Elicitor Induction of Cytochrome P-450 Monooxygenases in Cell Suspension Cultures of Chickpea (*Cicer arietinum* L.) and Their Involvement in Pterocarpan Phytoalexin Biosynthesis

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A yeast glucan elicitor causes the accumulation of the pterocarpan phytoalexins medicarpin and maackiain in chickpea *(Cicer arietinum)* cell suspension cultures established from seeds. A cell culture line from a chickpea cultivar resistant against its main fungal pathogen *Ascochyta rabiei* accumulates large amounts (944 nmol/g fr. wt.) whereas a cell culture line from a susceptible cultivar accumulates only low amounts (38 nmol/g fr. wt.) of the phytoalexins. This is consistent with differential accumulation of pterocarpan phytoalexins in intact plants [1]. The first reactions in the pterocarpan-specific branch of biosynthesis are hydroxylation of the isoflavone intermediate formononetin in position 2′ or 3′, catalyzed by microsomal cytochrome P-450 monooxygenases. Upon elicitation formononetin 2′-hydroxylase undergoes a strong transient induction in the cell suspension culture of the resistant cultivar, whereas in the cell culture from the susceptible cultivar it is only slightly induced. In both cell suspension cultures the induction of cinnamic acid 4-hydroxylase and of formononetin 3′-hydroxylase does not show a clear correlation with phytoalexin accumulation. Experiments with different elicitor concentrations confirm that formononetin 2′-hydroxylase is much more induced in cell cultures from the resistant cultivar than from the susceptible one. It is concluded that the massive difference in phytoalexin accumulation between cell suspension cultures from the resistant and susceptible cultivar is determined mainly by the differential induction of formononetin 2′-hydroxylase activity.

Introduction

Cytochrome P-450 monooxygenases (EC 1.14.14.1) are widely distributed in animals, plants and microorganisms. In eukaryots these enzymes are localized in microsomes where these membrane proteins occur together with cytochrome P-450 reductases (EC 1.6.2.4). The structure of the plant cytochrome P-450 system is highly analogous to the enzymes from animals [2, 3]. However, the plant cytochrome P-450 monooxygenases do not possess such a broad substrate specifity as has been observed with the hepatic enzymes [4–6].

Plant cytochrome P-450 monooxygenases are involved in a number of biochemical processes such as biosynthesis of gibberellic acid [7], sterol biosynthesis [8], ω-hydroxylation of fatty acids [9] as well as in pesticide and herbicide metabolism [10, 11]. In plant secondary product metabolism they catalyze reactions in the general phenylpropanoid pathway [12] and in the biosynthesis of cardenolides [13], monoterpene alkaloids [14, 15] and flavonoids [16, 17].

In addition, a number of cytochrome P-450 monooxygenases are involved in phytoalexin biosynthesis. Examples are psoralen synthase known from the biosynthesis of furanocoumarins in *Petroselinum hortense* and *Ammi majus* [18, 19], the dihydroxypterocarpan 6a-hydroxylase involved in the biosynthesis of the glyceollins in soybean [20] and isoflavone 2′- and 3′-hydroxylases demonstrated in the formation of the pterocarpans medicarpin and maackiain in chickpea (*Cicer arietinum*) [21]. Phytoalexins accumulate in plants after stress, wounding or microbial infection and are an integral part of plant antimicrobial defence reactions [22, 23].

Medicarpin and maackiain (Fig. 1) are synthesized in chickpea *via* the general phenylpropanoid

Abbreviations: C4H, cinnamic acid 4-hydroxylase; CO, carbon monoxide; F2H, formononetin 2′-hydroxylase; F3H, formononetin 3′-hydroxylase; PAL, phenylalanine ammonia lyase.

