Elicitor-Induced Formation of Pterocarpan Phytoalexins in Chickpea (*Cicer arietinum* L.) Cell Suspension Cultures from Constitutive Isoflavone Conjugates upon Inhibition of Phenylalanine Ammonia Lyase*

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After inhibition of phenylalanine ammonia lyase by L-α-aminooxy-β-phenylpropionic acid, the constitutively formed formononetin 7-O-glucoside-6″-O-malonate is metabolized with the isoflavone aglycone being used as an intermediate in the elicitor-induced formation of pterocarpan phytoalexins in chickpea cell suspension cultures. In elicited cultures not treated with the inhibitor phytoalexins are synthesized *de novo* from phenylalanine. Therefore, in chickpea cells the constitutive isoflavone conjugate metabolism and the elicitor-induced pterocarpan formation show metabolic linkage under specific physiological conditions.

Introduction

The main phenolic compounds of chickpea (*Cicer arietinum* L.) plants and cell suspension cultures are the isoflavones biochanin A and formononetin which predominantly occur as the 7-O-glucoside-6″-O-malonates [3]. The enzymology of isoflavone conjugation and metabolism in *Cicer arietinum* has intensively been studied [4–7].

Numerous investigations have amply demonstrated the increasing importance of malonyl conjugates in plant metabolism [8] because several classes of secondary constituents, D-configurated amino acids, end products of pesticide degradation and intermediates of phytohormone production occur in malonylated form. In addition to chickpea and other previously analyzed plants [3] very recent investigations also demonstrated that the isoflavones in soybean and alfalfa mainly accumulate as the 7-O-glucoside-6″-O-malonates [9, 10]. Such malonyl conjugates are clearly deposited in vacuoles [11, 12] where they are thought to be stored as stable end products of cellular metabolism [2].

In contrast to this assumption the malonylglucoside of the 5-deoxyisoflavone formononetin (structure Fig. 4) in chickpea tissue is in a state of permanent turnover because substantial rates of metabolism with a biological half-life of approx. 70–120 h have been measured [13]. Interestingly, the co-occurring biochanin A malonylglucoside was shown to be metabolically inert. The adherent aspects of selective vacuolar influx and efflux have been reviewed [2].

Upon infection with the phytopathogenic fungus *Ascochyta rabiei* or treatment with elicitors, chickpea plants and cell suspension cultures accumulate the two pterocarpan phytoalexins medicarpin and maackiain (structures Fig. 4) [14, 15] which are important components of the plant defense system.

These pterocarpans essentially as other phytoalexins [16] are synthesized *de novo* from early precursors of primary metabolism [15]. The biosynthetic sequence leading from phenylalanine to the pterocarpans (Fig. 4) has recently been fully elucidated in this laboratory [15] and the essential enzymes which are all formed by *de novo* synthesis [17] have been characterized from chickpea plants and cell cultures [18].

Although the isoflavone formononetin acts as a central intermediate in the biosynthesis of medi-

Abbreviations: L-AOPP, L-α-aminooxy-β-phenylpropionic acid; PAL, phenylalanine ammonia lyase; PAX, phytoalexins; CHS, chalcone synthase; FGM, formononetin 7-O-glucoside-6″-O-malonate; BGM, biochanin A 7-O-glucoside-6″-O-malonate.

* The data of this contribution are from the Doctoral Thesis of U. M. [1] and have partly been presented earlier [2].

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carpin and maackiain (Fig. 4) the pool of the constitutively accumulating formononetin malonylglucoside remained unaltered at times of massive pterocarpan formation in chickpea cell cultures. We therefore concluded that no metabolic link exists between the constitutive routes of formononetin metabolism and the elicitor-induced pterocarpan biosynthetic pathway which might draw the isoflavone from the conjugate pool into phytoalexin formation.

We have now extended our investigations on the regulatory pattern of constitutive isoflavone conjugate turnover and elicitor-caused pterocarpan biosynthesis in chickpea cell suspension cultures. Using the specific PAL inhibitor L-aminooxy-β-phenylpropionic acid (L-AOPP) [19, 20] in combination with feeding experiments we tried to demonstrate whether the formation of the pterocarpans will always proceed completely *de novo* or whether the demand for the isoflavone intermediate may not be covered from the constitutive pool of the formononetin malonylglucoside. The results obtained indeed show that under the specific conditions of PAL inhibition the pterocarpans may be synthesized from the preformed isoflavone pool. Thus a metabolic linkage between the two formally independent routes could be demonstrated. The adherent aspects of the regulatory mechanism of this linkage are discussed.

