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A methyltransferase for synthesis of the phytoalexin 6-methoxymellein in carrot cells

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Carrot (*Daucus carota* L.) roots treated with 2-chloroethylphosphonic acid or uronide elicitor accumulated the phytoalexin 6-methoxymellein. The extracts of these cells catalyzed methylation of 6-hydroxymellein to 6-methoxymellein with *S*-adenosyl-L-methionine as a methyl donor. Activity of the *O*-methyltransferase was not found in fresh carrot roots but increased when they were treated with either type of elicitor in parallel with the increase in 6-methoxymellein content. The enzyme did not methylate 6-methoxymellein, 3,4-dehydro-6-methoxymellein, and some coumarin derivatives, though 3,4-dehydro-6-hydroxymellein accepted methyl moiety at the same rate as in 6-hydroxymellein.

O-Methyltransferase; 6-Methoxymellein; Phytoalexin; Dihydroisocoumarin; (*Daucus carota* L.)

1. INTRODUCTION

It has been reported that treatment of plant cells of Leguminosae with fungal elicitors caused marked increase in the activities of many enzymes, such as phenylalanine ammonia-lyase, 4-coumarate:CoA ligase and flavanone synthase [1–3], which participate in the early stages of biosynthesis of pterocarpan phytoalexins. Recently, the occurrence of enzymes catalyzing the later stages of biosynthesis of these phytoalexins, *O*-methyltransferase [4], isoflavone oxidoreductase [5], dimethylallyltransferase [6] and hydrolases [7], has also been demonstrated in elicitor-treated cells. However, little is known about enzymes in the biosynthetic route of phytoalexins other than pterocarpan derivatives.

Carrot phytoalexin, 6-methoxymellein (fig.1, **3**), is one of the very few known 3-methylisocoumarins from higher plants which accumulates in the cells upon treatment with uronide elicitor or other stress. From ^{13}C-NMR analyses, it has been proposed [8] that the compound is synthesized from acetyl-CoA and malonyl-CoA via pentaketide (fig.1, **1**). However, the sequence of modification of the putative intermediate **2**, and enzymes catalyzing these reactions have not been elucidated. We now report that carrot cells possess an inducible *O*-methyltransferase which catalyzes the terminal step in the biosynthesis of 6-methoxymellein.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

6-Methoxymellein was isolated from infected carrot roots [9], and 6-hydroxymellein was prepared by demethylating it by boron tribromide in anhydrous methylene chloride as described in detail [10]. 2-Chloroethylphosphonic acid (2-CEPA), umbelliferon (7-hydroxycoumarin), scopoletin (7-hydroxy-6-methoxycoumarin) and esculetin (6,7-dihydroxycoumarin) were obtained from Sigma (St. Louis, MO). 3,4-Dehydro-6-methoxymellein was isolated from culture filtrate of *Ceratocystis fimbriata* by preparative TLC [11] and identified by ^{1}H-NMR (δ 2.26 3H singlet, δ 3.87 3H singlet; δ 6.18 1H broad singlet; δ 6.29 1H doublet, J = 2 Hz; δ 6.47 1H doublet, J = 2 Hz). 3,4-Dehydro-6-hydroxymellein (fig.1,**2**) was prepared from the isolated compound by demethylation with boron tribromide as described above. *S*-[^{14}C]Adenosyl-L-methionine (spec. act. 58.6 mCi/mmol) was purchased from New England Nuclear (Boston).

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2.2. *Preparation of uronide elicitor*

Uronide elicitor for 6-methoxymellein production was prepared from the pectin fraction of carrot cells. Pectic substances (4.5 mg) were partially hydrolyzed by pectinase (100 units) for 1 h at 37°C in Na-acetate buffer (0.1 M, pH 5.2) and the resultant hydrolysates were fractionated on a DEAE-Sephacel column to obtain elicitor-active substances. Details of the procedure will be published elsewhere [12].

2.3. *Plant material*

Carrot roots were purchased from the local market. The surface of the roots was sterilized by ethanol, and disks (1.8 cm diameter and 2 mm thickness) were prepared by a sterilized cork borer. They were placed in Petri dishes and 50 μl aliquots of filter-sterilized 2-CEPA solution (10 mg/ml in 0.1 M Na-citrate buffer, pH 5.0) or autoclaved uronide elicitor solution (25 μg galacturonic acid equivalent/ml as determined by the carbazole-sulphuric acid method [13]) were dropped onto the disks. They were incubated at 26°C, and, at regular intervals, 8 disks were harvested (4 disks for the determination of 6-methoxymellein and 4 for the *O*-methyltransferase assay). Control disks received Na-citrate buffer instead of the elicitors.

2.4. *Enzyme preparation*

Elicitor-treated carrot disks were frozen in liquid N_2 and ground in a cold motor. They were further homogenized with a Waring Blendor in 3 ml of 20 mM Na-phosphate buffer (pH 7.5) containing 0.1% mercaptoethanol (v/v) and 0.5 g polyvinylpyrrolidone. The homogenates were filtered through double layered gauze, and centrifuged at 10000 × *g* for 20 min. The resultant supernatants were mixed with 0.2 g of AG 1-X8 resin (Bio-Rad) and stirred for 3 min. They were passed through a glass fibre filter paper (Whatman GF/C), and supplied as an enzyme preparation.

