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ROLE OF A CYTOCHROME P450-DEPENDENT MONOOXYGENASE IN THE HYDROXYLATION OF 24-*EPI*-BRASSINOLIDE*

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Key Word Index—*Lycopersicon esculentum*; Solanaceae; brassinosteroids; cell suspension cultures; cytochrome P450 inhibitors; cytochrome P450 inducers; hydroxylation; inhibition of protein biosynthesis.

Abstract—24-*epi*-Brassinolide, exogenously applied to cell suspension cultures of *Lycopersicon esculentum* is hydroxylated at C-25 and C-26, respectively, followed by glucosylation of the newly formed hydroxyl group. Treatment of the cell cultures with the specific cytochrome P450 inhibitors, clotrimazole and ketoconazole, resulted in a strong decrease of only the C-25 hydroxylation, whereas hydroxylation at C-26 was not affected. The common cytochrome P450 inducers, ethanol, $MnCl_2$, phenobarbital, pregnenolone 16α-carbonitrile or clofibrate, did not induce hydroxylation activity at C-25 or at C-26. In addition, substrate analogues (22*S*,23*S*-homobrassinolide, 24-*epi*-castasterone, ecdysone, and 20-OH-ecdysone) were not accepted. Only application of 24-*epi*-brassinolide and brassinolide resulted in an increased activity of both the C-25- and C-26-hydroxylases. For further examination of the molecular level of this inducing effect, the influence of the protein biosynthesis inhibitor cycloheximide has been studied. Thus, increase of both hydroxylase activities is obviously based on gene expression by action of the substrates, 24-*epi*-brassinolide and brassinolide.

INTRODUCTION

Brassinosteroids are a group of steroidal phytohormones with a multitude of physiological activities [1]. Recently, it has been shown that *Arabidopsis* mutants could be restored to phenotype wildtype by application of a variety of these brassinosteroids. Up till now all efforts to attach the mutations to specific enzyme activities have failed [2–5]. However, it has been suggested that different cytochrome P450 hydroxylases are involved in the biosynthesis of brassinosteroids [3, 6, 7]. We have shown that in cell suspension cultures of *Lycopersicon esculentum* 24-*epi*-brassinolide is converted to its 25- and 26-β-D-glucopyranosyloxy derivatives via the corresponding hydroxy intermediates, which have not been found endogenously up to now. It has been suggested that one of these hydroxylation steps is cytochrome P450 dependent [6]. In this paper we report on the effect of the specific P450 inhibitors clotrimazole and ketoconazole [8], as well as of various P450 inducers, such as ethanol, phenobarbital, $MnCl_2$ [9], pregnenolone 16α-carbonitrile [10] and clofibrate [11], to the hydroxylation of 24-*epi*-brassinolide in *L. esculentum* cell cultures. Furthermore, with the analogues 22S,23S-homobrassinolide, 24-*epi*-castasterone, ecdysone, or 20-hydroxyecdysone and with the substrates brassinolide and its 24-epimer 24-*epi*-brassinolide, the specificity of induction of this metabolic process has been studied.

RESULTS AND DISCUSSION

The time course of metabolism of exogenously applied 24-*epi*-brassinolide in cell suspension cultures of *Lycopersicon esculentum* has been studied over 72 hr. [5,7,7-^{3}H]24-*epi*-Brassinolide was used in final concentrations of 1.5 μM. The absorption of radioactivity by cultured cells proceeds very rapidly. After 2 hr 60% of the supplied radioactivity was taken up. The conversion to the 25- and 26-β-D-glucopyranosyloxy derivatives, respectively (Fig. 1), occurs between 8 and 24 hr after administration of 24-*epi*-brassinolide (Table 1). After 24 hr the substrate was totally converted in equal ratio of either product that did not change significantly up to 72 hr. To investigate