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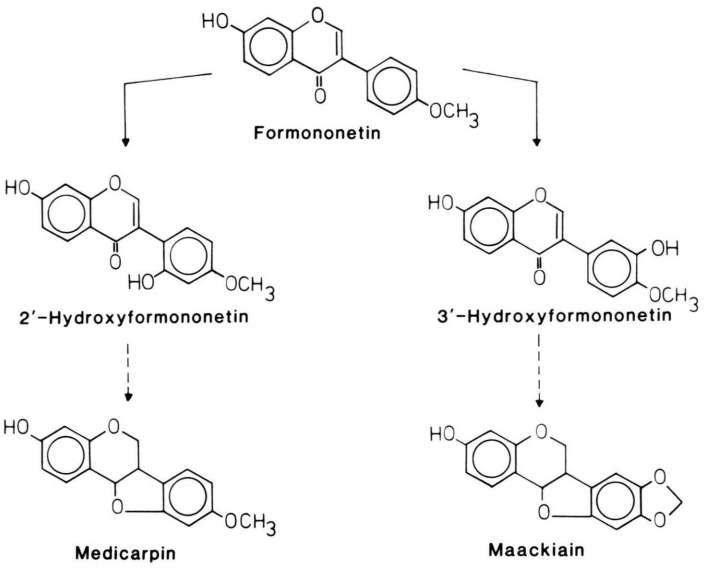

Fig. 1. Hydroxylation reactions of the isoflavone intermediate formononetin in the positions 2′ and 3′, catalyzed by microsomal cytochrome P-450 monooxygenases as involved in medicarpin and maackiain biosynthesis.

pathway with the isoflavone formononetin as an intermediate [24]. The subsequent enzymes of the pterocarpan specific branch of phytoalexin formation have been recently detected with isoflavone 2′- and 3′-hydroxylase [21], NADPH:isoflavone oxidoreductase [25] and pterocarpan synthase [26], respectively.

Up to now only limited knowledge is available concerning quantitative differences in the induction of phytoalexin biosynthetic enzymes expressed by plants which are either susceptible or resistant against their natural microbial pathogens [27, 28]. Earlier investigations had indicated [29, 30] that the chickpea cultivars greatly differed in the expression of the isoflavone 2′-hydroxylase. Therefore, we now report on the differential elicitor induction of cytochrome P-450 monooxygenases (*i.e.* cinnamic acid 4-hydroxylase, formononetin 2′- and 3′-hydroxylase) in chickpea cell suspension cultures established from different cultivars which are resistant and susceptible against the natural fungal pathogen *Ascochyta rabiei* [31].

Materials and Methods

Cell suspension cultures

Cell suspension cultures, derived from seeds of *Ascochyta rabiei* resistant (ILC 3279) and susceptible (ILC 1929) cultivars of chickpea [31] were grown on a PRL-4c medium [32] with the addition of 2 mg/ml glycine instead of yeast extract. The culture conditions were those of Keßmann and Barz [31].

Preparation of elicitor

The preparation of a yeast glucan elicitor was performed according to Schumacher *et al.* [33]. The final preparation was lyophilized and stored as a dry powder until use. The glucose content of the elicitor preparation was determined with the anthrone method [34]. For elicitation an appropriate amount of this powder was dissolved in water (2 ml), autoclaved for 20 min and applied under sterile conditions to the cell cultures at day 3 of the culture period.

Extraction of phytoalexins

Phenolic compounds containing medicarpin and maackiain were extracted from the culture medium [31] and the cells [35] with ethyl acetate.

Preparation of microsomes

Microsomes were prepared from cell suspension cultures of chickpea according to Hinderer *et al.* [21].

Enzyme assays

Cinnamic acid 4-hydroxylase was assayed in a total volume of 1 ml and incubated for 30 min at 25 °C. The assay mixture contained 25 to 100 µl microsomal preparations (0.1 to 0.3 mg protein), 50 µM cinnamic acid (in 25 µl MeOH; Roth, Karlsruhe), 1 mM NADPH and buffer (100 mM potassium phosphate, pH 7.0, with 400 mM sucrose). The reaction was terminated and the products were extracted with ethyl acetate (3 × 2 ml). The organic phases were collected, dried with a rotary evaporator (30 °C) and redissolved in 150 µl MeOH. Aliquots of 20 µl were analyzed by HPLC methods. Formononetin 2′- and 3′-hydroxylase were determined according to Hinderer *et al.* [21] with the above mentioned modifications.

