDMBOA-Glc	OMe	Glc	OMe
6	4-*O*-Me-DIBOA-Glc	OMe	Glc	H
7	HMBOA	H	H	OMe
8	HBOA	H	H	H
9	HMBOA-Glc	H	Glc	OMe
10	HBOA-Glc	H	Glc	H

Fig. 1. Structures of benzoxazinones and their glucosides.

The effects of various compounds on the induction of **5** were studied next (Fig. 3). Leaf segments from 7-day-old wheat seedlings were floated on solutions of $CuCl_2$, penta-*N*-acetylchitopentaose [$(GlcNAc)_5$], chitopentaose [$(GlcN)_5$], and JA. After 48-h incubation, the Bx contents were analyzed. In leaves treated with 0.5 mM $CuCl_2$, the amount of **5** (1.4 μmol g^{-1} fr. wt) increased to 690% and 210% of that in intact leaves and water-treated leaves, respectively, whereas the amount of **3** decreased to 30 and 40%. The amount of **5** accumulated in 0.75 mM $(GlcNAc)_5$-treated and $(GlcN)_5$-treated leaves was not significantly different from that in water treated leaves.

In the treatment experiments, **5** increased 2–3-fold relative to intact leaves.

2.3. Induction of activity of DIMBOA-Glc 4-O-methyltransferase in JA-treated leaves

In view of the possibility that a specific methyltransferase is involved in the JA-induced conversion of **3** to **5**, the detection of enzyme activity was attempted. Wheat and maize leaves treated with 50 μM JA were extracted with 50 mM Tris–HCl buffer, and the enzyme activity was examined using **3** and *S*-adenosyl-L-methionine (SAM) as substrates. When the crude extract from maize leaves treated with JA was added to the reaction mixture, no formation of **5** was detected even after 3-h incubation at 35 °C. On the other hand, in the reaction mixture that contained the extract from JA-treated wheat leaves, the formation of **5** was detected after 15-min incubation. The reaction proceeded linearly up to 1.5 h, and thereafter the rate of formation decreased gradually. **5** was not formed when boiled extract or extraction buffer was added to the reaction mixture. Addition of an inhibitor of β-glucosidases, castanospermine (Saul et al., 1983) at 0.2 mM was effective in preventing the degradation of the substrate and product by endogenous β-glucosidase activity. Based on these findings, we established a standard assay method for DIMBOA-Glc 4-*O*-methyltransferase, and investigated the mode of induction of the enzyme activity in wheat leaves.

Fig. 4A shows the effect of duration of JA treatment on the induction of enzyme activity in wheat leaves

Table 1
Changes in benzoxazinone glucoside content after treatment with 50 μM jasmonic acid (JA) for 48 h in poaceous plants (μmol g^{-1} fr. wt)

		DIMBOA-Glc (**3**)	HDMBOA-Glc (**5**)	HMBOA-Glc (**9**)	DIBOA-Glc (**4**)	4-*O*-Me-DIBOA-Glc (**6**)	HBOA-Glc (**10**)
Wheat	Water	2.76	0.63	0.47	n.d.[a]	n.d.	n.d.
	JA	0.05	3.51	0.30	n.d.	n.d.	n.d.
Rye	Water	n.d.	n.d.	n.d.	3.19	n.d.	0.22
	JA	n.d.	n.d.	n.d.	3.50	n.d.	0.28
Job's tears	Water	7.93	2.55	0.74	n.d.	n.d.	n.d.
	JA	6.62	3.97	0.93	n.d.	n.d.	n.d.
Maize	Water	6.44	0.67	0.69	n.d.	n.d.	n.d.
	JA	3.66	4.65	0.50	n.d.	n.d.	n.d.

[a] Not detected.