2.5. *Methyltransferase assay*

The standard assay mixture consisted of the enzyme preparation (approx. 50 μg protein), 250 μM 6-hydroxymellein, 0.03 μM *S*-[^{14}C]adenosylmethionine, 10 mM cysteine, 1 mM $MgCl_2$, 0.1% mercaptoethanol and 100 mM Na-phosphate buffer (pH 7.5) in a total volume of 250 μl. After a 1 h incubation at 37°C, the reaction was terminated by adding 50 μl of 6 N HCl. The radioactive product was extracted with 200 μl hexane by vigorous shaking on a vortex-mixer, and a 100 μl aliquot was removed to determine the radioactivity. In some experiments, the aliquots were applied to a TLC plate (Merck, silica gel 60 F 254) and developed in benzene with appropriate authentic samples to identify the reaction products.

2.6. *Other assay*

The 6-methoxymellein accumulated in carrot disks was extracted, partially purified and developed on a TLC plate as above, and the content of the compound was determined by the scanning method as described in [9]. Protein was determined according to Lowry et al. [14].

3. RESULTS AND DISCUSSION

In 2-CEPA-treated carrot disks, an appreciable increase in 6-methoxymellein production was observed after a 2 day lag (fig.2a). The content of the phytoalexin increased linearly for about 48 h, and the rate declined thereafter. *O*-Methyltransferase activity in the induced cells was determined by the methylation of 6-hydroxymellein. After the enzyme preparation was incubated with the substrate in the presence of *S*-[^{14}C]adenosylmethionine, the radioactive reac-

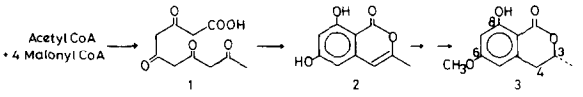

Fig.1. Possible pathway of biosynthesis of 6-methoxymellein. Pentaketide chain **1** from acetyl-CoA and malonyl-CoA condenses to form isocoumarin **2**. After reduction and *O*-methylation, **2** is converted to 6-methoxymellein **3**.

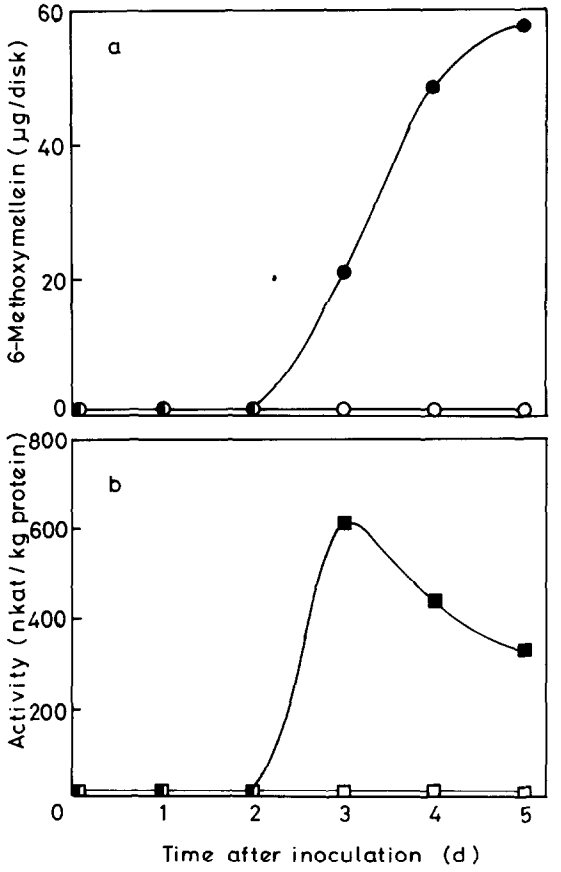

Fig.2. Changes in 6-methoxymellein content (a) and 6-hydroxymellein *O*-methyltransferase activity (b) in 2-CEPA-treated carrot disks. Closed symbols represent the results of treated cells and open symbols of controls. Repeated experiments gave similar results.

tion product was extracted with hexane and applied to a TLC plate. More than 85% of the total radioactivity of the extract co-migrated with authentic 6-methoxymellein. Since no other distinct spot was found on the TLC plate, 6-methoxymellein is regarded as the sole product of the reaction system. It was also confirmed that the 6-hydroxymellein-dependent reaction continued linearly for at least 1 h. The activity of *O*-methyltransferase was not detectable in untreated carrot root disks (fig.2b). When they were treated with 2-CEPA, the enzyme activity increased markedly. The activity began to increase after a 2 day lag period and attained a maximum 3 days after the addition of the elicitor. The enzyme activity was then decreased gradually.

