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Constitutive and elicitation induced metabolism of isoflavones and pterocarpans in chickpea (*Cicer arietinum*) cell suspension cultures

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Abstract

Constitutive phenolics of chickpea cell suspension cultures are the isoflavones formononetin and biochanin A, the isoflavanones homoferreirin and cicerin and the pterocarpans medicarpin and maackiain. They accumulate as vacuolar malonylglucosides. The biosynthetic pathways to isoflavones, pterocarpans and malonylglucoside conjugates together with their enzymes are explained. Elicitation of cell cultures leads to pronounced increases in the activities of biosynthetic enzymes with differential effects on the enzymes involved in conjugate metabolism. Low elicitor doses favour pterocarpan conjugate formation whereas high doses lead to pterocarpan aglycone accumulation accompanied by vacuolar efflux of formononetin and pterocarpan malonylglucosides. Elicitor-induced changes in enzyme activities and vacuolar efflux of conjugates are prevented by application of 10^{-3}M concentrations of cinnamic acid. Cinnamate is alternatively metabolized to a glucose ester, a S-glutathionyl conjugate and to cell wall bounds forms; these reactions are intensified by elicitation. Isoflavone and pterocarpan biosynthesis and conjugate metabolism as regulated by elicitation and cinnamate is depicted in a metabolic grid to explain the complex regulatory pattern of phenolic accumulation in chickpea cell cultures.

Abbreviations: AOPP – L-α-aminooxy-β-phenylpropionic acid, BGM – biochanin A 7–0-glucoside–6″–0-malonate, FGM – formononetin 7–0-glucoside–6″–0-malonate, HPLC – high performance liquid chromatography, MaGM – maackiain 3–0-glucoside–6′–0-malonate, MeGM – medicarpin 3–0-glucoside–6′–0-malonate

Introduction

Higher plants when challenged by pathogenic microorganisms express a series of active defense mechanisms. They all aim at the inhibition of microbial growth, isolation of the pathogens in lesions and finally death of the invading microbe by the accumulation of antibiotic compounds (Lamb et al. 1989; Barz et al. 1990a; Dixon & Lamb 1990a). Detailed analyses of the chickpea (*Cicer arietinum* L.) – *Ascochyta rabiei* (teleomorph: *Mycosphaerella rabiei* Kovachevski) interaction have demonstrated that the hyersensitive response, expression of pathogenesis-related proteins together with chitinases and β–1,3-glucanases, formation of the pterocarpan phytoalexins medicarpin and maackiain together with increased polyphenol deposition are essential elements of the plant defence responses (Höhl et al. 1990; Daniel & Barz 1990; Vogelsang & Barz 1993; Barz & Welle 1992). Furthermore, constitutively produced isoflavones ('preinfectional inhibitors') are of interest for chickpea resistance due to the localisation of these fungi-toxic compounds in the outer tissue layers of the plant (Barz & Hösel 1978). Investigations on the biosynthesis of constitutive isoflavones and de-novo synthesized antimicrobial pterocarpan phytoalexins clearly demonstrated a tight metabolic linkage between these two classes of phenolic constituents (Barz & Welle 1992). The analyses revealed an interesting regulatory pattern in operation in chickpea cells between constitutive and de-novo synthesized phenolic compounds, i.e. metabolic activation of vacuolar phenolic conjugates for rapid infection-induced accumulation of phytoalexins.

Elucidation of isoflavone and pterocarpan biosynthetic pathways, characterization of enzymes and mechanisms of gene activation as well as determina-

tion of adherent regulatory pattern have been investigated in chickpea cell suspension cultures. Infection-induced changes of cellular metabolism can very efficiently be simulated in such cultures by the application of fungal polysaccharide elicitors (Barz et al. 1990a; Dixon & Lamb 1990a).

Materials and methods

Cell cultures

Chickpea (cultivar ILC 3279) cell suspension cultures (40 ml medium in 250 ml Erlenmeyer flasks) were grown as previously described (Keßmann & Barz 1987; Mackenbrock et al. 1992). Elicitation and AOPP/cinnamic acid inhibition experiments were performed with cells 3 days after transfer into new medium using published procedures (Mackenbrock et al. 1993).

Elicitor

The preparation of yeast elicitor has been described (Gunia et al. 1991).

Reference compounds

The isoflavone, pterocarpan and phenylpropanoid compounds used in the experiments described in this paper were from the institute's collection.

Quantitation of phenolics

The extraction of phenolics from cultured cells and the preparation of fractions for chromatography have been described (Keßmann & Barz 1987; Mackenbrock & Barz 1991).

Chromatographic analyses

Isoflavones and pterocarpans were analyzed by HPLC using previously described methods (Gunia et al. 1991; Keßmann & Barz 1987).

TLC analysis of cinnamic acid conjugates were performed as described by Edwards et al. (1990) and Edwards & Dixon (1991).

Enzyme assays

Cinnamic acid glucosyltransferase and glutathione S-cinnamoyltransferase were measured as described (Edwards et al. 1990; Edwards & Dixon 1991). Assays for all other enzymes mentioned in this paper have previously been described (Daniel et al. 1990; Gunia et al. 1991; Mackenbrock et al. 1992, 1993).

Protein concentrations were determined by the Bradford method with bovine serum albumin (Cohn fraction V, Sigma, Munich) as reference.

Results and discussion

Constitutive accumulation of phenolics in cell cultures

Heterotrophic chickpea cell suspension cultures established from various cultivars have turned out to be a rich source of phenolic constituents (Keßmann & Barz 1987; Weidemann et al. 1991; Barz & Welle 1992). In principle, the phenolics belong to the following classes, a) 5-hydroxyisoflavones (i.e. biochanin A), b) 5-deoxyisoflavones (formononetin), c) 2′-methoxy–5-hydroxyisoflavanones (homoferreirin, cicerin), and d) pterocarpans (medicarpin, maackiain) (structures Fig. 1).