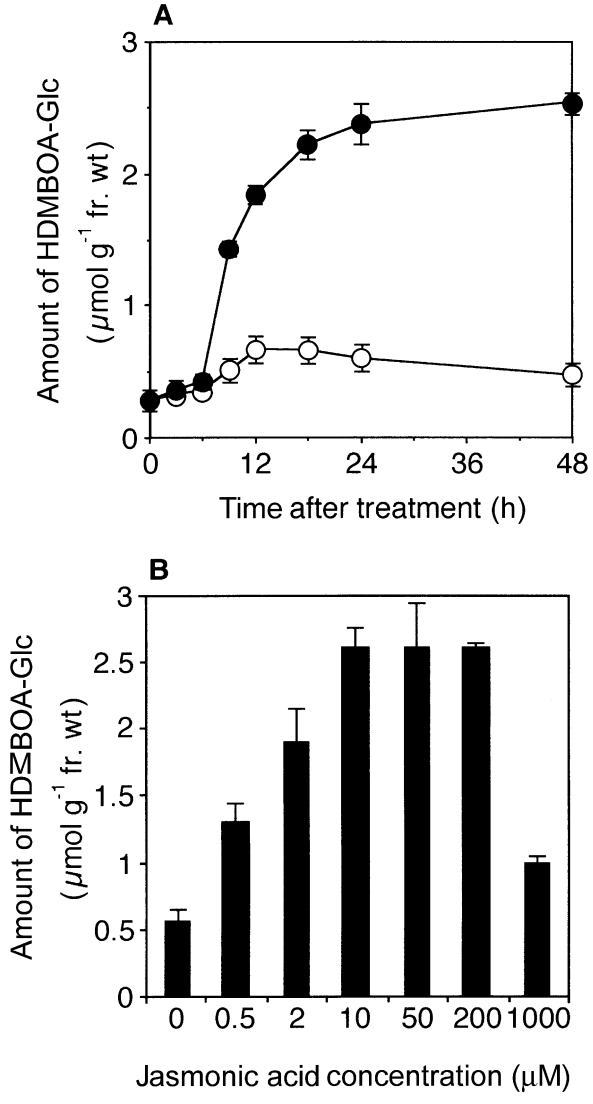


Fig. 2. Changes in amounts of HDMBOA-Glc (**5**) following treatment with 50 μM jasmonic acid (●) and water (○) (A). Effect of jasmonic acid concentration on induction of (**5**) (B). The amounts of (**5**) were determined 24 h after treatment. Error bars indicate standard deviations of four replicates.

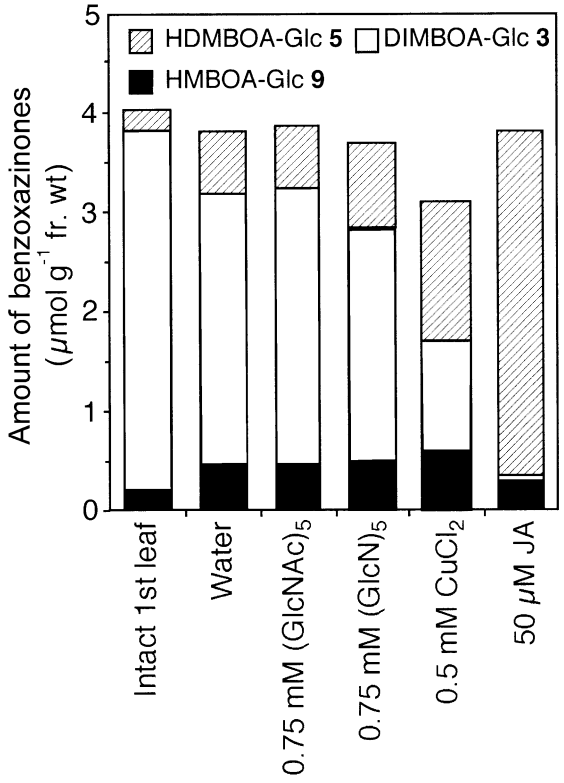


Fig. 3. Effects of treatment with various compounds on benzoxazinones levels in wheat leaf segments. The segments were prepared from the first leaf of 7-day-old wheat seedling. After 48 h incubation on the solutions, benzoxazinone levels were measured.

treated with 50 μM JA. The activity was hardly detectable before treatment. The activity increased 3 h after treatment, reached a maximum after 9 h, and then decreased gradually. The effect of concentration of JA on the induction of enzyme activity was also examined (Fig. 4B). The activity was detected 9 h after treatment when maximal activity was detected in the time-course experiment. An increase in methyltransferase activity was detected in leaves treated with 0.5 μM JA, and the activity increased along with the JA concentration up to

200 μM. The activity decreased at higher concentrations, probably due to the toxicity of JA.