Chromatographic analyses

Phytoalexins were separated on a Merck LiChrosorb RP select B HPLC column (5 µm, 250 × 4 mm i.d.) according to Köster *et al.* [35, 36]. Separation of substrate and product from the assays for cinnamic acid 4-hydroxylase was achieved on a Merck LiChrosher 100 RP select B HPLC column (5 µm, 250 × 4 mm i.d.). A linear gradient was run from 25% to 65% eluent B in 12 min (eluent A: 1.5% phosphoric acid; eluent B: 84% acetonitrile in water) with a flow rate of 0.8 ml/min at room temperature. Cinnamic acid and *p*-coumaric acid were detected at 290 nm wavelength and quantitated with external standards.

Formononetin, 2′- and 3′-hydroxyformononetin were separated on a WATERS µBondapak C18 column (10 µm; 300 × 3.9 mm i.d.) at room temperature with a flow rate of 1 ml/min and a linear gradient from 35% to 60% eluent B in 25 min (eluent A: 1.5% phosphoric acid; eluent B: 80% acetonitrile in water). The isoflavones were monitored at 248 nm wavelength and their amounts calculated with external standards.

Protein determination

Microsomal protein was determined by the method of Bradford [37] with bovine serum albumin (Cohn fraction V, Sigma, Munich) as reference.

Results

Upon elicitation cell suspension cultures of chickpea accumulate the pterocarpan phytoalexins medicarpin and maackiain in essentially the same manner as intact plants when infected with the fungal pathogen *Ascochyta rabiei* [1, 31]. Therefore we used such chickpea cell suspension cultures to determine biochemical differences between resistant and susceptible cultivars.

Upon elicitation with yeast extract cell suspension cultures from the resistant cultivar ILC 3279 accumulated large amounts of the phytoalexins and concomitantly formononetin 2′- and formononetin 3′-hydroxylase activities were strongly induced [21]. In our present study we changed to a yeast glucan preparation as a more defined source of elicitor which mainly consists of glucose and mannose residues [33, 38, 39]. The glucose content of our elicitor preparation was determined to be 450 nmol glucose residues per mg dry powder.

In Fig. 2A the effect of this elicitor preparation on phytoalexin accumulation is demonstrated. The cell suspension culture from the resistant cultivar accumulated phytoalexins with a maximum of 944 nmol/g fresh weight (in cells and medium) 12 h after elicitation. Medicarpin was the predominant phytoalexin as has been previously observed with an elicitor preparation from the natural pathogen *Ascochyta rabiei* [31]. In contrast, cell suspension cultures from the susceptible cultivar ILC 1929 accumulated only low amounts of both phytoalexins (38 nmol/g fresh weight in cells and medium) after elicitation with an identical quantity of the glucan elicitor. But in case of the latter culture both phytoalexins reached maximum levels earlier namely maackiain 4 h and medicarpin 8 h after addition of the elicitor.

Further experiments were designed to measure the ability of the cells from the susceptible cultivar to possibly produce larger amounts of the phytoalexins upon addition of increasing amounts of the elicitor. We, therefore, compared both cell suspension cultures with respect to the dose response of the elicitor on the accumulation of phytoalexins (Fig. 2B). In cell suspension cultures from the resistant cultivar ILC 3279 medicarpin accumulation responded very sensitively to elicitation even at the lowest elicitor concentration (1 mg per 40 ml cell suspension culture) and reached its maximum