These compounds acccumulate under normal culture conditions (Keßmann & Barz 1987) in a mainly growth-linked pattern (Barz et al. 1990b) with the 5-hydroxyisoflavonoids being the major components. All phenolics predominantly occur as 0-glucoside–6″–0-malonate conjugates (Fig. 1) which are exclusively stored in vacuoles (Mackenbrock et al. 1992). Such polar, hydrophilic conjugates of isoflavones and various other plant phenolics are well known widely occuring constituents (Barz et al. 1985). Isoflavone/isoflavanone/pterocarpan conjugate accumulation in these cultures is highly responsive to auxin regulation because cultivation under auxin-free conditions may lead to a ca. 20-fold increase in phenolic material. The bulk of these additional compounds is again being represented by 5-hydroxy-isoflavone/-isoflavanones (Vogelsang 1993).

The biosynthesis of isoflavones has extensively been studied using chickpea cultured cells so that the essential enzymes of the general phenylpropanoid pathway and chalcone synthase, chalcone isomerase as well as isoflavone synthase together with the 4′–0-methylation step have well been characterized (Barz et al. 1990a; Barz & Welle 1992). In comparison to biochanin A the pathway to formononetin (Fig. 2, upper part) involves an additional independent enzyme, chalcone reductase (CHR) which coacts with chalcone synthase (CHS) and

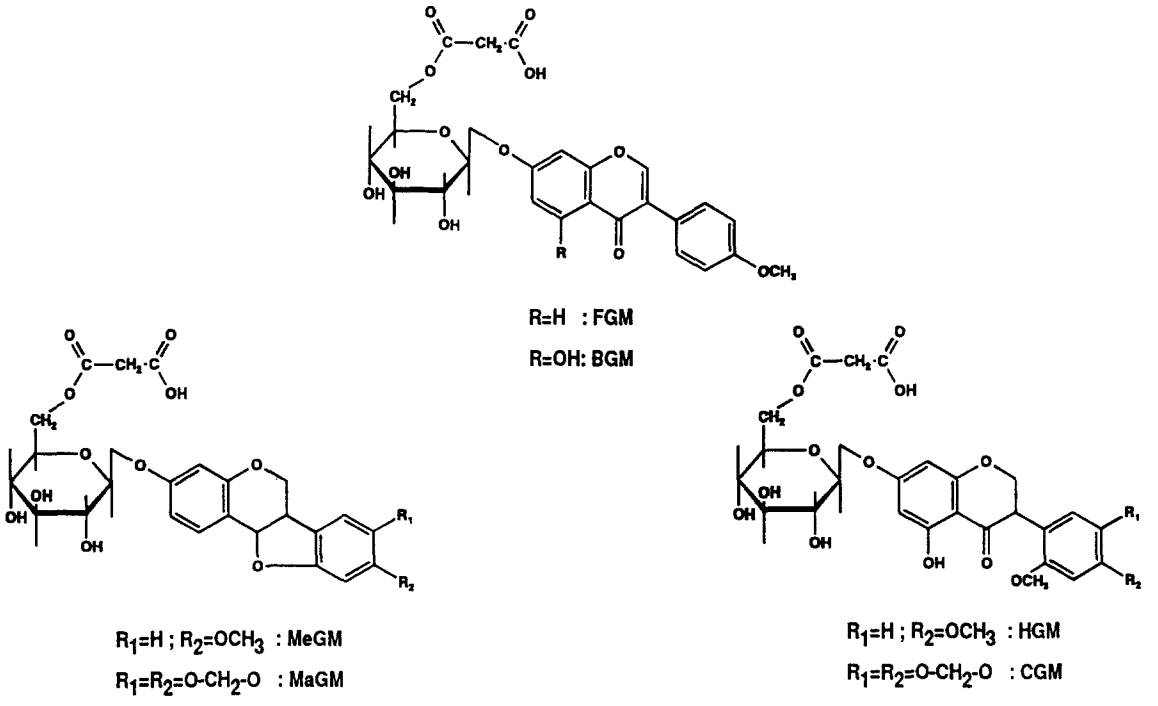

Fig. 1. Structures of malonylglucosides of the isoflavones formononetin (FGM) and biochanin A (BGM), the isoflavanones homoferreirin (HGM) and cicerin (CGM) and the pterocarpans medicarpin (MeGM) and maackiain (MaGM) constitutively formed in chickpea cell suspension cultures.

NADPH as cofactor in the formation of the intermediate 2′–4′–4-trihydroxychalcone isoliquiritigenin. This enzyme may be regarded as an important regulatory step for the channelling of substrates into the two competing pathways leading to either 5-deoxyisoflavones (daidzein, formononetin) and pterocarpans (medicarpin/maackiain) on one hand or 5-hydroxyisoflavones (biochanin A) and -isoflavanones (homoferreirin, cicerin) on the other. In chickpea CHS and CHR occur in multiple isoforms which are induced to a different extent upon elicitation of the cell cultures (Bless 1992).

Under normal growth conditions chickpea cell suspension cultures accumulate the (6aR : 11aR) pterocarpans, medicarpin and maackiain (Fig. 2), in form of the aglycones to only a very low extent if at all. However, considerable quantities of the 3–0-glucoside–6′–0-malonate conjugates (Fig. 1) are regularly formed (Weidemann et al. 1991). This has previously been interpreted as an indication for a state of partial induction of these cultures due to the specific conditions of culture growth. However, medicarpin and maackiain malonylglucosides have also been detected as normal constituents of older chickpea roots and furthermore these conjugates always co-occur with the aglycones when these are expressed as a phytoalexin response. The biosynthetic pathway leading from the formononetin intermediate to the pterocarpans (Fig. 2, lower part) has been characterized using elicited chickpea cells (see below). In other pterocarpan producing plants (glyceollin/soybean; pisatin/pea; medicarpin/alfalfa) identical sequences for such phytoalexins have been detected which also involve enzyme systems with a high degree of homology (Barz & Welle 1992; Dixon et al. 1992).