Materials and Methods

Cell suspension cultures

Cell suspension cultures of *Cicer arietinum* L. cultivar ILC 3279 were grown in a modified PRL 4c medium [21] under conditions previously described [22].

Chemicals

[U-^{14}C]Phenylalanine (spec. radioact. 1.66×10^{10} Bq/mmol) and side chain [3-^{14}C]cinnamic acid (spec. radioact. 2×10^{10} Bq/mmol) were purchased from Amersham Buchler (Braunschweig, F.R.G.). Ring A [^{14}C]formononetin (spec. radioact. 0.9×10^{7} Bq/mmol) was obtained from the institute's collection. The translation and transcription inhibitors cycloheximide and actinomycin D were purchased from Sigma (Munich, F.R.G.), L-α-aminooxy-β-phenylpropionic acid (L-AOPP) was a gift from Prof. N. Amrhein (ETH Zürich, Switzerland).

Application of actinomycin D, cycloheximide and fungal elicitor

The required amounts of actinomycin D and cycloheximide, dissolved in ethanol and filter sterilized, were applied to the cell cultures on day 3 of the growth cycle. For cell culture elicitation 5 mg of an elicitor preparation from the phytopathogenic fungus *Ascochyta rabiei* [23], dissolved in 1 ml sterile water, were added aseptically. The control flasks were supplied with 1 ml sterile water or 5 mg elicitor in 1 ml water. 10 h after elicitation the cells and the growth media were extracted for quantitation of the phenolic compounds [22].

Application of L-AOPP

Cell cultures were treated with 5×10^{-4} M L-AOPP 3 days after subculture. The inhibitor was dissolved in water and filter-sterilized before application.

After an incubation period of 4 h, 5 mg of an elicitor preparation from *Saccharomyces cerevisiae* [24], dissolved in 1 ml sterile water, were added aseptically to the cell cultures. 10 h after elicitation the phenolic compounds recovered from the cells and the culture media were quantitated by HPLC.

As controls were used: a) untreated cell suspension cultures of chickpea, b) cell suspension cultures treated with 5×10^{-4} M L-AOPP and c) cell cultures treated with 5 mg yeast elicitor without previous L-AOPP treatment.

Feeding experiments

[U-^{14}C]Phenylalanine (1.5 μCi) was added to chickpea cell suspension cultures on day 3 of the growth cycle. 8 h after application of the radioactive compound, 5×10^{-4} M L-AOPP was also added to the cell cultures and 4 h after this addition of the PAL inhibitor phytoalexin formation was induced by application of 5 mg yeast elicitor. Controls were elicited without a previous treatment with L-AOPP. The phenolic compounds were extracted and quantitated 10 h after elicitor treatment.

Ring A [^{14}C]formononetin (200,000 dpm) was applied to the chickpea cell cultures also on day 3 of the growth cycle. The PAL inhibitor L-AOPP

(5×10^{-4} M) was added 8 h after feeding of ring A [^{14}C]formononetin. After a further incubation period of 4 h, the cell cultures were elicited by application of 5 mg yeast elicitor.

10 h after elicitation the phenolic compounds in the cells and the culture media were extracted and quantitatively analyzed. As controls were used: a) normal untreated cell cultures, b) cell cultures after application of 200,000 dpm ring A [^{14}C]formononetin and c) cell cultures elicited after feeding of ring A [^{14}C]formononetin without previous L-AOPP treatment.

Extraction and quantitation of isoflavones and phytoalexins

Extraction of isoflavones, isoflavone conjugates and phytoalexins from cells and media was carried out according to Köster *et al.* [25]. The isoflavones and phytoalexins were quantitated by HPLC techniques as described [3, 25].

For the additional determination of radioactivity of the isoflavones and the phytoalexins an HPLC apparatus coupled with a radioactivity counter (RAMONA 5, ray test) was used.