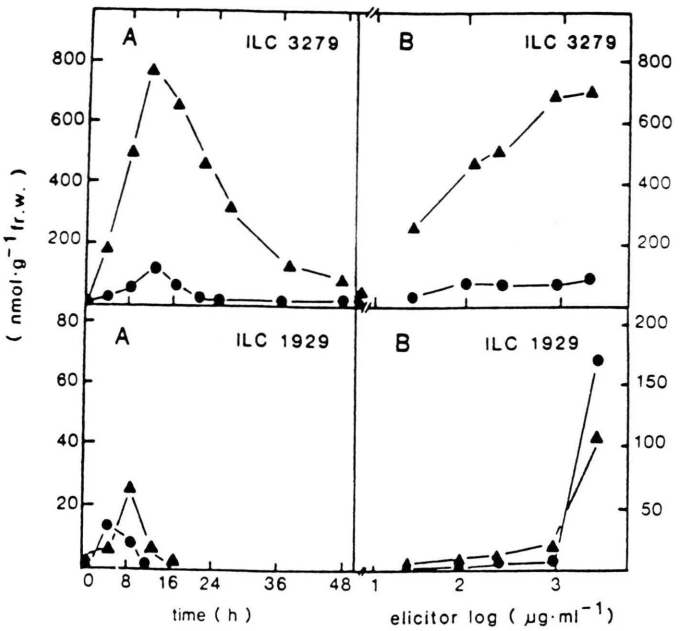

Fig. 2. Accumulation of the pterocarpan phytoalexins medicarpin (▲—▲) and maackiain (●—●) in chickpea cell suspension cultures upon elicitation. A: Time course of accumulation in cell cultures from the *A. rabiei* resistant cultivar ILC 3279 and the susceptible cultivar ILC 1929 after application of 40 mg yeast elicitor per 40 ml cell suspension. B: Accumulation 12 h after elicitation with different yeast glucan elicitor concentrations (1 to 100 mg per 40 ml cell suspension).

level at 40 mg. Maackiain accumulation rose only slightly. In contrast, the cell suspension culture from the susceptible cultivar ILC 1929 responded very weakly to elicitation with the exception of the highest elicitor concentration (100 mg per 40 ml cell culture). However the amounts of medicarpin (87 nmol/g) did not reach those (147 nmol/g) obtained with the lowest elicitor concentration (1 mg per 40 ml) in cell suspension cultures from the resistant cultivar ILC 3279. These findings indicate that the significantly higher phytoalexin accumulation in cell suspension cultures from the resistant cultivar is due to a higher sensitivity towards the elicitor.

Higher accumulation rates should require higher enzyme activities. Therefore we assayed three microsomal cytochrome P-450 monooxygenases involved in the biosynthesis of medicarpin and maackiain: cinnamic acid 4-hydroxylase from the general phenylpropanoid pathway as well as formononetin 2′-hydroxylase and formononetin 3′-hydroxylase. As shown in Fig. 1 medicarpin biosynthesis only requires formononetin 2′-hydroxylase, whereas maackiain biosynthesis involves both formononetin 3′-hydroxylase and subsequently formononetin 2′-hydroxylase.

In time course experiments (Fig. 2A) cell suspension cultures from the resistant and the susceptible cultivar were inoculated with 40 mg yeast elicitor per 40 ml culture and water as control respectively. The activities of the above mentioned monooxygenases were monitored for 48 h (Fig. 3).

Cinnamic acid 4-hydroxylase started from a basal level varying between 2 and 6 µkat/kg and has been induced after elicitor application up to 13 µkat/kg in cell suspension cultures from the resistant cultivar. In cell suspension cultures from the susceptible cultivar (ILC 1929) C4H has also been induced to nearly the same maximum activity (10.9 µkat/kg). In the controls there are minor differences in C4H activity. Cell suspension cultures from the resistant cultivar ILC 3279 seemed to respond more sensitively to the handling of flasks and injection of elicitor solution than those from the susceptible cultivar. This has led to a clear but lower induction in the controls compared with the elicitor-treated cultures (Fig. 3).

While C4H is involved not only in phytoalexin biosynthesis the isoflavone monooxygenases are exclusively involved in the biosynthetic pathway leading to the two pterocarpan phytoalexins medicarpin and maackiain (Fig. 1). Formononetin 2′-