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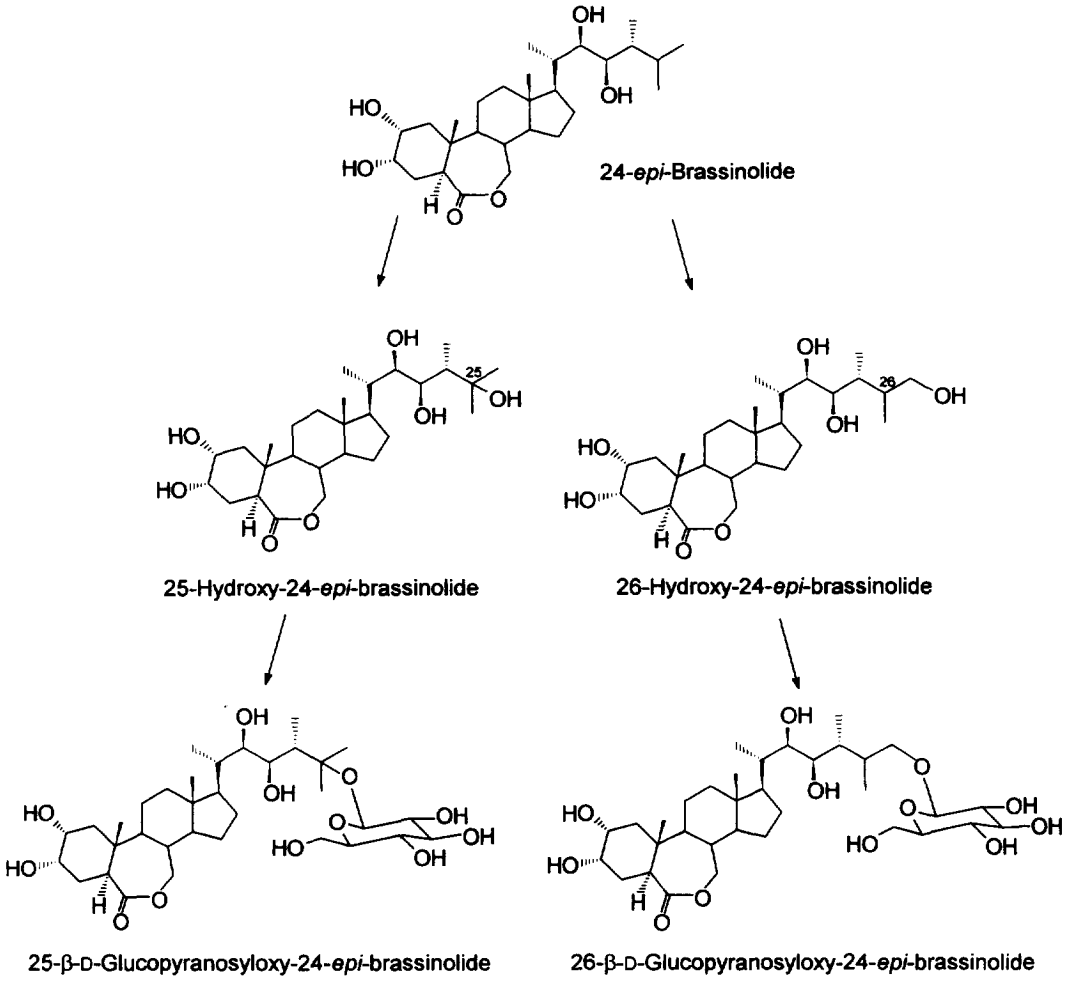

Fig. 1. Hydroxylation and glucosylation of 24-*epi*-brassinolide in cell cultures of *Lycopersicon esculentum*.

whether the metabolism of 24-*epi*-brassinolide is dose-dependent, experiments with different amounts of the substrate were carried out. It could be shown that raising the concentration of the substrate from 2 to 7 μM and 20 μM was followed by a dramatic decrease of the conversion to the 25- and 26-metabolites. The conversion rate with 7 μM was only 50% compared with the value obtained with 2 μM. With a substrate concentration of 20 μM no products were detectable.

Table 1. Time dependent metabolism of exogenously applied [5,7,7-^{3}H]24-*epi*-brassinolide to cell suspension cultures of *Lycopersicon esculentum*. The C-25- and C-26-metabolites were detected as their glucosides

Time (hr)	25-OH- %	26-OH- %	24-*epi*-brassinolide %
8	n.d.	n.d.	100
12	34	42	24
16	40	51	9
24	37	63	n.d.
48	46	54	n.d.
72	44	56	n.d.

n.d.: not detectable.