Homoferreirin and cicerin (Fig. 1) together with their malonylglucosides are synthesized from biochanin A in sequences which are highly analogous to the formation of medicarpin and maackiain from formononetin (Fig. 2). The reactions are hydroxylation of biochanin A in positions 2′ and 3′, respectively, closing of the methylenedioxyring, reduction of the intermediate isoflavones to isoflavanones with the terminal step of 2′–0-methylation. From a structural point of view this 0-methylation reaction may well be compared with the formation of a 2′-methoxychalcone (Dixon et al. 1992) in that very similar hydroxyketo substrates are being involved. The addition of the 2′–0-methylgroup

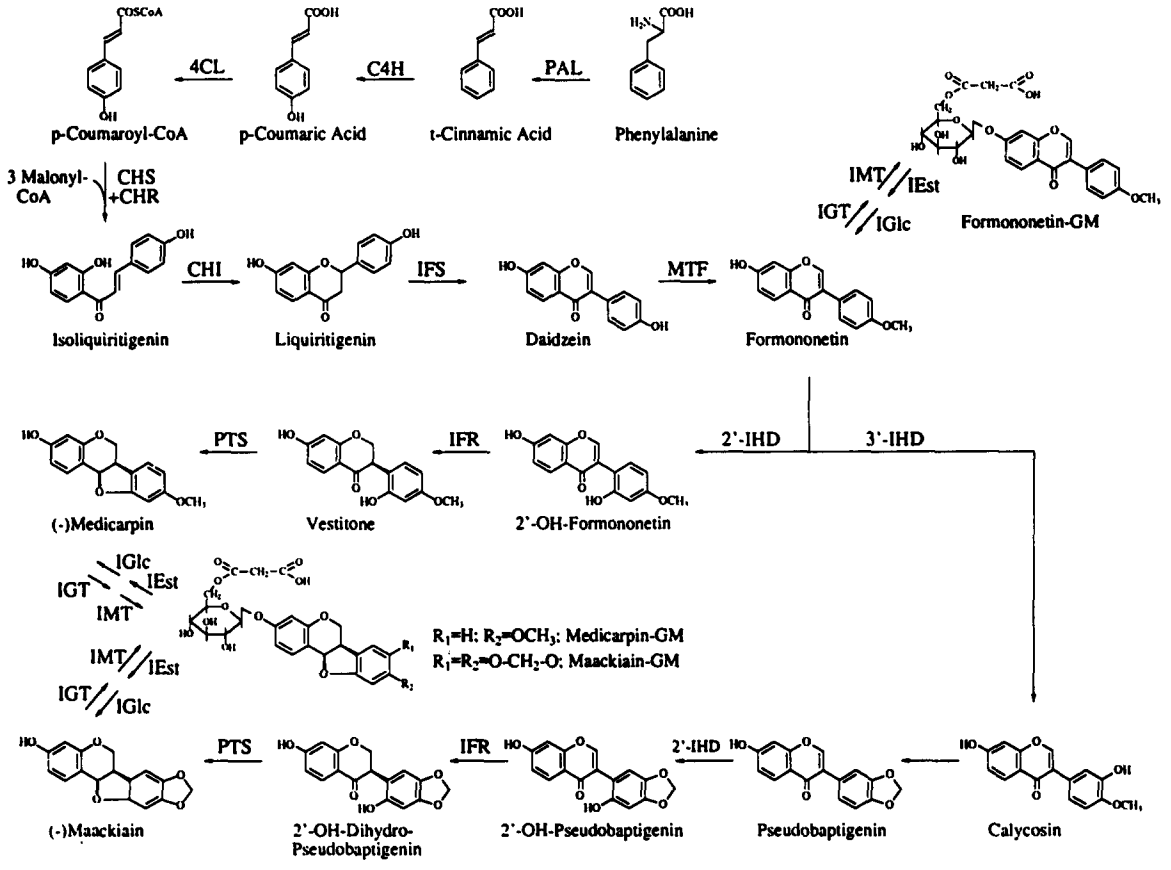

Fig. 2. Biosynthetic pathway to the isoflavone formononetin (upper half) and the pterocarpan phytoalexines medicarpin and maackiain as well as their malonylglucosides in chickpea. The enzymes are: PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, p-coumaric acid CoA-ligase; CHS, chalcone synthase; CHR, chalcone reductase; CHI, chalcone isomerase; IFS, isoflavone synthase; MTF, isoflavone methyltransferase; IGT, isoflavone 7–0-glucosyltransferase; IMT, isoflavone glucoside malonyltransferase; IEST, isoflavone malonylglucoside malonylesterase; IGLC, isoflavone glucoside glucosidase; 2′-IHD, isoflavone 2′-hydroxylase; 3′-IHD, isoflavone 3′-hydroxylase; IFR, 2′-hydroxyisoflavone oxidoreductase; PTS, pterocarpan synthase.

in homoferreirin and cicerin formally prevents formation of pterocarpan structures; a pterocarpan synthase specific for 5-hydroxyisoflavanones and relevant products, i.e. 1-hydroxypterocarpans have so far not been detected in the chickpea system.

Great care has been taken in the characterization of the enzymes involved in the formation and the hydrolysis of the isoflavone and pterocarpan malonylglucosides (Fig. 2). These are isoflavone 7–0-glucosyltransferase (IGT), isoflavone glucoside malonyl transferase (IMT), a highly substrate-specific isoflavone malonylglucoside malonylesterase (IEST) and isoflavone glucoside glucosidase (IGLC) (Barz et al. 1990a, 1990b; Barz & Welle 1992). These enzymes were first characterized in connection with the isoflavone substrates but were later on shown to act with the pterocarpan structures also. For IEST an association with the tonoplast membrane has recently been demonstrated whereas the other three proteins appear to be soluble, cytosolic proteins (Mackenbrock et al. 1992). These four enzymes appear to act in a metabolic cycle because, as shown for the formononetin moiety, vacuolar influx and efflux of the isoflavone occur simultaneously (Barz et al. 1990b). This turnover system based on these enzymes obviously participates in regulating the pool size of the vacuolar malonylglucoside of formononetin. Therefore, a strict metabolic and spatial separation of these anabolic and catabolic enzymes must exist.