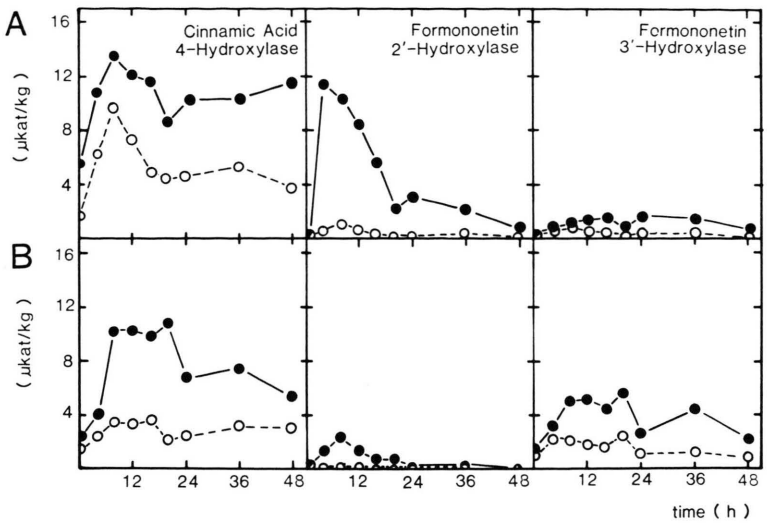

Fig. 3. Time course of the enzyme activities of cinnamic acid 4-hydroxylase and formononetin 2'- and 3'-hydroxylase after elicitation with 40 mg yeast elicitor per 40 ml cell suspension (●—●) and controls (○—○). Enzyme activities were measured with microsomes from cell suspension cultures of the *A. rabiei* resistant cultivar ILC 3279 (A) and of the susceptible cultivar ILC 1929 (B).

hydroxylase (F2H) was nearly absent in non-induced cell suspension cultures of both cultivars. After elicitation F2H activity rose very rapidly in cell cultures from the resistant cultivar to reach a maximum (11.4 µkat/kg) 4 h after elicitation but declined equally rapidly (Fig. 3A). In contrast F2H activity in cell suspension cultures from the susceptible cultivar was induced only very weakly (2.4 µkat/kg) with a maximum 8 h after elicitation (Fig. 3B). Formononetin 3'-hydroxylase (F3H) which is only involved in maackiain biosynthesis has been assayed over the same period after elicitation (Fig. 3). This enzyme showed a totally different induction pattern. In cell suspension cultures from the resistant cultivar F3H activity started from a very low level and was induced very weakly without any sharp maximum (Fig. 3A). Cell suspension cultures from the susceptible cultivar contained appreciable constitutive F3H activity. After elicitation this activity rose to a plateau lasting from 8 to 20 h and then declined (Fig. 3B). Although F3H activity was significantly lower in cell suspension cultures from the resistant cultivar (1.5 µkat/kg) than in the cells of the susceptible one (5.0 µkat/kg) the former cell cultures accumulated much more maackiain (145 nmol/g) than cell suspension cultures from the susceptible cultivar (14 nmol/g; Fig. 2). This discrepancy has to be examined in connection with F2H activity which is additionally required for maackiain biosynthesis. As shown before this enzyme was strongly induced in cell suspension cultures from the resistant cultivar, but very weakly in those from the susceptible cultivar (Fig. 3).

To confirm the assumption that F2H represents an important gap for phytoalexin accumulation we investigated the three monooxygenases (Fig. 4) in relation to the corresponding phytoalexin accumulation (Fig. 2B) after application of different elicitor concentrations (1 to 100 mg per 40 ml suspension). In both cell suspension cultures C4H activity rose nearly identically with the application of increasing amounts of yeast glucan elicitor (Fig. 4). F2H activity significantly differed as a consequence of increasing amounts of elicitor. While enzyme activity in cell suspension cultures from the susceptible cultivar remained very low, this F2H activity (Fig. 4A) rose in parallel to medicarpin accumulation (Fig. 2B) in cell suspension cultures from the resistant cultivar. Essentially as observed in the time course experiments (Fig. 3) the behaviour of F3H activity was again totally different. F3H activity in cell suspension cultures from the resistant cultivar (Fig. 4A) was elevated to a constant level even at the lowest elicitor concentration with no further increase. In cell suspension cultures from the susceptible cultivar (Fig. 4B) the monooxygenase activity rose with increasing amounts of elicitor. Finally in both cell suspension cultures F3H activity declined at the highest elicitor concentration.