The biosynthesis of brassinosteroids is characterized by the introduction of various hydroxyl groups to the phytosterol precursor [12, 13]. At least some of these hydroxylations are assumed to be cytochrome P450 dependent [2, 3, 6, 7], strongly suggesting a similar biosynthetic pathway as described for the ecdysteroid metabolism [14]. To demonstrate the cytochrome P450 characteristics of both hydroxylases, cell suspension cultures were treated with the specific imidazole-based P450 inhibitors clotrimazole and ketoconazole. Both compounds only inhibited the conversion of 24-*epi*-brassinolide to the 25-hydroxy metabolite, whereas hydroxylation at C-26 was not negatively affected (Table 2).

Cytochrome P450 enzymes are involved in biosynthesis and in detoxification processes of numerous substrates [15, 16]. Therefore, studies of the metabolism of brassinosteroids with exogenously applied precursors bear the risk of inducing detoxification pathways. To exclude such an unspecific metabolism,

Table 2. Effect of cytochrome P450 inhibitors (50 μM each) on the C-25- and C-26-hydroxylation of exogenously administered [5, 7, 7-^{3}H]24-*epi*-brassinolide in cell suspension cultures of *Lycopersicon esculentum*. The C-25- and C-26-metabolites were detected as their glucosides. The control values were arbitrarily set 100% for each metabolite

Inhibitor	Hydroxylase activity (%) C-25-	C-26-
None (control)	100	100
+ Clotrimazole	43	149
+ Ketoconazole	34	154

both enzyme activities involved in the hydroxylation at C-25 and C-26 of 24-*epi*-brassinolide have been investigated for their inducibility by substances with diverse inducing spectra. Ethanol, $MnCl_2$ and phenobarbital are known for their ability to unspecifically induce numerous cytochromes P450 [9, 15, 17, 18]. Thus, these compounds exhibit a broad spectrum of induction, in contrast to pregnenolone 16α-carbonitrile and clofibrate, which only have inducing effects on distinct cytochromes P450 [15, 17] and, therefore, show a narrow spectrum.

As shown in Table 3 both enzyme activities could not be increased by these commonly used P450-inducers. Furthermore, no substrate analogues, such as ecdysteroids (ecdysone, 20-hydroxyecdysone) [19], nor even the brassinosteroids 22S,23S-homobrassinolide and 24-*epi*-castasterone showed any positive effect on hydroxylase activities. Only the substrates 24-*epi*-brassinolide and brassinolide increased the activity of the C-25- and C-26-hydroxylase, respectively. Thus, the induction of both hydroxylases seems to be highly specific and requires essentially the combination of the lactone and the (22R, 23R)-diol side-chain moiety. On the other hand, the configuration of the 24-methyl group seems not to be important, because both 24-epimers, brassinolide and 24-*epi*-brassinolide, induced both hydroxylase activities. These structural requirements for an increase of C-25- and C-26-hydroxylase activities indicate a strict regulation. Such a conclusion is also verified by the observation that no hydroxylase activity could be detected after inhibiting protein biosynthesis by cycloheximide.

Furthermore, the possible hydroxylation by an unspecific cytochrome P450-dependent lauric acid-ω-hydroxylase could be excluded since no activity of this enzyme was detectable, employing [1-^{14}C]lauric acid. This also indicates that both hydroxylation reactions are involved in the natural pathway of these compounds. In addition, the inhibitory effect of substrate concentrations in a low micromolar range (7 μM; 20 μM) supports this assumption.

As suggested earlier [6] our studies showed that hydroxylation at C-25 and C-26 is catalysed by two different enzymes. To confirm this observation, inhibitory studies were done, which demonstrated clearly that the specific cytochrome P450 inhibitors only affect hydroxylation at C-25. The increase in hydroxylase activity at C-26 based on the availability of more substrate, since the competing enzyme, C-25-hydroxylase, is inhibited. These data and those shown before indicate that the C-25-hydroxylase is a typical cytochrome P450-protein, and not, as assumed earlier, the C-26-hydroxylase [6]. Therefore, the latter enzyme could be a flavin-containing monooxygenase [20], which would explain the insensitivity to specific P450-inhibitors.