Elicitor-induced accumulation of pterocarpan phytoalexins

Chickpea cell suspension cultures treated with *A. rabiei* or yeast polysaccharide elicitor readily accumulate medicarpin and maackiain which are mainly secreted from the cells into the growth medium (Keßmann & Barz 1987; Gunia et al. 1991; Weidemann et al. 1991). Medicarpin and maackiain occur in the cell cultures in a ratio of ca. 5 : 1 and for the sake of clarity the sum of both pterocarpans will only be given in this paper. Phytoalexin formation is regarded as de-novo synthesis from early precursors of primary metabolism (Dixon & Lamb 1990; Mackenbrock & Barz 1991). Synthesis of the aglycones was found to be restricted to a period of ca. 6–20 h after elicitation followed by very rapid disappearance of the compounds from the medium. This decline in pterocarpan levels has been determined as peroxidative destruction catalyzed by extracellular peroxidases (Barz et al. 1990b). Furthermore, pterocarpan aglycone accumulation has been shown to be positively correlated with the amount of elicitor applied (Gunia et al. 1991). In contrast to this short burst of elicitor-induced aglycone formation a simultaneous and much longer lasting accumulation of medicarpin and maackiain malonylglucosides has also been found to occur (Weidemann et al. 1991). In fact, low doses of polysaccharide elicitor almost exclusively induced accumulation of the vacuolar phytoalexin conjugates (Fig. 3) and this process continued for a considerable period of time. Elicitor dose studies on the ratio of accumulating phytoalexin aglycones to pterocarpan malonylglucosides (Fig.4) revealed that low or moderate doses (5–10 mg/flask) favoured accumulation of the conjugates whereas high doses (30–80 mg) steadily led to increasing amounts of the aglycones. Figure 4 also depicts various investigations which showed that the formononetin moiety of the vacuolar-localized FGM (comp. Fig. 2) can be consumed and funnelled into pterocarpan phytoalexin biosynthesis if sufficiently high elicitor doses are being applied (Mackenbrock & Barz 1991). Furthermore, this vacuolar efflux appears to be highly substrate-specific because the pool sizes of the vacuolar BGM were not affected by elicitation (Fig. 4). The contribution of formononetin derived from FGM to phytoalexin biosynthesis became even more prominent when the introductory enzyme of pterocarpan formation, PAL, was inhibited by L-α-aminooxy-β-phenylpropionic acid (L-AOPP) during elicitation. Since under these conditions no phenylpropanoid precursors were available for de-novo pterocarpan formation the elicitor-induced accumulation of phytoalexins was now quantitatively covered by formononetin derived from FGM (Mackenbrock & Barz 1991).

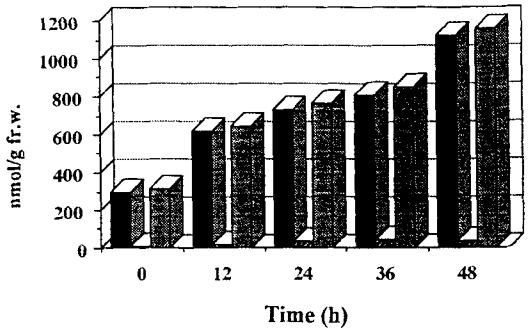

Fig. 3. Time course of elicitor-induced (10 mg per flask) accumulation of pterocarpan phytoalexin aglycones and pterocarpan conjugates (the sum of medicarpin and maackiain) in chickpea cell suspension cultures. The bars represent:

■ phytoalexin conjugates,
▨ phytoalexin aglycones, and
■ total amount of pterocarpans

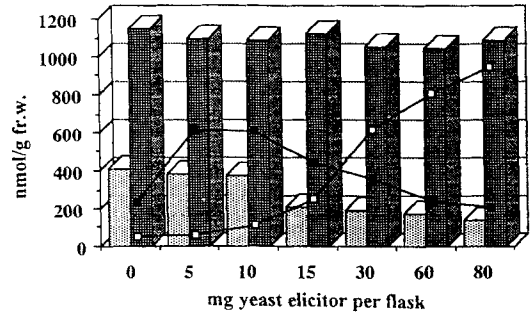

Fig. 4. Effect of elicitor dose on the accumulation of pterocarpan aglycones, pterocarpan conjugates and the malonylglucosides of formononetin and biochanin A in chickpea cell cultures 12 h after elicitation. The bars represent:

▒ formononetin malonylglucoside,
■ biochanin A malonylglucoside

The curves indicate:

–□– phytoalexin aglycones,
–■– phytoalexin conjugates.

The consumption of constitutive isoflavone conjugates for the induced formation of phytoalexins has also been observed in other systems than chickpea. In *Phytophthora megasperma* infected seedlings of soybean accumulation of the glyceollin phytoalexins is accompanied by a substantial turnover of a daidzein 7–0-glucoside–6″–0-malonate (Graham et al. 1990). The isoflavone aglycone is then thought to be used for glyceollin formation. Such data obtained for soybean and chickpea provide evidence for a tight metabolic

link between constitutive isoflavone metabolism and elicitor-induced pterocarpan formation. Depending on the physiological situation and the actual demand for phytoalexin material the process of de-novo synthesis can be sustained by the consumption of constitutive intermediates.

The pterocarpan malonylglucosides MeGM and MaGM synthesized constitutively in low amounts and inducibly in much higher quantities (Fig. 3) also represent a reservoir for elicitor-induced accumulation of pterocarpan aglycones. Thus, conjugate material formed under the impact of low elicitor doses is readily consumed for aglycone formation with subsequent secretion of the phytoalexins into the growth medium when high elicitor doses are applied (Fig. 4) (Mackenbrock et al. 1993). Again, the elicitor quantity, i.e. the intensity of the elicitation effect, determines the ratio of conjugate to aglycone formation and alternatively leads to either vacuolar influx or efflux. Experiments with L-AOPP in moderately elicited cell cultures clearly showed that a subsequent high elicitor dose leads to almost complete release of the vacuolar pterocarpan malonylglucosides (Mackenbrock et al. 1993). These data indicate that the vacuolar conjugates are metabolically linked to the process of phytoalexin synthesis and that the elicitation-induced pattern of metabolic regulation also comprises the vacuole and the tonoplast transport systems.

Elicitor-induced changes of enzyme activities

Elicitor-treated chickpea cell suspension cultures have been very helpful in the elucidation of the enzymology of pterocarpan biosynthesis.