Beside formononetin (7-hydroxy-4'-methoxy-

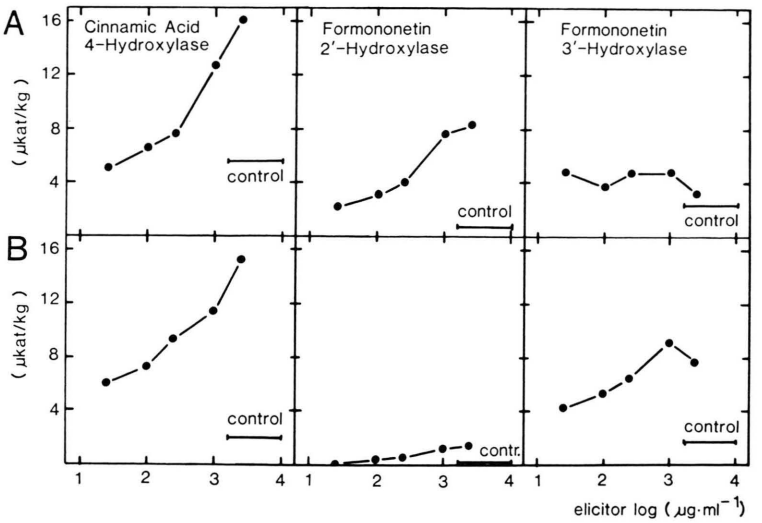

Fig. 4. Enzyme activities of cinnamic acid 4-hydroxylase and formononetin 2'- and 3'-hydroxylase 12 h after elicitation with different amounts of yeast glucan elicitor (1 to 100 mg per 40 ml cell suspension). Enzyme activities were measured with microsomes from cell suspension cultures of the *A. rabiei* resistant cultivar ILC 3279 (A) and of the susceptible cultivar ILC 1929 (B).

isoflavone) chickpea plants contain as a second major phenolic compound biochanin A (5,7-dihydroxy-4'-methoxyisoflavone; [35, 36]). Nevertheless no pterocarpan phytoalexin derived from biochanin A as an intermediate has so far been observed. Surprisingly the microsomal fraction of chickpea cell suspension cultures from the resistant cultivar ILC 3279 was able to form 2'- and 3'-hydroxybiochanin A [21]. The latter compound has been detected as a naturally occurring constituent in chickpea [40]. Therefore we also examined the hydroxylation of biochanin A in both sets of experiments (Gunia and Barz, unpublished). In short biochanin A 2'-hydroxylase showed similar values for specific activity and induction kinetics as F2H. Biochanin A 2'-hydroxylase was strongly induced in cell suspension cultures from the resistant cultivar, but not in those from the susceptible cultivar (weak induction, data not shown). Similar differences were observed with the biochanin A 3'-hydroxylase in both cell culture lines. This leads us to the assumption that there exists one isoflavone 2'-hydroxylase (F2H) which is able to convert formononetin as well as biochanin A to the corresponding 2'-hydroxy compounds. With isoflavone 3'-hydroxylase such a decision cannot yet be made.

Discussion

Chickpea plants accumulate the pterocarpan phytoalexins medicarpin and maackiain (Fig. 1) upon infection with its main fungal pathogen *Ascochyta rabiei* [41]. The pathway of medicarpin and maackiain biosynthesis has been elucidated in experiments with labeled precursors using $CuCl_2$-treated *Medicago sativa* and *Trifolium pratense* seedlings [24] and with elicitor-treated cell suspension cultures from which all relevant enzymes have been isolated [42]. Investigations by Weigand *et al.* [1] revealed that the resistant cultivar ILC 3279 was able to accumulate considerably larger amounts of these phytoalexins than the susceptible cultivar ILC 1929. This difference has consistently been found in various later investigations with chickpea plants and cell cultures [31, 42].

The chickpea cell suspension cultures from the two above mentioned cultivars have turned out to be an excellent system to investigate the antifungal defence reactions of this plant and the enzymes involved in these pathways. An important advantage of cell cultures is the exact exposure of nearly all cells to the inducing agents [23] and even enables the measurement of trace amounts of enzymes.

An acceptable procedure to mimick fungal infection in cell suspension cultures is the application of elicitors, preferentially biotic elicitors from fungal mycelia [31, 33, 43, 44]. In the present study we used an elicitor prepared from the cell walls of commercially available baker's yeast. This elicitor has been successfully used to induce glyceollin accumulation in soybean [38, 39] and benzophenanthridine alkaloid biosynthesis in *Escholtzia califor-*

nica and *Thalictrum rugosum* cell suspension cultures [33, 38, 45, 46].