EXPERIMENTAL

Radiochemicals and measurement of radioactivity. The synthesis of [5,7,7-^{3}H]24-*epi*-brassinolide was recently described [21]. The compound with a sp. act. of 80 MBq mmol^{-1} was a kind gift from Dr A. Kolbe

Table 3. Effect of cytochrome P450-inducing compounds on the C-25- and C-26-hydroxylation of exogenously administered [5, 7, 7-^{3}H]24-*epi*-brassinolide in cell suspension cultures of *Lycopersicon esculentum*. The C-25- and C-26-metabolites were detected as their glucosides. The control values were arbitrarily set 100% for each metabolite

Compound	Concentration	Hydroxylase activity (%) C-25-	C-26-
Ethanol	300 mM	110	91
Phenobarbital	5 mM	70	84
$MnCl_2$	5 mM	60	63
Clofibrate	4 μM	71	110
Pregnenolone 16α-carbonitrile	4 μM	108	113
Ecdysone	4 μM	88	94
20-Hydroxyecdysone	4 μM	90	101
24-*epi*-Castasterone	4 μM	77	107
22*S*,23*S*-homobrassinolide	4 μM	83	77
24-*epi*-Brassinolide	4 μM	154	167
Brassinolide	4 μM	227	210

(Halle). [1-^{14}C]Lauric acid with a sp. act. of 2.15 GBq mmol^{-1} was obtained from Amersham. Radioactivity was measured by liquid scintillation counting (L.S.C.). TLC plates were analysed for radioactive compounds with an automatic TLC linear analyser. For quantitation of metabolites the ratios of peak areas from TLC radioscans were used. All measurements were performed at least twice. The data shown came from one representative experiment.

Biochemicals. Clotrimazole, pregnenolone 16α-carbonitrile, cycloheximide, and 12-hydroxydodecanoic acid were obtained from Sigma; lauric acid, phenobarbital, clofibrate, ecdysone, 20-hydroxyecdysone and $MnCl_2$ from Fluka; ketoconazole from ICN, and brassinolide from Beak Technologies Inc. 24-*epi*-Brassinolide, 24-*epi*-castasterone and 22S,23S-homobrassinolide were a kind gift from Dr B. Voigt (Halle).

Cell culture. Plant cell cultures of *Lycopersicon esculentum* Mill. were obtained from the cell culture laboratory of Prof. Zenk (Munich, Germany). The suspended cells were grown in Linsmaier–Skoog medium [22] at 23° on a gyratory shaker under constant diffuse light (4.4 μmol m^{-2} sec^{-1}) in 140 ml conical flasks containing 40 ml cell suspension.

Administration of labelled 24-epi-*brassinolide.* EtOH solns of [5,7,7-^{3}H]24-*epi*-brassinolide were administered to the cell cultures 72 hr after subculturing under sterile conditions.

Administration of [1-^{14}C]*lauric acid.* Cell suspension cultures were incubated for 2 hr with 2 mM of an *n*-hexane soln of [1-^{14}C]lauric acid (132 kBq mmol^{-1}).

Isolation of metabolites. The brassinosteroids were purified as described [6, 23]. Lauric acid and 12-hydroxydodecanoic acid were isolated according to [24, 25] by TLC (Et_2O–*n*-hexane–HCOOH 60 : 30 : 1).

Time-dependency. Cell suspension cultures were incubated with [5,7,7-^{3}H]24-*epi*-brassinolide for increasing time periods up to 72 hr. The incubation was stopped by filtration of the cells which were subsequently extracted with MeOH.

Inhibition and induction. Cell suspension cultures were incubated with 50 μM of clotrimazole and ketoconazole, respectively, 8 hr prior to the application of [5,7,7-^{3}H]24-*epi*-brassinolide. The incubation time was 12 hr, which seemed to be optimal for detecting the substrate and the products. Thus, inhibitory effects could be well investigated. For inhibiting protein biosynthesis cycloheximide (7 mg in EtOH) was added to the cells 1 hr prior to the application of the inducing compounds. Induction experiments were carried out by treating the cells with the inducing substance (dissolved in DMSO or EtOH) 24 hr prior to the application of [5,7,7-^{3}H]24-*epi*-brassinolide. The incubation period was 8.5 hr, which seemed to be optimal for investigating inducing effects, since not too much of the substrate was metabolized.

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