The pterocarpan-specific branch leading from formononetin to medicarpin and maackiain (Fig. 2) starts with cytochrome P_{450} monooxygenases for isoflavone 2′- and 3′-hydroxylation (2′-IHD, 3′-IHD). These inducible, microsomal enzyme activities have thoroughly been characterized in cell cultures and plants (Clemens et al. 1993) and they were recognized as distinct enzymes for the two position-specific reactions. Expression of isoflavone 2′-hydroxylase has been found to be closely related to phytoalexin accumulation and it may be regarded as an important regulatory step (Gunia et al. 1991; Clemens et al. 1993).

Reduction of the two 2′-hydroxyisoflavones shown in Fig. 2 is catalyzed by a NADPH : isoflavone oxidoreductase (IFR) leading to chiral 2′-hydroxyisoflavanones. This enzyme has meanwhile been cDNA-cloned from chickpea and alfalfa and its elicitor-induced expression was studied at the mRNA level (Tiemann et al. 1993).

The terminal enzyme reaction for the formation of the dihydrofuran ring of pterocarpans (PTS) (Fig. 2) only accepts the 3 R-configurated isoflavanone intermediates and it is together with 2′-IHD, 3′-IHD and IFR coordinately induced upon elicitation of cell cultures or infection of plants (Barz & Welle 1992).

Elicitation experiments in chickpea cell suspension cultures revealed that the enzymes of the complete biosynthetic sequence (Fig. 2) comprising the general phenylpropanoid pathway, chalcone synthase, isoflavone synthase, including the 4′–0-methylation step and the pterocarpan specific branch from formononetin to medicarpin and maackiain are all subject to induction (Daniel et al. 1990). Although formononetin biosynthesis proceeds constitutively in the chickpea cultures a pronounced increase in the enzyme activities of these enzymes has also been measured. The adherent aspects of metabolic regulation and the involvement of isoforms at certain reactions steps (CHS, CHR, see above) have been reviewed (Barz & Welle 1992). This pattern of a coordinate induction of a long and complex biosynthetic pathway appears to be a general feature of phytoalexin formation from early precursor of primary metabolism (Hahlbrock & Scheel 1989).

In view of the elicitor dose-dependent, differential accumulation versus metabolism of formononetin and pterocarpan malonylglucosides during phytoalexin aglycone formation (Fig. 4) more detailed investigations on the elicitation of essential biosynthetic enzymes (PAL, C4H, CHS, 2′-IHD and 3′-IHD) and the enzymes involved in conjugate metabolism (IGT, IMT, IEST, IGLC) have been performed. Using a wide range of elicitor doses (10–80 mg/flask) changes in enzyme activities during the first 10–12 h in comparison to the controls were recorded. Elicitor doses of ca. 80 mg/flask had previously been shown to result in maximum expression of elicitor-induced changes in enzyme activities in the chickpea cultures (Gunia et al. 1991; Mackenbrock et al. 1993).

In case of the 5 key biosynthetic enzymes a coordinated and linear increase in enzyme activities over this range of elicitor doses could be measured. The data are shown in Table 1 in the columns 'control' and 'elicitor'. Although the absolute values of elicitor-induced levels of enzyme activities vary the results clearly showed that these enzymes are subject to strong induction. As mentioned before this process is accompanied by the formation of either pterocarpan malonylglucosides or

pterocarpan aglycones (Fig. 4). These data will later be used for the discussion of the phenomenon of cinnamate-caused modulation of enzyme activities.

The analyses of the enzymes involved in the metabolism of malonylglucoside metabolism (Table 2) revealed a more complex pattern of elicitor-caused changes in enzyme activities. Low elicitor doses (10 mg/flask) appeared to increase the activities of IGT and IMT whereas IEST and IGLC were repressed in their activities. In contrast, high elicitor doses (80 mg/flask) caused strong repression of IGT and IMT below the control values with IEST and IGLC being induced to significantly higher values. These fluctuations of enzyme activities over the indicated range of elicitor doses should be evaluated in comparison to the data shown in Fig. 4. Low elicitor doses which lead to the preferential formation of pterocarpan malonylglucosides also increase the activities of IGT and IMT. Thus, the biosynthetic branch of conjugate formation and vacuolar influx appears to be intensified. On the other hand, a strong elicitation effect represented by high elicitor doses (60–80 mg/flask) results both in an increase of IEST and IGLC activities and also in increased rates of conversion of pterocarpan and formononetin malonylglucosides to pterocarpan aglycones; in essence, vacuolar efflux appears to be favoured. The differential elicitor-caused behaviour of the enzymes of conjugate metabolism (Table 2) perfectly agrees with the data (Fig. 4) obtained for the differential accumulation of the various phenolic compounds. However, it is presently unknown whether enzyme induction or repression processes are involved in the modulation of the four enzyme activities.

The regulatory system for the differential accumulation of aglycones versus conjugates obviously involves the enzymes responsible for conjugate formation and hydrolysis and also comprises those factors which control the storage and efflux capability of the vacuole. The mechanisms for uptake, storage and release of secondary products presently known favour the assumption that specific tonoplast carrier systems exist (Wink 1993). In case of the isoflavonoid malonylglucosides such carriers will then be linked with the enzymes involved in conjugate formation and hydrolysis.

Regulation of isoflavonoid metabolism by cinnamic acid

The mobilisation of the formononetin and the pterocarpan moieties of the vacuolar conjugates for pterocarpan aglycones accumulation is presently described as a metabolic grid with the vacuolar conjugates being reservoir pools for phytoalexin formation (Fig. 2). As a suitable model explaining the regulation of vacuolar efflux of malonylglucosides and the changes in enzyme acitivities (Table 1 and 2) the regulatory potential of cinnamic acid was thought to be of importance. This acid is known to participate in the regulation of enzymes of the phenylpropanoid pathway (Dixon et al. 1980; Bolwell et al. 1986, 1988), to mediate the metabolic flux through this sequence (Shields et al. 1982) and to affect gene expression by influencing the translational process of PAL and CHS (Bolwell et al. 1986, 1988; Dixon & Lamb 1990b). Therefore, the cytoplasmic concentration of cinnamic acid may also represent a regulatory component both for controlling vacuolar influx and efflux (Fig. 2) as well as for the modulation of the enzyme activities involved in the synthesis and the hydrolysis of pterocarpan and formononetin conjugates (Table 2).