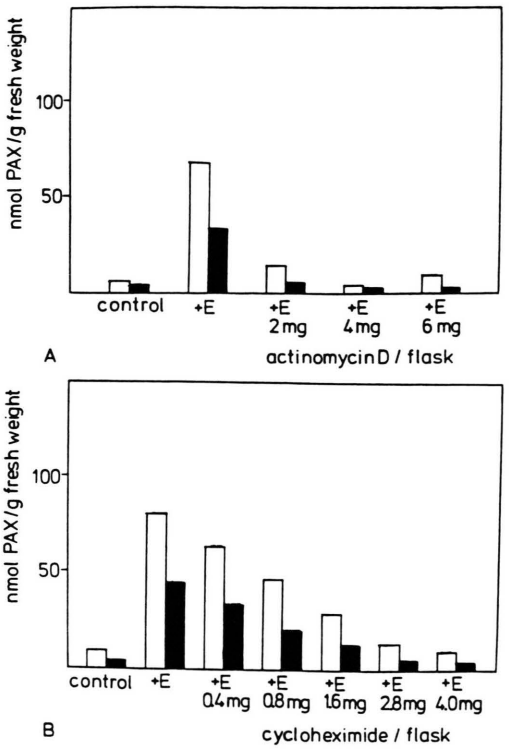


Fig. 1. Effect of different amounts of the transcription inhibitor actinomycin D (panel A) and the translation inhibitor cycloheximide (panel B) on the elicitor-induced accumulation of phytoalexins in chickpea cell suspension cultures. The control represents untreated cells. The bars (□ medicarpin; ■ maackiain) marked "+E" show the effect of elicitor only. 5 mg elicitor per 40 ml cell suspension culture were given and phytoalexins were quantitated 10 h after elicitation.

Results

Inhibition of pterocarpan formation with actinomycin D and cycloheximide

Chickpea cell suspension cultures established from cultivar ILC 3279 constitutively accumulate substantial amounts of the malonylglucosides of formononetin and biochanin A [22]. Without elicitation the phytoalexins medicarpin and maackiain are only formed in trace amounts if at all. Elicitation of such cultures leads to rapid induction of pterocarpan biosynthetic enzymes [17, 26] and to accumulation of the phytoalexins (Fig. 1a and 1b). The maximum of this accumulation is reached after some 10 h with the bulk of the phytoalexins being found in the cell culture medium. In this report data for phytoalexin accumulation are always given as the sum of medicarpin (approx. 80% of the total) and maackiain.

This elicitor-induced phytoalexin biosynthesis is substantially suppressed by the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide (Fig. 1a and 1b).

Simultaneous application of elicitor and either 10^{-5} M actinomycin D (0.5 mg/flask) or 10^{-4} M cycloheximide (1.2 mg/flask min) respectively, to the cell suspension cultures led to a 50% inhibition of phytoalexin accumulation. Treatment of the cell cultures with 0.8×10^{-4} M actinomycin D (4 mg/flask) or 3.5×10^{-4} M cycloheximide (4 mg/flask) blocked the elicitor-induced phytoalexin accumulation almost completely. This data again supports the earlier reports that elicitation leads to both *de novo* synthesis of enzymes required for phytoalexin biosynthesis and of the corresponding mRNAs [17, 27].

In these elicitation and inhibitor experiments (Fig. 1a, 1b) the level of the constitutively formed formononetin conjugate remained completely un-

altered and even in the controls without molecular inhibitor FGM appeared to be no substrate for phytoalexin formation (data not shown).

Experiments with [^{14}C]phenylalanine and L-AOPP

In elicitor-treated chickpea cell suspension cultures the newly synthesized pterocarpan phytoalexins are derived from phenylalanine as indicated by the efficient conversion of [^{14}C]phenylalanine into the pterocarpans (Fig. 2a). For this labelling experiment the [^{14}C]amino acid was applied to the cell cultures a few hours prior to elicitation. However, this labelling of the pterocarpans by [^{14}C]phenylalanine was completely inhibited when the elicitor-treated cells were simultaneously applied with 5×10^{-4} M L-AOPP (Fig. 2b). Though pterocarpan phytoalexins were still formed under these conditions (Fig. 2b) the aforementioned concentration of L-AOPP obviously completely blocked the PAL enzyme reaction in the chickpea cells.