2.4. Purification of DIMBOA-Glc 4-O-methyltransferase

DIMBOA-Glc 4-*O*-methyltransferase was purified from JA-treated wheat leaves by anion exchange chromatography, hydrophobic chromatography and size exclusion chromatography, with a purification factor of 95-fold and 1.44% yield of enzyme activity (Table 2). In all chromatographic steps, only one peak of activity was detected. The anion exchange chromatography resulted in a low recovery of activity (8.9%) with 2.9-fold purification. Although other chromatographic processes were also employed as the first step of purification, they resulted in similar or worse recovery of the activity, probably because of instability of the enzyme in the crude extract. The enzyme activity in crude extract dropped around 80% within 12 h at 4 °C.

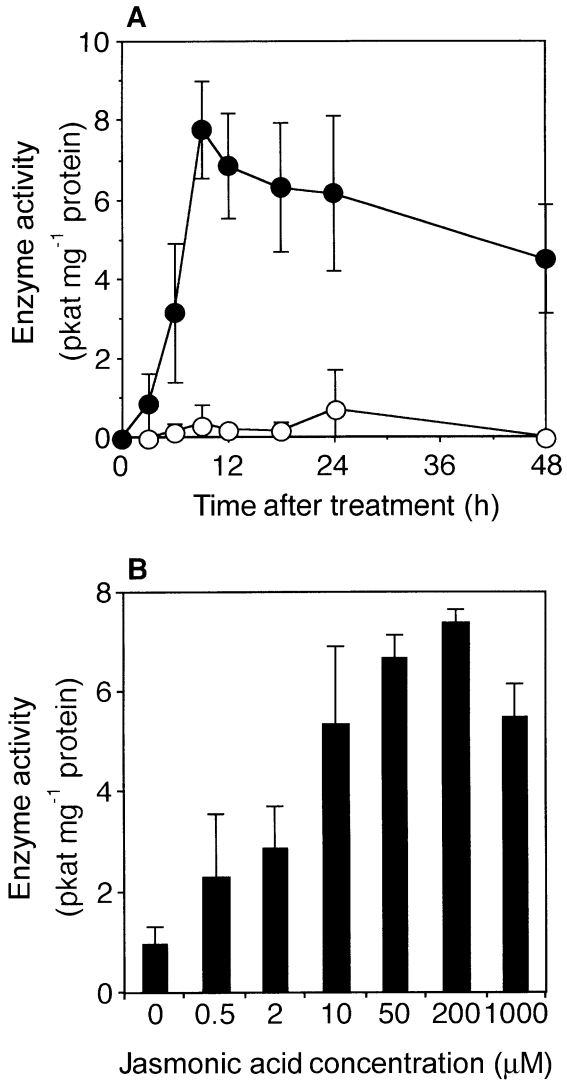


Fig. 4. Changes in activity of DIMBOA-Glc 4-*O*-methyltransferase following treatment with 50 μM jasmonic acid (●) and water (○) (A). Effects of jasmonic acid concentration on the induction of DIMBOA-Glc 4-*O*-methyltransferase activity (B). The enzyme activity was determined 9 h after treatment. Error bars indicate standard deviations of four replicates.

2.5. Characterization of DIMBOA-Glc 4-O-methyltransferase

The enzyme purified 95-fold showed an optimum pH at 7.5 with 50% activity at pH 6.5 and 8.5, and an optimum temperature at 35 °C. The molecular weight of the enzyme was estimated to be about 66 kDa by size exclusion chromatography. The activity was completely inhibited by the presence of 5 mM Mn^{2+}, Cu^{2+} or Zn^{2+}. The addition of 5 mM Mg^{2+} and Ca^{2+} resulted in around 19 and 24% inhibition, respectively. The presence of 5 mM Na^{+} and K^{+} did not affect the enzyme activity. The activity decreased 33% without EDTA.

Kinetic parameters were determined with the set of compounds shown in Table 3. The apparent K_m value and maximum velocity (V_{max}) for **3** were determined to be 0.12 mM and 11.6 nkat mg^{-1} protein, respectively. **4** was also methylated by the wheat DIMBOA-Glc 4-*O*-methyltransferase. Although the V_{max} value was somewhat higher for **4** than for **3**, the ten times larger K_m value yielded a lower catalytic efficiency (V_{max}/K_m) for the methylation of **4** compared with **3**. Furthermore, the addition of **4** to a reaction solution resulted in an inhibition of the methylation of **3** ($K_i = 0.24$ mM). Neither **1** nor **2** served as substrates for the enzyme. The apparent K_m value for SAM was 0.06 mM. *S*-Adenosyl-L-homocysteine (SAH) effectively inhibited DIMBOA-Glc 4-*O*-methyltransferase activity ($K_i = 0.025$ mM).