Experiments to test this hypothesis are depicted in Fig. 5. Chickpea cell suspension cultures were treated with a high elicitor dose (80 mg/flask) and concomitantly with increasing amounts of cinnamic acid (10^{-4} to 10^{-3}M). Elicited control cells (Fig. 5 B) showed in comparison to non-treated cells (Fig. 5 A) a pronounced accumulation of pterocarpan aglycones and a substantial decrease of the constitutively formed pterocarpan conjugates and FGM. This elicitor effect is quantitatively reversed upon application of increasing levels of exogenous cinnamic acid (Fig. 5 C-E). Finally, 10^{-3} M cinnamic acid completely inhibited the strong elicitor effect because a situation as seen with the non treated cells (Fig. 5 A) has again been measured. Essentially as seen in the previous experiments (Fig. 4) the vacuolar pool of BGM was not affected.

Inhibition of both vacuolar efflux and hydrolysis of malonylglucoside conjugates by high cinnamic acid concentrations was further substantiated by elicitation experiments in the presence of cinnamic acid and L-AOPP for PAL inhibition. Again, no phytoalexin aglycones were formed under these conditions indicating that cinnamate appears to act at the vacuolar efflux system (data not shown).

To further elucidate the inhibitory mechanism of high cinnamic acid concentrations on conjugate metabolism possible modifications of elicitor-induced modulations of enzyme activities by cinnamate were also recorded. The data in Table 1 show that a high elicitor dose (80 mg/flask) strongly increases the activities of PAL, C4H, CHS, 2′-IHD,3′-IHD, IEST and

Table 1. Changes of enzyme activities (μkat/kg protein) in chickpea cell suspensions after application of 80 mg yeast elicitor/flask and reversion of enzyme induction/representation by simultaneous application of cinnamic acid (10^{-3}M).

Enzyme	Control	Elicitor	Elicitor + Cinnamic acid
Phenylalanine Ammonialyase (PAL)	10	28	11
Cinnamic acid 4-Hydroxylase (C4H)	7	17	8
Chalcone Synthase (CHS)	2,5	4,8	3
Isoflavone 2'-Hydroxylase (2'-IHD)	0,8	7,8	2,2
Isoflavone 3'-Hydroxylase (3'-IHD)	1,8	5,3	2,6
Isoflavone 7-0-Glucoside Malonyltransferase (IMT)	1.800	1.100	1.500
Isoflavone Malonyl-Glucoside Malonyl-Esterase (IEST)	20.000	35.000	20.000
Isoflavone Glucoside Glucosidase (IGLC)	300	480	300

Table 2. Changes of enzyme activities (μkat/kg protein) involved in Isoflavone/Pterocarpan Malonylglucoside metabolism in chickpea cell suspensions after application of different amounts of yeast elicitor. Enzymes were assayed 10 h after elicitation.

Enzyme	Elicitor dose (mg)/flask			
	0	10	30	80
Isoflavone-7-0-Glucosyl-Transferase (IGT)	30	46	21	18
Isoflavone Glucoside Malonyltransferase (IMT)	2.000	2.450	1.300	1.080
Isoflavone Malonyl-Glucoside Malonyl-esterase (IEST)	18.000	13.000	19.000	26.000
Isoflavone Glucoside Glucosidase (IGLC)	100	100	120	158

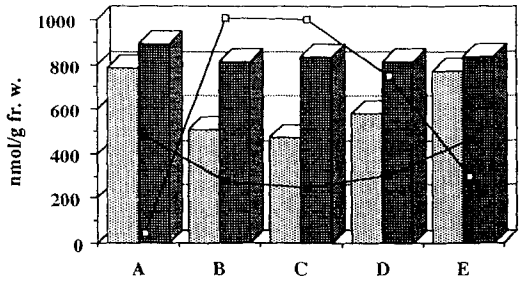

Fig. 5. Effect of cinnamic acid on the elicitor (80 mg per flask) induced metabolism of isoflavone and pterocarpan conjugates measured 12 h after treatment. Experimental conditions were: A: unelicited cells; B: elicitor-treated cells; C: elicitor + 10^{-4} M cinnamate; D: elicitor + 5×10^{-4} M cinnamate; E: elicitor + 10^{-3} M cinnamate. The bars represent:

▒ formononetin malonylglucoside
■ biochanin A malonylglucoside

The curves indicate:

-□- phytoalexin aglycones
-■- phytoalexin conjugates.

IGLC whereas the activities of IGT and IMT were found to be reduced. Upon simultaneous application of 10^{-3} M cinnamic acid and elicitor to such cells (Table 1, column 'elicitor + cinnamic acid') these changes of enzyme activities were all quantitatively prevented. These results indicate a pronounced regulatory potential of cinnamic acid on pterocarpan biosynthetic enzymes, proteins involved in the metabolism of malonylglucosides and on vacuolar efflux systems. Furthermore, the data again support the assumption that the various enzymes of the pterocarpan biosynthetic pathway (Fig. 2) and the vacuolar pools of FGM, MeGM and MaGM together with the relevant enzymes all belong to one metabolic grid.

The effects here described for cinnamic acid appear to be highly specific for this particular compound because similar experiments as presented in Fig. 5 and Table 1 using appropriate concentrations of p-coumaric acid failed to show any significant changes exerted by this phenylpropanoid intermediate.

Cinnamate metabolism in elicited cell cultures

Application of cinnamic acid to chickpea cell cultures in comparatively high doses (Fig. 5) caused pronounced regulatory effects on isoflavone/pterocarpan metabolism but failed to lead to any significant toxic effects or cell necrosis. Therefore, metabolic reactions for the detoxification of excess quantities of the exogenously applied acid are most likely functioning in these cells.

In agreement with studies by Edwards et al. (1990) and Edwards & Dixon (1991) on French bean cell cultures metabolism of cinnamate fed to cultured chickpea cells mainly resulted in

a) the accumulation of wall-bound, ethanol-insoluble material,
b) formation of cinnamic acid glucoside and
c) glutathionyl-S-cinnamic acid; respectively.