In Fig. 2 the inducing effect of this yeast glucan elicitor is well documented and the results resemble experiments obtained with chickpea cell suspension cultures and an elicitor preparation from mycelia of the natural chickpea pathogen *Ascochyta rabiei* [47–49]. The quantitative difference in phytoalexin accumulation observed with intact chickpea plants [1] of both cultivars after infection has again been confirmed with this cell culture system after elicitation with the yeast glucan elicitor.

Upon elicitation the phytoalexins accumulated in the cells as well as in the culture medium with the major portion in the latter. For medicarpin about 66% and for maackiain about 75% of the total amount have been found in the medium of cell suspension cultures from the resistant cultivar ILC 3279. This secretion of phytoalexins into the medium is a widespread phenomenon in cell suspension cultures [44, 50].

In addition to phytoalexins enzymes are also secreted from the cells into the medium [51]. In chickpea cell suspension cultures extracellular peroxidases have been shown to metabolize medicarpin and maackiain [52]. However, careful measurements revealed no significant differences in peroxidase activity in the medium of the cell cultures of both chickpea cultivars (Gunia and Barz, unpublished; [52]). This implies that the different levels of phytoalexin accumulation in cell suspension cultures from resistant and susceptible cultivars are not caused by different rates in their catabolism, but are a result of differences in the rate of medicarpin and maackiain biosynthesis.

The quantity of phytoalexins being synthesized depends on the presence and sufficient activity of all enzymes involved in the biosynthetic pathway. The fact that both pterocarpan phytoalexins also accumulated in cell suspension cultures from the susceptible cultivar is evidence for the expression of all enzymes involved in their biosynthesis during the accumulation period. Therefore special attention has to be directed at those enzymes being absent or having extremely low activity in non-induced cell cultures, but are active just before or during highest phytoalexin accumulation after elicitation. It has been shown that PAL, chalcone synthase and chalcone isomerase [29, 47, 48] as well as cinnamic acid 4-hydroxylase (this work) are inducible in cell suspension cultures from both cultivars. Rapid increase of the key enzymes of the phenylpropanoid pathway upon elicitation has also been observed in other plant systems such as bean [53–55] by quantitating the corresponding mRNAs and in alfalfa [56].

In the terminal biosynthetic sequence leading to the phytoalexins medicarpin and maackiain isoflavone reductase and pterocarpan synthase were both present in the non-induced cell suspension cultures from both cultivars with appreciable activity, although both activities rose after elicitation [26, 29, 48].

In contrast, formononetin 2′-hydroxylase (F2H) was nearly absent in non-induced cell cultures of both cultivars (Fig. 3). Formononetin 3′-hydroxylase (F3H) has been detected in the cell suspension cultures from the susceptible cultivar with low activity in the non-induced state (1.3 to 1.5 μkat/kg). After addition of yeast glucan elicitor F3H activity has been induced only moderately (Fig.3) whereas F2H showed a sharp increase in its activity only in cell suspension cultures from the resistant cultivar (Fig. 3A) and not in those from the susceptible cultivar (Fig. 3B).

Recently a short transient induction of cytochrome P-450 monooxygenases in cell suspension cultures from alfalfa has also been reported [56]. The enzyme activities of isoflavone 2′-hydroxylase increased and disappeared after elicitation with an elicitor from *Colletotrichum lindemuthianum* in a similar manner as has been observed in chickpea cell cultures from the resistant cultivar ILC 3279 (this work).

The increase and subsequent decrease of F2H behaved like a typical transient induction with a short transcription period and a high mRNA turnover. The term "induction" used in the following text will only be understood in the meaning of increase of enzyme activity. At present we cannot exclude the possibility of enzyme activation, but it is very probable that the induction results from *de novo* biosynthesis of the enzymes involved in phytoalexin biosynthesis [54, 57]. *In vitro* translation studies with mRNA isolated from chickpea cell suspension cultures revealed that two new isoforms of chalcone synthase appeared after elicitation with a cell wall preparation from *Ascochyta rabiei* [57]. In addition *de novo* enzyme synthesis has indirectly been shown by inhibition experi-

ments with actinomycin D or cycloheximide [52] and by an elicitor-caused increase of the cytochrome P-450 content in chickpea microsomes as calculated from CO difference spectra (Gunia, Wittkampf, and Barz, unpublished).

