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RAPID METABOLISM OF ISOFLAVONOIDS IN ELICITOR-TREATED CELL SUSPENSION CULTURES OF *PUERARIA LOBATA*

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Key Word Index—*Pueraria lobata*; Leguminosae; cell suspension cultures; elicitation; isoflavonoids; malonylglucosides; biotransformation.

Abstract—Upon treatment with yeast extract as an elicitor to cell suspension cultures of *Pueraria lobata*, the constitutive isoflavonoid conjugates (7-*O*-glucoside-6″-*O*-malonyl esters) showed a rapid decline in their contents within 4 hr, followed by both reaccumulation of the conjugates and formation of isoflavones. Simultaneous treatment of a potent translation inhibitor, cycloheximide (200 μM), could not suppress the elicitor-inducible decrease of the pre-existing isoflavonoids, although it resulted in almost complete inhibition of both reaccumulation of the conjugates and formation of isoflavones. Exogenously applied ^{14}C-labelled isoflavones were readily converted to their respective malonyl glucosides in the cells. When yeast extract was applied to the cell cultures in which [^{14}C]-daidzein had been incorporated into its malonyl glucoside, the methanol-soluble radioactivity decreased rapidly within 4 hr and it was not recovered for 48 hr, indicating that most of the conjugate was incorporated into an insoluble cell wall fraction. The rapid response upon elicitation was selective for isoflavonoids and not for flavonoids.

INTRODUCTION

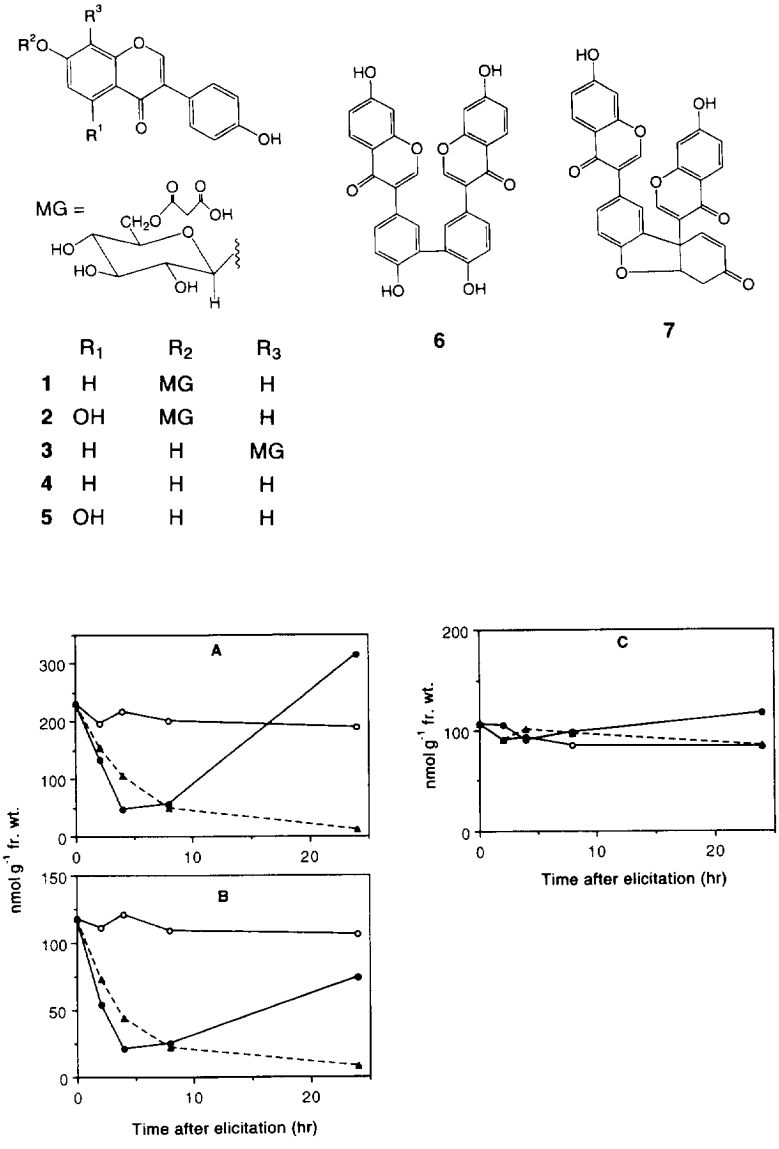
Cell cultures of *Pueraria lobata* have been studied for elicitor-induced enzymatic and genetic activation of isoflavonoid production [1–4]. Addition of yeast extract (YE) to the cell cultures stimulates the accumulation of isoflavones and daidzein-dimers [5]. We have recently reported that constitutive isoflavone glucosides exist as their 6″-*O*-malonyl esters in both cultured cells and intact plant [6]. On the other hand, recent investigation with soybean has shown that precursors of the pterocarpan phytoalexin glyceollins are present in tissues as isoflavone malonylated conjugates [7]. Mackenbrock and Barz [8] have reported that the constitutively accumulated formononetin-7-*O*-glucoside-6″-*O*-malonyl ester might be metabolized into the isoflavone aglycone being used as precursor in the elicitor-induced formation of pterocarpan phytoalexins in chickpea (*Cicer arietinum*) when *de novo* biosynthesis was suppressed by the PAL inhibitor, L-α-aminooxy-β-phenylpropionic acid (L-AOPP). A similar result was shown by Dixon and co-workers [9], that treatment of ^{14}C-labelled, elicited cells of alfalfa (*Medicago sativa*) with L-AOPP resulted in the initial appearance of labelled medicarpin, indicating that the phytoalexin could be released from preformed conjugates under these conditions.