Experiments with [^{14}C]formononetin and L-AOPP

In order to investigate whether the formononetin-malonate ester can indeed be metabolized with the aglycone subsequently being used as an intermediate for the elicitor-induced phytoalexin formation, feeding experiments with [^{14}C]formononetin and inhibitory studies with L-AOPP were carried out.

When [^{14}C]formononetin is applied to the chickpea cell suspension cultures the isoflavone is readily taken up and funnelled into the pool of FGM. After some 8 h nearly 90% of the applied radioactivity could be found in the malonyl glucoside of formononetin (data not shown).

These results demonstrate that the vacuolar pool of FGM can efficiently and selectively be labelled by the incorporation of [^{14}C]formononetin. This observation provided the basis for the following experiments.

If elicitor-induced phytoalexin accumulation will occur despite of a complete inhibition of the introductory enzyme of the phenylpropane pathway by L-AOPP treatment, the observed phytoalexins must have been synthesized from the pool of labelled formononetin malonylglucoside. Quantitative determinations of compounds together with total and specific radioactivity will clearly prove this assumption.

Chickpea cell suspension cultures pretreated with [^{14}C]formononetin were first incubated with 5×10^{-4} M L-AOPP and then elicited with an elicitor preparation from *Saccharomyces cerevisiae* [24] (5 mg/20 ml cell suspension). In parallel assays such pretreated chickpea cultures were elicited without the additional application of L-AOPP. The phenolic compounds from the medium and the cells were separately extracted 10 h after elicitation. Isoflavones and pterocarpans were analyzed and quantitated by HPLC techniques coupled with a radioactivity counter. Table I summarizes the results of these experiments.

After feeding of [^{14}C]formononetin the amount of the isoflavone malonyl conjugate had increased from 150 nmol/g fr. wt. up to 420 nmol/g fr. wt. Without elicitation only very small amounts of phytoalexins could be detected. Elicitation of the cells labelled with [^{14}C]formononetin led to an accumulation of 260 nmol phytoalexins/g fr. wt., while the content of the formononetin conjugate had decreased to 200 nmol/g fr. wt. Determination of the radioactivity of the compounds showed that

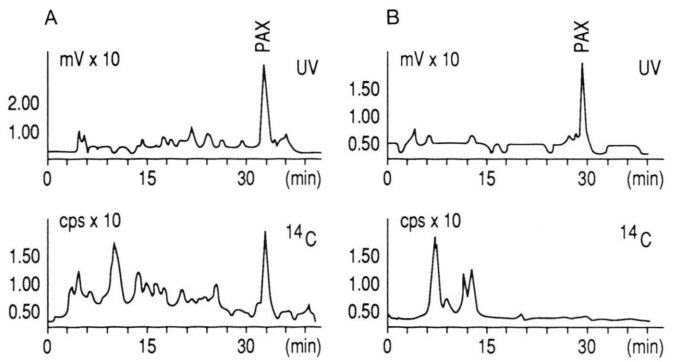


Fig. 2. HPLC chromatograms recorded by UV absorption (upper panels) and ^{14}C radioactivity (lower panels) of the pterocarpan phytoalexins (PAX) isolated from the culture medium of chickpea cell suspension cultures. A: after application of [^{14}C]phenylalanine followed by elicitation; B: after application of [^{14}C]phenylalanine, subsequent elicitation but pretreatment of cells with 5×10^{-4} M L-AOPP.

Table I. Amount, total radioactivity and specific radioactivity of the cellular formononetin 7-O-glucoside-6″-O-malonate (FGM) and of the elicitor-induced phytoalexins (PAX) medicarpin and maackiain after different feeding of [^{14}C]formononetin, application of L-AOPP and of elicitor (n.m.: not measurable).