3. Discussion

In the present study, it was demonstrated that the conversion of **3** to **5** is induced by JA in leaves of wheat and Job's tears, in addition to maize in which a similar JA-induced conversion of **3** has been found (Oikawa et al., 2001). On the other hand, the presence of **5** was detected in neither JA-treated nor control leaves of rye. Rye has little **3**, **4** being the major Bx. Thus, the 4-*O*-methylation product of **4** was expected to accumulate following JA treatment. However, the compound was not detected. The occurrence of **5** has been indicated in some poaceous plants (Hofman et al., 1970; Nagao et al., 1985; Kluge et al., 1997), while **6** has not been detected in plants. These findings suggest that the *O*-methylation at position 4 of Bxs is specific to **3** and that a specific methyltransferase is involved in this reaction.

JA has been found to induce the formation of many secondary metabolites in plants. In the Poaceae, JA has

Table 2
Purification of DIMBOA-Glc 4-*O*-methyltransferase

	Total protein (mg)	Specific activity (pkat mg^{-1} protein)	Purification (-fold)	Recovery (%)
Crude	65.0	11.3	1	100
DEAE Sepharose	1.99	32.6	2.89	8.85
Butyl Sepharose	0.070	181	16.0	1.73
Superdex 200	0.010	1074	95.3	1.44

Table 3
Substrate specificity and inhibition of DIMBOA-Glc 4-*O*-methyltransferase

Compound	K_m (mM)	V_{max} (nkat mg^{-1} protein)	K_i (mM)
DIMBOA-Glc **3**	0.12	11.6	–
DIBOA-Glc **4**	1.20	14.5	0.24
DIMBOA **1**	n.d.[a]	n.d.	n.d.
DIBOA **2**	n.d.	n.d.	n.d.
SAM	0.06	6.04	–
SAH	–	–	0.025

[a] Not detectable.

been shown to evoke the production of defensive low molecular weight compounds like phytoalexins in rice (Nojiri et al., 1996) and hydroxycinnamic acid amides in barley (Lee et al., 1997). Together with these results, our finding that the accumulation of HDMBOA is induced by JA in Bx-accumulating poaceous plants suggests the generality of the role of JA in defense reactions in poaceous plants.

Compound **5** has been indicated to accumulate in wheat leaves in certain combinations between moderately resistant cultivars and races of the stem rust fungus (Bücker and Grambow, 1990). It was proposed that **5** plays a role in defense reactions as a phytoalexin in wheat based on the inducible properties and toxicity of **5**. A decrease in the amount of **3** plus **9** was also observed in infected leaves, but the decrease was not limited to the combinations where **5** accumulated. The conversion of **3** to **5** is probably activated only in specific combinations.

The treatment with $CuCl_2$ resulted in an enhanced accumulation of **5** in wheat leaves, while treatment with $(GlcNAc)_5$ and $(GlcN)_5$ did not affect the **5** content. In maize, the accumulation of **5** was observed in leaves treated with $(GlcNAc)_5$ and $(GlcN)_5$ as well as in leaves treated with $CuCl_2$ (Oikawa et al., 2001). Wheat may not possess the signal transduction system leading from oligosaccharide elicitors to JA accumulation, and probably recognizes elicitors different from maize.

Methylation of the hydroxyl group of a compound is considered to modify the biological activity of the compound by altering chemical and physical properties. Indeed, **5** has unique features among Bx glucosides due to its methylated hydroxamic acid unit. For instance, **5** is relatively lipophilic among Bxs (Hofman et al., 1970; Kluge et al., 1997). This property may affect the absorption of the compound by pathogens at the site of interaction. **5** serves as an efficient substrate for constitutively present glucosidase activity in wheat leaves as does **3** (data not shown). The released aglycone, HDMBOA, is highly unstable, and degrades quickly into 6-methoxy-benzoxazolinone (MBOA), since the methoxide ion is a good leaving group (Grambow et al., 1986). In addition, MBOA is produced from **5** when the benzoxazinone is brought into contact with fungal cell walls that have β-glucosidase activity (Bücker and Grambow, 1990). Since MBOA has a number of biological activities (Virtanen et al., 1957; Elnaghy and Linko, 1962), the quick degradation of HDMBOA into MBOA may be important in the defense reaction of wheat, although intermediate species or other degradation products may also be involved in inhibiting pathogens, as in the case of **1** (Woodward et al., 1978). In this context, it is interesting that the accumulation of **6** was not detected in rye that accumulates **4** as the major Bx, because 4-*O*-Me-DIBOA is much stabler than HDMBOA, and not easily degraded into the corresponding benzoxazolinone, BOA (Escobar et al., 1997).