In the light of these observations, it could be expected that isoflavonoid malonyl conjugates are involved in a defence mechanism of legumes against pathogens in a direct or indirect manner. But there is no information on the metabolic relationship between the pre-existing isoflavonoids (malonyl conjugates) and the elicitor-induced compounds in *P. lobata* cells, which prompted us to elucidate the elicitor-induced metabolism of the constitutive isoflavonoids. In this report, we show that the constitutive isolfavonoids are rapidly metabolized to associate with an insoluble cell wall fraction in elicited *P. lobata* cells, and also describe that this type of response to elicitation is selective for isoflavonoids.

RESULTS

Rapid decrease of isoflavone malonyl glucosides by elicitation

Cell suspension cultures of *P. lobata* were elicited with YE (1 mg ml^{-1} medium), and at appropriate times the methanol extract was quantitatively analysed for isoflavonoids by HPLC. In control cells, the amounts of daidzein malonyl glucoside (**1**) and genistein malonyl glucoside (**2**) were *ca* 200 nmol and 150 nmol g^{-1} fresh weight cells, respectively. Upon elicitation, both of the malonyl glucosides began to decrease and their amounts lowered to 10–15% of those in control cells in 4 hr (Fig. 1). In contrast, a malonyl conjugate of the isoflavone-*C*-glucoside, puerarin malonyl ester (**3**), showed no change, compared to those of control cells. The medium of elicited cells was recovered and analysed by HPLC, but no significant difference between the chromatograms of the



	R_1	R_2	R_3
1	H	MG	H
2	OH	MG	H
3	H	H	MG
4	H	H	H
5	OH	H	H

Fig. 1. Elicitor-induced metabolism of constitutive isoflavone malonyl glucosides in *P. lobata* cell suspension cultures. (A); daidzein malonyl glucoside (**1**), (B); genistein malonyl glucoside (**2**), (C); puerarin malonyl ester (**3**). For elicitation, YE (1 mg ml^{-1}) was administered to the cells. ○—○, Control cells; ●—●, YE-treated cells; ▲—▲, cells treated with YE plus cycloheximide (200 μM).

elicited cells and control was observed, indicating that the rapidly decreased isoflavonoids were not excreted into the medium (data not shown). Moreover, since no alternative turnover product could be detected in the methanol extract within the first 4 hr after elicitation, the isoflavonoids which disappeared on elicitation seemed to be converted to an insoluble product or bound to some insoluble cell wall fraction. Eight hr after elicitation **1** and **2** began to reaccumulate in the cells, along with the accumulation of daidzein (**4**), genistein (**5**) and daidzein-dimers (**6**, **7**) [Fig. 2]. At 24 hr after elicitation, the amount of malonyl glucosides reached almost the same level as those of control cells.