	Amount [nmol/g fr. wt.]		Total radioactivity [dpm]		Spec. radioactivity [dpm/μmol]	
	FGM	PAX	FGM	PAX	FGM	PAX
Pool in untreated cells	150	n.m.	–	–	–	–
Pool after uptake of [^{14}C]formononetin	420	21	120,000	n.m.	107,000	n.m.
Pool after uptake of [^{14}C]formononetin and addition of elicitor	200	260	70,500	54,800	103,000	66,700
Pool after uptake of [^{14}C]formononetin and addition of elicitor and L-AOPP	154	210	66,500	56,000	116,000	94,000

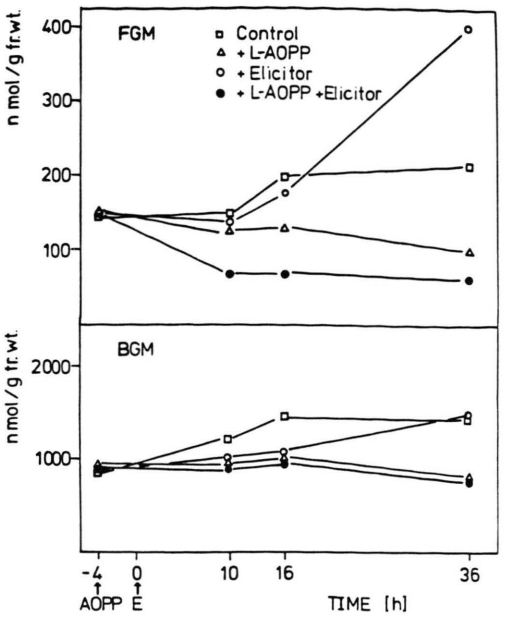
the synthesized phytoalexins were radioactive. Although the phenylpropane pathway had not been blocked by L-AOPP a portion of the phytoalexins was not synthesized *de novo* but rather formed from the formononetin-malonate ester pool. The radioactivity data show that some 70% of the phytoalexins had been derived from the formononetin conjugate with the residual 30% being synthesized *de novo*. One reason for this observation may have been the unphysiologically high formononetin-malonate ester content of the cells due to the feeding of [^{14}C]formononetin.

Cells which were elicited after feeding of [^{14}C]formononetin and L-AOPP treatment accumulated nearly 210 nmol phytoalexins/g fr. wt. (Table I). Elicitation resulted in the formation of highly ^{14}C-labelled phytoalexins. This amount of phytoalexins must have been derived from the malonate ester pool exclusively because simultaneously to phytoalexin accumulation the concentration of the malonyl conjugate decreased from 420 nmol (measured after [^{14}C]formononetin application) to 154 nmol/g fr. wt. 10 h after elicitor application. Quantitation of total and specific radioactivity of the formononetin-malonate ester and the synthesized phytoalexins (Table I) provided conclusive evidence that the formononetin conjugate was the source for phytoalexin formation. This is clearly corroborated by the fact that the phytoalexins accumulating under these experimental conditions showed nearly the same specific radioactivity as the formononetin conjugate.

Elicitation of chickpea cell suspension cultures after L-AOPP treatment

In order to eliminate any possible negative influence of the pretreatment of cells with [^{14}C]formononetin on isoflavone and phytoalexin metabolism, a second experiment without labelling of the formononetin-malonyl conjugate pool was performed. Cell cultures at the 3rd day of growth were treated with 5×10^{-4} M L-AOPP for 4 h and then elicited with the elicited preparation from *S. cerevisiae*. As controls were included a) normally grown cell cultures, b) cell cultures only treated with L-AOPP and c) elicited cell cultures without previous L-AOPP application. In these parallel assays the cellular levels of FGM and BGM together with phytoalexin formation were measured over a period of 36 h. The results are shown in Fig. 3.

In the untreated control cells the expected growth-linked increase of FGM and BGM [22] with no phytoalexin formation was recorded. L-AOPP treatment of cells showed no inducing effect on phytoalexin formation but resulted in complete cessation of the accumulation of FGM and BGM obviously due to PAL inhibition. Elicitation of the cells in the absence of L-AOPP led to an accumulation of approx. 170 nmol phytoalexins/g fr. wt. which were obviously not derived from FGM because the pool of this conjugate showed no significant change. In the later phases of the experiment both FGM and even BGM significantly increased most likely as a result of stimulation of isoflavone biosynthesis.



Phytoalexin accumulation according to treatment

a. + L-AOPP: 9 nmol/ g fr.wt.
b. + elicitor: 170 nmol/ g fr.wt.
c. + L-AOPP: 72 nmol/ g fr.wt.
+ elicitor

Fig. 3. Changes in the cellular level of formononetin 7-O-glucoside-6″-O-malonate (FGM) and biochanin A 7-O-glucoside-6″-O-malonate (BGM) together with phytoalexin accumulation in chickpea cell suspension cultures after L-AOPP treatment, elicitor application and simultaneous treatment with L-AOPP and elicitor. Phytoalexins were quantitated 10 h after elicitation. Arrows indicate time of application of inhibitor (AOPP) and elicitor (E).