DIMBOA-Glc 4-*O*-methyltransferase activity was detected in JA-treated wheat leaves. The changes in activity following JA treatment and the dependency of the induction on JA concentration showed a good correlation with those for the accumulation of **5**. In addition, the enzyme showed strict substrate specificity for **3**. Accordingly, the inducible 4-*O*-methyltansferase is considered to be responsible for the accumulation of **5** in leaves treated with JA. The strict substrate specificity of the methyltransferase for **3** unequivocally indicates the direct conversion of **3** to **5**, together with the incorporation of the labeled methyl group from [$^2H_3$-Me]L-methionine into **5** in maize (Oikawa et al., 2001). The enzyme activity was not detected in the crude extracts from maize leaves treated with JA, and this might be attributable to the lability of the enzyme.

DIMBOA-Glc 4-*O*-methyltransferase exhibited a high affinity for SAM with a K_m value of 0.06 mM and a high level of inhibition by SAH ($K_i = 0.025$ mM), which is consistent with methyltransferases in other plants (Poulton, 1981). Moreover, this enzyme showed weaker

affinity for the methyl acceptor, **3**, with a K_m value of 0.12 mM. These results imply that DIMBOA-Glc 4-*O*-methyltransferase is regulated by SAM/SAH ratio, as suggested for the caffeic acid *O*-methyltransferase in spinach (Poulton and Butt, 1975) and alfalfa (Edwards and Dixon, 1991).

In that DIMBOA-Glc 4-*O*-methyltransferase transfers a methyl group to the *N*-hydroxyl group, this methyltransferase appears to be unique. Compounds that have the *N*-*O*-Me structure have occasionally been found in the plant kingdom, e.g. oxindole alkaloids in *Gelsemium* spp. (Wenkert et al., 1972; Schun and Cordell, 1986), and wasalexins (Pedras et al., 1999), sinalbins (Pedras and Zaharia, 2000), and indole glucosinolates (Gmelin and Virtanen, 1962) in cruciferous plants. However, the corresponding *O*-methyltransferases have not been reported to date. Since the compounds found in plants that have the *N*-*O*-Me structure are largely indole derivatives, methyltransferases similar to DIMBOA-Glc 4-*O*-methyltransferase may be involved in the formation of their *N*–*O*–Me structure.

4. Experimental

4.1. Plant materials

Seeds of wheat (*Triticum aestivum* L. cv. Asakazekomugi) were kindly provided by Dr. Koichiro Tsunewaki. Other plant seeds were purchased from Yukijirushi Seeds and Plants Co., Sapporo, Japan. Wheat and rye (*Secale cereale* L. cv. Haruka) seeds were sown on wet filter paper and incubated at 20 °C with a 12-h period of illumination under fluorescent lamps (15 W m^{-2}). Job's tears (*Coix lacryma-jobi* var. *frumentacea* Makino) seeds were sown on vermiculite and incubated at 25 °C under the same conditions of illumination. Maize (*Zea mays* L. cv. Snowdent 108) seeds were sown according to the method described previously (Oikawa et al., 2001). The plants germinated about 24 h after sowing. The first leaves of 7-day-old seedlings of wheat and rye with 8–10 cm in height and the third leaves of 10-day-old seedlings of Job's tears and maize were used for experiments.

4.2. Chemicals

Compounds **3**, **9**, **1**, **7**, **4**, **10**, **2**, and **8** were prepared by the method described previously (Sue et al., 2000). The preparation of **5** was carried out according to the method of Oikawa et al. (2001). Synthesized **6** was kindly provided by Dr. Dieter Sicker. MBOA and *S*-adenosyl-L-methionine *p*-toluenesulufonate salt were purchased from SIGMA. Chitopentaose and penta-*N*-acetylchitopentaose were obtained from Seikagaku Kogyo, Tokyo, Japan. All other chemicals were from Wako Pure Chemical Industries, Osaka, Japan.