The transient induction of F2H preceded the accumulation of phytoalexins by about 4 h (Fig. 2A) especially for medicarpin. Though appreciable F3H activity has been induced in cell suspension cultures from the susceptible cultivar (Fig. 3B) the very low F2H activity appears to be responsible for the formation of the small amounts of maackiain and medicarpin. As mentioned before medicarpin biosynthesis requires only one hydroxylation step (F2H) while maackiain biosynthesis first involves 3′-hydroxylation and then 2′-hydroxylation.

In medicarpin and maackiain biosynthesis the isoflavone hydroxylases are of special interest because of their location at a branch point (Fig. 1) which is often subject to metabolic regulation and to differential induction [46, 47, 53]. In chickpea cell suspension cultures it is probable that hydroxylation of formononetin in positions 2′ and 3′ is catalyzed by either the same or two distinct enzymes. With regard to the results shown in Fig. 3 and Fig. 4 we obtained evidence for the assumption that these hydroxylations of formononetin are catalyzed by two distinct P-450 enzymes. During the time course experiment formononetin 2′-hydroxylase (F2H) activity and formononetin 3′-hydroxylase (F3H) activity behaved quite differently upon elicitation. In cell suspension cultures from the resistant cultivar ILC 3279 (Fig. 3A) F2H was strongly increased while F3H showed only low activation. In cell suspension cultures from the susceptible cultivar ILC 1929 (Fig. 3B) F2H was only slightly induced whereas F3H activity increased significantly. The existence of two formononetin hydroxylases was also indicated by experiments with cytochrome P-450 monooxygenase inhibitors (Wittkampf and Barz, unpublished).

Upon elicitation with increasing amounts of yeast elicitor F2H activity increased concomitantly whereas F3H activity remained nearly constant (Fig. 4). The different induction behaviour of 2′- and 3-isoflavone hydroxylases after elicitation has also been observed with biochanin A as substrate (data not shown). If the hydroxylation at position 2′ or 3′ of the B-ring of formononetin would have been catalyzed by only one enzyme we should have expected a similar induction pattern for both hydroxylase activities, this is clearly not the case. We therefore conclude from these results that formononetin 2′- and 3′-hydroxylase are different cytochrome P-450 monooxygenases.

The differential induction behaviour of C4H, F2H and F3H (Fig. 3 and 4) also demonstrates that there is no general induction mechanism of cytochrome P-450 monooxygenases. Recent work by Zimmerlin and Durst [58] on the differential induction of C4H, lauric acid in-chain hydroxylase and diclofop aryl hydroxylase confirmed the view, that a sophisticated regulation for different cytochrome P-450 monooxygenases exists in plants.

In conclusion the large differences in phytoalexin accumulation between cell suspension cultures from the resistant (ILC 3279) and the susceptible (ILC 1929) cultivar (Fig. 3 and 4) are mainly due to large differences in the expression of formononetin 2′-hydroxylase activity. This insufficient activity of formononetin 2′-hydroxylase together with the small number and low expression of various pathogenesis-related proteins [29, 57] appears to be a characteristic feature of chickpea cultivars susceptible to *A. rabiei*.

At this stage of knowledge we do not possess sufficient experimental data to decide whether the different F2H activity after elicitation of cell cultures from both cultivars is caused by differences in gene activation or by expression of enzymes which differ in their kinetic properties with regard to formononetin conversion. To obtain an insight into this mechanism our present experiments are designed to quantitate the amount of F2H specific mRNA which will be synthesized in both cell suspension cultures after elicitation. In our future research we will also have to prove our hypothesis that only formononetin 2′-hydroxylase is responsible for the different amounts of phytoalexins accumulated after induction. This work will also include measurements of the corresponding enzyme activities in intact plants from both cultivars after infection with spores from *A. rabiei*.

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