Inhibition of the PAL reaction by L-AOPP treatment and subsequent elicitation of the cells resulted in the formation of approx. 72 nmol phytoalexins/g fr. wt. This phytoalexin accumulation is correlated with a strong decrease of the formononetin 7-O-glucoside-6″-O-malonate ester from approx. 150 nmol/g fr. wt. at the beginning of the experiment to approx. 80 nmol/g fr. wt. 10 h after elicitor application. This decrease of 70 nmol quantitatively provides for the 72 nmol phytoalexins formed in this period. Therefore, inhibition of phenylalanine ammonia lyase in elicited cells leads to a consumption of constitutive formononetin conjugate for pterocarpan biosynthesis.

In these latter investigations the cellular content of the other constitutive isoflavone of chickpea, the biochanin A 7-O-glucoside-6″-O-malonate was not affected by the joint action of elicitation and L-AOPP treatment. This result indicates that there must be a very selective regulatory mechanism involving the pool of the formononetin conjugate only.

Discussion

Phytoalexins are defined as antimicrobial compounds which are synthesized *de novo* upon infection or elicitor treatment [16]. The adherent processes of gene activation and synthesis of specific mRNA molecules and of enzyme proteins involved in phytoalexin biosynthesis have repeatedly been reported [18, 28–30].

Our experiments with the transcription and translation inhibitors actinomycin D and cycloheximide again show that in chickpea cell suspension cultures *de novo* mRNA and protein synthesis are involved in the elicitor-induced formation of phytoalexins. Under normal growth conditions the pterocarpan phytoalexins, medicarpan and maackiain are formed *de novo* from phenylalanine. Under such conditions the pool of the constitutive formononetin-malonyl glucoside which might have been used for phytoalexin biosynthesis remains totally unaffected (Fig. 2a and 3).

Upon application of the PAL inhibitor L-AOPP we have, however, shown that in chickpea cells a regulatory mechanism seems to exist which allows the consumption of a constitutively formed formononetin conjugate for pterocarpan phytoalexin biosynthesis (Fig. 2a and 3). This conversion of the isoflavone moiety of FGM to the pterocarpans (Fig. 4) has unequivocally been demonstrated by the feeding experiments presented in Table I and the comparative investigations summarized in Fig. 3. These data taken together show that inhibition of PAL, *i.e.* the formation of *t*-cinnamic acid is prevented, is a prerequisite for FGM metabolism directed at pterocarpan formation (Fig. 2a, 3) and furthermore (Fig. 3, Table I) that the decrease of FGM and phytoalexin accumulation practically proceed in a stochiometric relation. Only if the FGM pool appears to be overloaded with comparatively high doses of formononetin elicitor-caused consumption of FGM occurs upon mere elicitation of cells without L-AOPP treatment (Table I). An interesting feature of the regulatory pattern in the depicted pathway (Fig. 4) is the observation

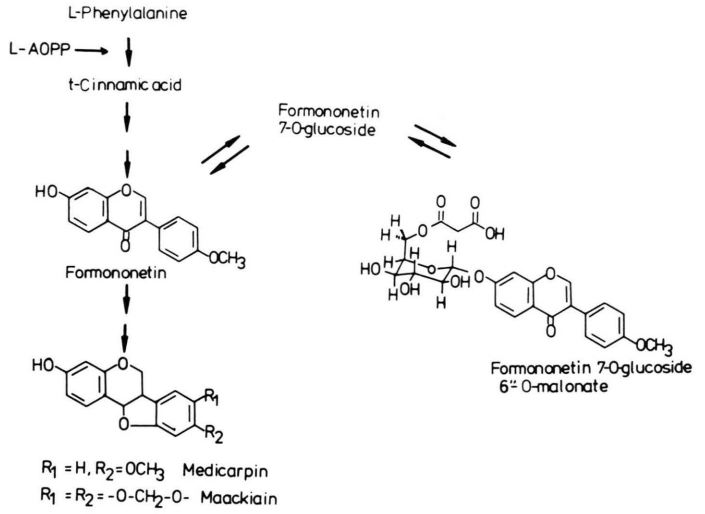


Fig. 4. Simplified scheme of metabolic linkage between the constitutive isoflavone and the elicitor-induced phytoalexin metabolism in chickpea (*Cicer arietinum* L.) cell suspension cultures. A complete scheme of the biosynthetic sequences is shown in [15, 17, 18].