4.3. Treatment with chemicals

Eight leaf segments (about 5 mm along the longitudinal axis) excised from the first leaf of 7-day-old wheat or rye seedlings were floated on a soln. (500 μl) containing the chemicals of interest in a tissue culture plate (1 cm i.d.). For maize and Job's tears, one leaf segment (about 2 cm along the longitudinal axis) excised from the third leaf of 10-day-old seedlings was floated on the soln. (1 ml) in a Petri dish (4 cm i.d.). Four segments were taken from one plant. Incubation was performed at 20 °C (for wheat and rye) or 25 °C (for maize and Job's tears) with a 12-h period of illumination under fluorescent lamps (15 W m^{-2}). More than five replicates were performed for each treatment.

4.4. Analysis of benzoxazinones

Bxs were analyzed by HPLC (Shimadzu 10Avp) with a 4.6×150 mm metal-free Wakosil II 5C18 HG column (Wako). A two-element system was used to generate the mobile phase: eluant A was HOAc-H_2O (1:1000) and eluant B was MeCN. The flow rate was 0.8 ml/min. The mobile phase at the initiation of each run was A:B at a ratio of 92.5:7.5. After injection, a 20-min linear gradient to 75:25 (A:B) was applied. The elution of compounds was monitored at 280 nm.

4.5. Purification of methyltransferase

All operations were carried out at 1–4 °C. Wheat leaves (3 g) treated with 50 μM JA were frozen in liquid nitrogen, and ground to a powder with sea sand (30–50 mesh). The powder was suspended in 12 ml of 50 mM Tris–HCl (pH 8.5) containing 10 mM 2-mercaptethanol, 1 mM EDTA, 10 μM APMSF and 10% (v/v) glycerol (buffer A), and homogenized after adding PVPP (0.3 g). The homogenate was centrifuged at 17,000 *g* for 30 min following filtration through a miracloth (Calbiochem). The supernatant was loaded on a DEAE Sepharose column (5 ml, Pharmacia) equilibrated with buffer A. The proteins were eluted with a linear NaCl gradient (0–250 mM) of buffer A, at a flow rate of 1 ml/min. The fractions having methyltransferase activity were collected and concentrated to 0.5 ml by ultrafiltration, and then the buffer of the soln. was exchanged to buffer A containing 1.5 M $(NH_4)_2SO_4$ (buffer B), using a PD-10 column (Pharmacia). The resulting soln. was applied onto a HiTrap Butyl Sepharose 4 FF column (Pharmacia) equilibrated with buffer B. Proteins were eluted with a linear gradient to buffer A, at a flow rate of 0.1 ml/min. The active fractions were combined and subjected to size exclusion chromatography on a Superdex 200 HR 10/30 column (Pharmacia), and the methyltransferase was eluted with buffer A containing 150 mM NaCl at a flow rate of 0.25 ml/min. For the estimation

of molecular mass on the size exclusion column, the following proteins were used as standards: IgG (160 kDa), transferrin (81 kDa), ovalbumin (43 kDa) and myoglobin (17.6 kDa). Protein contents were estimated by the method of Bradford (1976) using bovine serum albumin as standard.

4.6. Enzyme assay

The activity of methyltransferase was measured in buffer C [50 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 10 μM APMSF] with 10–100 μl of enzyme soln., 67 nmol **3**, 200 nmol *S*-adenosyl-L-methionine and 100 nmol castanospermine in a final volume of 500 μl. After 1 h incubation at 35 °C, the reaction was stopped by adding 50 μl of 1 *N* HCl, and the reaction mixture was subjected to HPLC analysis. Apparent Michaelis constants (K_m) and maximum velocities (V_{max}) were determined from the [s]/v versus [s] plot. The optimum pH for methyltransferase activity was determined using 50 mM Bis–Tris buffer (pH 5.5–7.5) and 50 mM Tris–HCl buffer (pH 7.0–9.0). In the reaction with **4**, the identity of the product, **6**, was confirmed by the comparison of UV spectrum, ion-spray MS, EI–MS, and chromatographic behavior on HPLC with the authentic compound.

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