In the next experiment, we examined the effect of uronide elicitor which is derived from plant cell walls and thought to be natural and a rather specific elicitor of 6-methoxymellein production in carrot. As shown in fig.3, uronide elicitor also induced 6-hydroxymellein-*O*-methyltransferase in carrot cells as well as the production of 6-methoxymellein. In this case, the enzyme activity became detectable, prior to the appearance of 6-methoxymellein, about a day after the addition of the elicitor, and began to decrease before the phytoalexin content reached a maximal value.

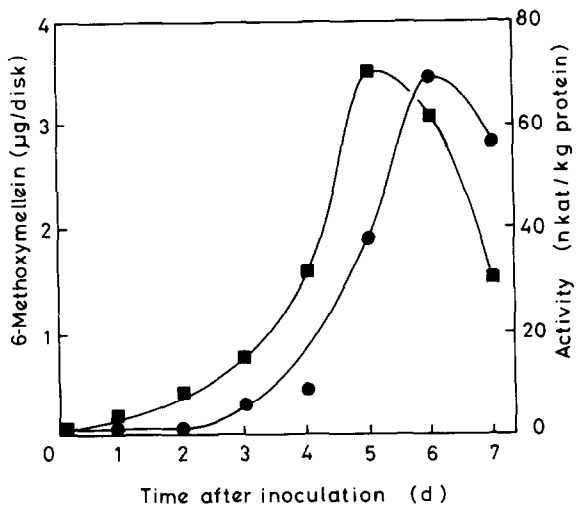

Fig.3. Changes in 6-methoxymellein and 6-hydroxymellein *O*-methyltransferase activity in uronide elicitor-treated carrot disks. Circles represent 6-methoxymellein content and squares *O*-methyltransferase. Repeated experiments gave similar results.

The substrate specificity of the enzyme to some coumarin and isocoumarin derivatives was tested and the results are summarized in table 1. The hydroxyl group of coumarin derivatives was not efficiently methylated by the enzyme. In isocoumarins, the enzyme catalyzed the incorporation of the methyl group of *S*-adenosylmethionine into 3,4-dehydro-6-hydroxymellein at the same rate as into 6-hydroxymellein. However, the hydroxyl moiety at the 8 position of 6-methoxymellein and 3,4-dehydro-6-methoxymellein did not accept the methyl group. In both 6-hydroxymellein and 3,4-dehydro-6-hydroxymellein, the corresponding 6-methoxyl compounds were detected as the sole product of the reaction by TLC analyses. These observations suggest that the *O*-methyltransferase detected in elicitor-treated carrot cells has a relatively high specificity, and catalyzes transfer of a methyl group to a hydroxyl moiety at the 6 position of isocoumarin compounds.

We expected that the enzyme preparation might also contain a reductase which catalyzes the con-

Table 1

Substrate specificity of the *O*-methyltransferase from elicitor-treated carrot cells

Substrate	Product	Relative activity (%)[a]
Coumarins		
Umbelliferone	–[b]	2
Scopoletin	–	1
Esculetin	–	8
Isocoumarins		
6-Hydroxymellein	6-methoxymellein	100
6-Methoxymellein	–	2
3,4-Dehydro-6-hydroxy-mellein	3,4-dehydro-6-methoxymellein	105
	6-methoxymellein	6
3,4-Dehydro-6-methoxy mellein	–	2
6-Hydroxymellein[c] + 3,4-dehydro-6-hydroxymellein	6-methoxymellein + 3,4-dehydro-6-methoxymellein	53 + 50

[a] Relative activity was expressed as percentage to the rate of the *O*-methylation of 6-hydroxymellein (0.5 μkat/kg protein)

[b] Not identified

[c] 6-Hydroxymellein and 3,4-dehydro-6-hydroxymellein (125 μM each) were mixed and supplied as substrate of the enzyme reaction. Products were analyzed by TLC

version of 3,4-dehydro-6-hydroxymellein into a saturated form, and, therefore, an attempt was made to obtain 6-methoxymellein as a radiolabeled product from 3,4-dehydro-6-hydroxymellein by incubating the compound with carrot cell homogenates in the presence of *S*-[^{14}C]adenosylmethionine and a possible reducing co-factor such as NADPH or NADH. In every case, however, effective conversion of the compound into 6-methoxymellein was not observed (not shown). At present, it is not clear whether reduction of the double bond at the 3,4-position precedes the *O*-methylation at 6-position, or vice versa.

4. CONCLUSION

The activity of *S*-adenosyl-L-methionine:6-hydroxymellein *O*-methyltransferase was found in carrot cell homogenates, being induced concomitantly with phytoalexin accumulation by the treatment with ethylene or uronide elicitor. The enzyme specifically catalyzes methylation of the hydroxyl moiety at the 6-position of isocoumarin, and, therefore, is considered to be involved in the biosynthesis of the carrot phytoalexin, 6-methoxymellein. This transferase also methylated 3,4-dehydro-derivative at the same rate as for 6-hydroxymellein. The purification and further characterization of the enzyme are in progress.

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