(Fig. 3) that both vacuolar efflux and the conversion reaction appear to be restricted to FGM. The simultaneously occurring conjugate of the 5-hydroxyisoflavone biochanin A is clearly not mobilized (Fig. 3) and furthermore, no increase in the very low levels of biochanin A and its 7-O-glucoside could be detected. Thus in accordance with our earlier studies [13] BGM is not subject to turnover. The mechanism regulating the differential vacuolar efflux of FGM in comparison to BGM is the subject of our present investigations.

The metabolic scheme shown in Fig. 4 requires the presence of the enzymes for the attachment and for the removal of the conjugating glucosyl and malonyl moieties of formononetin. These enzymes have all well been characterized [15, 18] and they were expressed in the chickpea cell cultures at high activities during our experiments (data not shown).

In soybean L-AOPP application leads to a complete inhibition of the accumulation of the glyceollin phytoalexins. This inhibition of phytoalexin biosynthesis even resulted in a concomitant loss of resistance against *Phytophthora megasperma* f. sp. *glycinea* [31, 32]. After completion of our investigations reported here Graham *et al.* [9] described that in *P. megasperma*-infected seedlings of soybean the accumulation of the glyceollin phytoalexins is preceded by a substantial turnover of a hitherto unknown daidzein 7-O-glucoside-6″-O-malonate. This 5-deoxyisoflavone functions as an intermediate in glyceollin biosynthesis and its transient accumulation from the malonyl conjugate at times of phytoalexin formation is interpreted as indicating that it is converted to the pterocarpans [9]. This assumption involved that in soybean tissue phytoalexin biosynthesis is regularly sustained both by *de novo* synthesis [31, 32] and by consumption of constitutive isoflavone conjugates [9]. This is in real contrast to the data obtained for chickpea cells in which *de novo* synthesis is the normal source for phytoalexin formation. Furthermore, in infected soybean tissue the co-occurring 5-hydroxyisoflavone genistein is also liberated from its malonylglucoside conjugate [9] though it cannot be used for phytoalexin formation. This is in further contrast to the situation in chickpea where a pronounced difference in the metabolism between 5-deoxy- and 5-hydroxyisoflavones has been determined ([13], Fig. 3).

Further evidence for a metabolic link between constitutive isoflavone metabolism and elicitor-induced isoflavone/pterocarpan biosynthesis is the observation (Fig. 3) that the cellular content of FGM is significantly increased when the rate of phytoalexin accumulation has again decreased. Such an effect has also been detected in infected chickpea tissue (Höhl and Barz, unpublished) and thus the use of the term "constitutive" for FGM should not be taken to exclude elicitor inducibility.

As a suitable model explaining the regulation of the vacuolar efflux of FGM upon L-AOPP treatment the regulatory potential of *t*-cinnamic acid is thought to be of importance. This acid is known to

participate in the activation and inactivation of enzymes of the phenylpropane pathway [33, 34], to regulate the metabolic flux through this sequence [35] and to affect gene expression by affecting translational activities of enzymes such as PAL and CHS [36, 37]. It is now postulated and the objective of present investigations that *t*-cinnamic acid is also involved in the regulation of transport mechanisms at the tonoplast membrane and that this effect will influence vacuolar efflux of the formononetin conjugate. Thus, the cytoplasmic concentration of *t*-cinnamic acid which is proportional to the rate of the PAL reaction represents a regulatory parameter which is thought to modulate vacuolar influx and efflux of FGM. It also remains to be shown whether the postulated regulatory pattern will not involve elicitor-caused changes in membrane potentials at the tonoplast [16] which are known to influence the intracellular distribution of amino acids and ions [38–40].

In general, the consumption of vacuolar, constitutively formed intermediates of pterocarpans for phytoalexin formation can be regarded as an anaplerotic sequence and thus a protective measure for the very rapid accumulation of defence compounds.

Acknowledgements

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