These results were obtained by feeding (10^{-5}–10^{-3}M) ^{14}C-cinnamic acid to the cultures (uptake ca. 85–90%/20h) with detailed TLC and HPLC analyses of the soluble conjugates formed using appropriate reference compounds. Incorporation of radioactivity into insoluble cell structures appeared to be the quantitatively most prominent portion (35–45%). Incorporation of ^{14}C-cinnamic acid into isoflavones and pterocarpans proceeded to a very low degree only whereas the two conjugates were formed at a level of 2–4%.

Subsequent enzymic investigations aimed at determination of the UDP-glucose dependent cinnamic acid glucosyltransferase (CGT) and the glutathione S-cinnamoyl transferase (GSCT) in the chickpea cultures. Furthermore, induction of these enzyme activities by elicitation and the possible regulatory effects of cinnamate were also measured. Enzyme assays using protocols by Edwards et al. (1990) and Edwards & Dixon (1991) showed that chickpea cell suspension cultures express both enzyme activities. Constitutive levels of CGT were found to be 7–8 μkat/kg protein and both elicitor and cinnamic acid were shown to exert a strong inducing effect independently from each other (Fig. 6). Maximum increase by approximately a factor 3 was measured 8 h after application of the elicitor and 12 h after treatment with cinnamic acid. Simultaneous treatment of cultures with combinations of elicitor (i.e. 80 mg/flasks and cinnamate (10^{-3} M) revealed a slight synergistic effect in the increase of CGT by a factor of 3.4. The data in Fig. 6 clearly document that high cinnamate levels (5×10^{-4}/10^{-3}M) may lead to an enhanced detoxification pathway for this regulatory compound. On the other hand such high cinnamate doses strongly repress elicitor-induced responses, namely, increase of biosynthetic enzyme activities (Table 1), phytoalexin accumulation (Fig. 5) as well as vacuolar efflux of formononetin and pterocarpan moieties from malonyl conjugates.

Assays for GSCT revealed a rather similar situation as seen for CGT because the constitutive levels (6 μkat/kg protein) were increased to 10–11 μkat/kg pro-

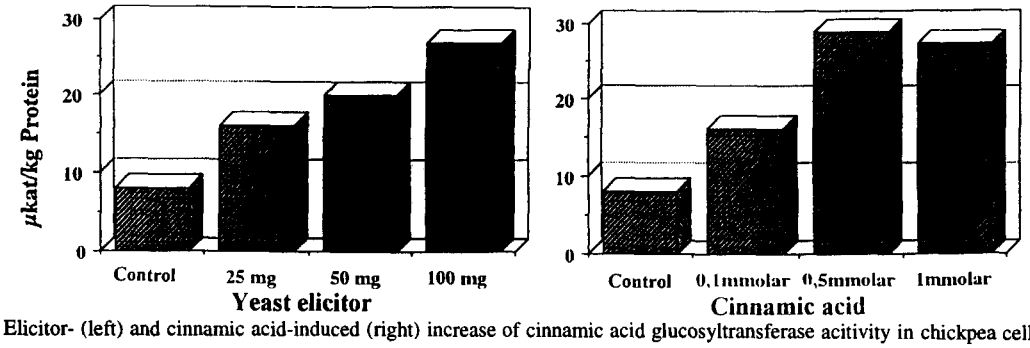

Fig. 6. Elicitor- (left) and cinnamic acid-induced (right) increase of cinnamic acid glucosyltransferase acitivity in chickpea cell suspension cultures.

tein both by yeast-elicitor (100 mg) or cinnamic acid (10^{-3}M). The maximum values were reached in ca. 12 h, but synergistic effects of the two inducers were much less pronounced. These GSCT data again reflect the metabolic function of cinnamate in that excess concentrations of this regulatory compound induce an increase in the detoxification reaction. Finally, elicitation of chickpea cell cultures (40–100 mg/flask; preincubation 8 h) increased the ability of the cells for transfer of exogenous cinnamic acid into insoluble wall-bound structures almost twofold (data not shown in detail).

In summary, chickpea cell suspension cultures were shown to posses at least three potent reaction sequences for the conversion/detoxification of cinnamic acid which are inducible both by elicitation and cinnamate itself. Elicitor-induction of glucosyltransferases (Cosio et al. 1985; Hahlbrock & Scheel 1989; Edwards et al. 1990) and glutathione S-cinnamoyl transferase (Edwards & Dixon 1991) together with induction of such enzymes by cinnamate itself (Edwards & Owen 1988) have also been shown in other plant systems. Furthermore, comparison of Fig. 5 with the data on CGT and GSCT document the existence of cinnamic acid repressible and inducible pathways in chickpea cells. Incorporation of cinnamate in wall-bound structures may on one hand lead to more resistent, less digestible wall structures and on the other hand lower the cytoplasmic concentration of this compound. This decrease in cinnamate level will in turn reduce the inhibition of phenylpropanoid and phytoalexin metabolism as caused by this acid.

Conclusions

In chickpea cell cultures the complete biosynthetic pathway from phenylalanine to the pterocarpans via the intermediate step of an isoflavone (Fig. 2), the branches leading to the vacuolar constituents FGM, MeGM and MaGM, respectively, together with the enzymes involved in malonylglucoside formation and hydrolysis as well as the reactions leading to cinnamic acid conjugates and cinnamate cell wall binding may be summarized in one metabolic grid (Fig. 7). This grid comprises a system of diverging reactions in which the enzymes are all highly responsive to elicitation and to regulation by cinnamic acid.

The intensity of elicitation by low or high doses influences the extent to which the conjugates FGM, MeGM and MaGM, respectively, are either preferentially formed (low/moderate doses) or consumed for the accumulation of pterocarpan aglycones. The elicitor-caused production of conjugates is favoured both by a simultaneous increase in the activities of the enzymes IGT and IMT and a reduction of the activities of IGLC and IEST. In contrast, high activities of the enzymes necessary for pterocarpan aglycone formation (PAL, C4H, CHS, 2′-IHD, 3′-IHD) as induced by high elicitor doses are accompanied by an intensified release of vacuolar conjugates and a reversion of the ratio of enzyme activities involved in conjugate formation versus hydrolysis. This regulatory pattern most likely also involves elicitor-caused changes in the affinity or the transport-rate of appropriate tonoplast carriers.