Cycloheximide (200 μM), a potent translation inhibitor, was simultaneously applied to the cell cultures with YE in order to confirm whether such elicitor-induced metabolism of isoflavonoids is regulated *de novo* or not. The rapid decrease of the constitutive isoflavonoids induced by elicitation was not significantly affected by the treatment of cycloheximide for 24 hr. In contrast, the elicitor-stimulated accumulation of isoflavones, e.g. **4**–**7**, together with the reaccumulation of the malonyl conjugates, e.g. **1** and **2**, was almost completely suppressed by the application of cycloheximide (Figs 1 and 2). These results show that elicitation leads to *de novo* synthesis of biosynthetic enzymes required for isoflavonoids and that the

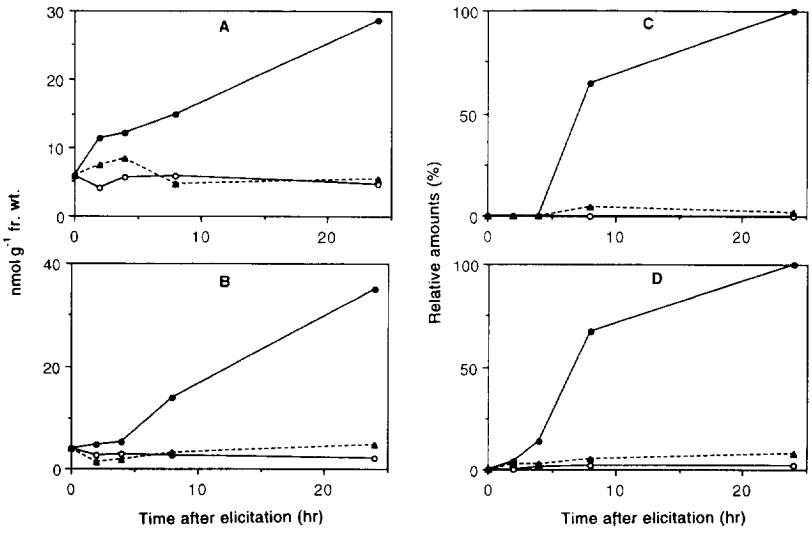


Fig. 2. Accumulation of inducible isoflavones in elicited cells of *P. lobata*. (A); daidzein (**4**), (B); genistein (**5**), (C); kudzuisoflavone A (**6**), (D); kudzuisoflavone B (**7**). Treatments and symbols are the same as those in Fig. 1.

Table 1. Metabolism of isoflavonoids in *Pmg* glycoprotein-treated *P. lobata* cells

Time after elicitation (hr)	Malonyl glucosides (nmol g^{-1} fr. wt)			Isoflavones (nmol g^{-1} fr. wt)			
	1	**2**	**3**	**4**	**5**	**6***	**7***
0	221	173	94	5	4	n.d.	n.d.
2	102	51	91	5	4	n.d.	n.d.
4	74	46	94	7	7	3	5
6	124	82	90	14	9	12	16
8	150	115	94	19	15	53	61
24	801	226	98	29	32	100	100

*Amounts relative to those in 24 hr-elicited cells.
For details of preparation and application of *Pmg* glycoprotein see Experimental.
n.d.: Not detected.

elicitor-triggered decrease of the constitutive isoflavonoids is not regulated by a *de novo* synthetic pathway. We also tested a fungal elicitor to evaluate its effect on these aspects of isoflavonoid metabolism. Treatment with a cell wall glycoprotein fraction (40 $\mu g\,ml^{-1}$) of *Phythophthora megasperma* f. sp. *glycinea* (*Pmg*) resulted in a similar metabolism of the isoflavonoids as observed in YE-elicited cells (Table 1). Cells treated with an excess ($> 200\,\mu g\,ml^{-1}$) amount of *Pmg* elicitor showed no reaccumulation of the constitutive isoflavonoids during 48 hr after their rapid disappearance, but accumulated the pterocarpan tuberosin (data not shown) as reported previously [5].