In addition to the previously described regulatory effects of cinnamic acid on the differential expression of PAL and CHS (Bolwell et al. 1986, 1988; Dixon & Lamb 1990b) our data now show that a similiar situation appears to exist with the cytochrome P_{450} monooxygenases C4H, 2′-IHD and 3′-IHD, respectively. Whether gene respression or translational processes are involved remains to be investigated. Especially interesting are the observations that high cytoplasmic concentrations of cinnamate (formed at times of high expression of PAL) also prevent the elicitor-caused modulations of the activities of IGT, IMT, IEST

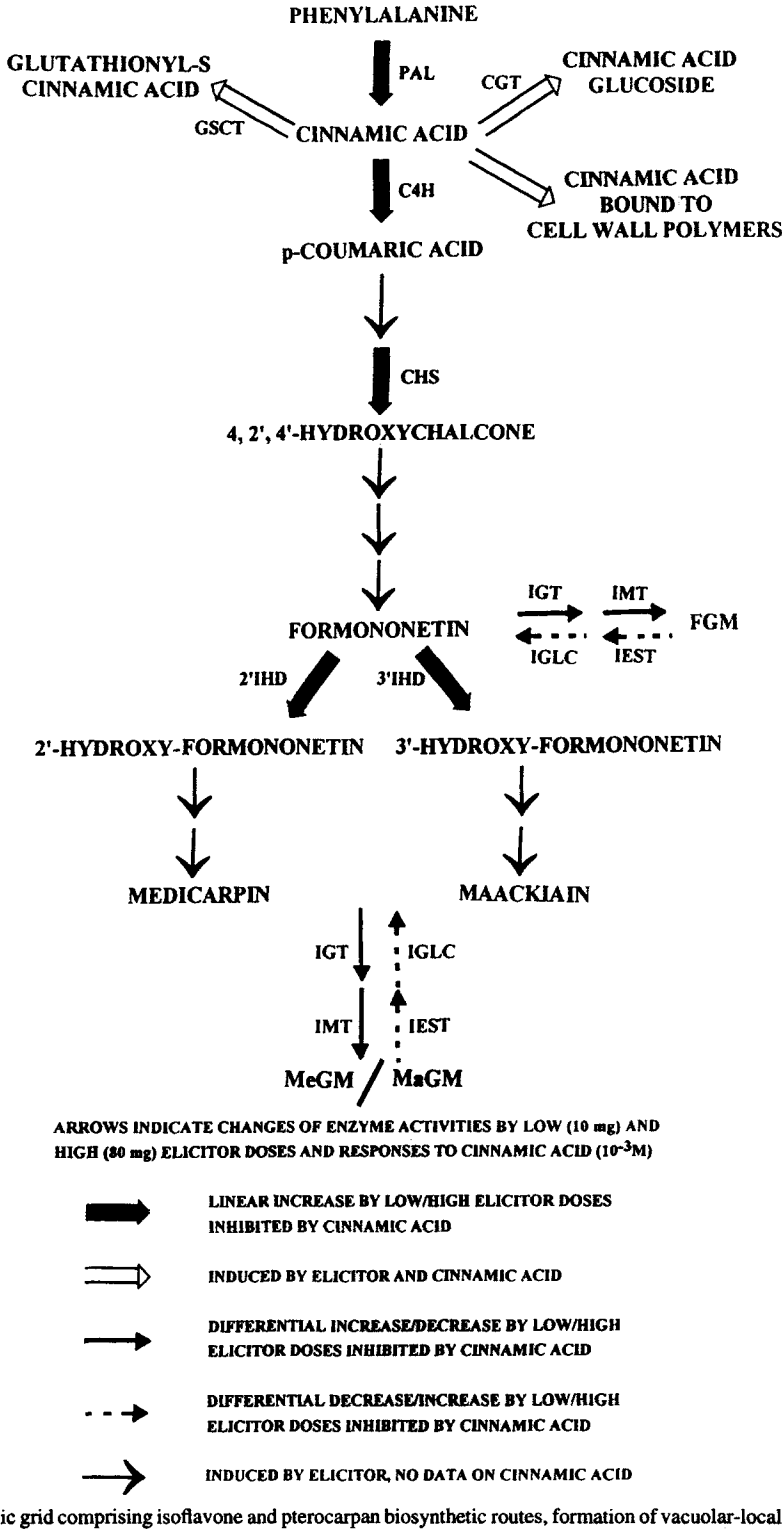

Fig. 7. Postulated metabolic grid comprising isoflavone and pterocarpan biosynthetic routes, formation of vacuolar-localized malonylglucosides of formononetin and medicarpin/maackiain as well as enzyme reactions for cinnamic acid conjugation and cell wall binding. Enzyme activities are differentially amenable by elicitation and/or cinnamic acid treatment. Enzymes are: GSCT, glutathione S-cinnamoyl transferase; CGT, cinnamic acid glucosyltransferase. For the other enzymes see legend of Fig. 2.

and IGLC (Table 2) and that the regulatory potential of cinnamic acid obviously also affects the postulated tonoplast carrier systems for the conjugates. Again, the exact mechanisms are presently unknown. Furthermore, the hypothetical metabolic grid (Fig. 7) also comprises three elicitor-amenable/cinnamate intensified diverging reactions (GSCT, CGT, wall-bound cinnamate) which allow for the conversion of high cytoplasmic concentrations of cinnamic acid to most likely non-active forms.

In essence, the investigations on medicarpin and maackiain accumulation in chickpea cell suspension cultures revealed that the biosynthetic pathway cannot be explained by a mere unidirectional, de-novo expressed synthetic route from precursors of primary metabolism but rather by a metabolic grid (Fig. 7) including constitutively formed conjugates and a regulatory pattern governed by differential changes of enzyme activities and pathway intermediates.

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