Uptake and elicitor-inducible metabolism of ^{14}C-labelled isoflavones

In order to investigate whether the isoflavone conjugates can indeed be metabolized to associate with insoluble fractions in response to elicitation, we carried out feeding experiments with ^{14}C-labelled isoflavones. [^{14}C]-Daidzein and [^{14}C]-genistein were biosynthetically prepared from L-[^{14}C]-phenylalanine (see Experimental). When [^{14}C] daidzein was applied to *P. lobata* cell suspension cultures in early growth phase, it was readily taken up and funnelled into the pool of daidzein malonyl glucoside (Fig. 3). After 12 hr incubation, nearly 70% of the applied radioactivity could be found in the malonyl glucoside of daidzein, **1**. These results demonstrate that the cellular pool of isoflavone malonyl glucosides can be efficiently and selectively labelled by the incorporation of ^{14}C-labelled isoflavones, providing the basis for the following experiments. The suspension cultured cells of *P. lobata* were pretreated for 12 hr with [^{14}C]-daidzein (3.6×10^5 dpm $flask^{-1}$) or [^{14}C]-genistein (3.0×10^4 dpm $flask^{-1}$), and elicited with YE. For the control ^{14}C-labelled isoflavone-treated cells were added with an equal volume of sterile water instead of YE. The total radioac-

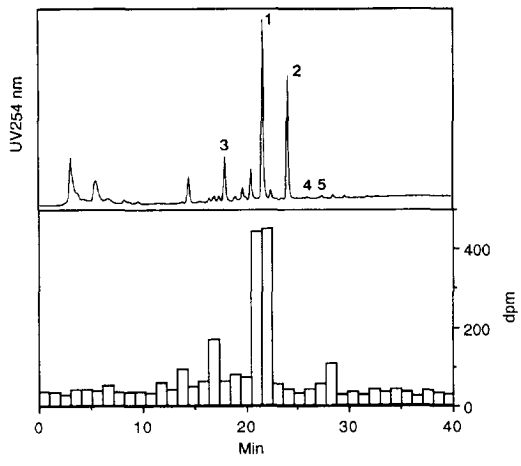


Fig. 3. Distribution of radioactivities in methanol extract of [^{14}C]-daidzein-fed cells of *P. lobata*. HPLC pattern at UV254 nm (upper trace) and radioactivities (lower trace); see Experimental. Each peak number corresponds to the compound number.

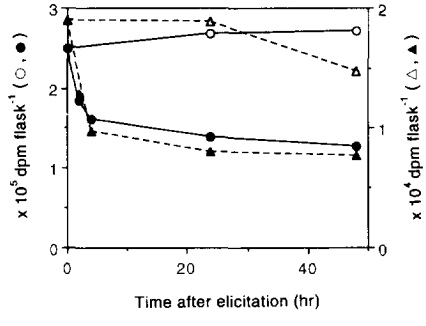


Fig. 4. Elicitor-induced decrease of methanol-soluble radioactivities in *P. lobata* cells treated with [^{14}C]-daidzein or [^{14}C]-genistein. Circles and triangles denote the radioactivities in methanol extract of the cells which had been treated with [^{14}C]-daidzein and [^{14}C]-genistein, respectively, and then elicited with YE (closed) or not (open).

tivity in the methanol extract began to decrease and after 14 hr the levels reached *ca* 40% of those of control cells (Fig. 4). No recovery of the decreased radioactivity was observed for 48 hr after elicitation, supporting our previous findings that elicitation triggers a rapid decrease in the content of the constitutive isoflavonoids resulting in an association with insoluble fractions.

Distribution of radioactivity in fractionated cell walls

We attempted to fractionate the cell wall of *P. lobata* which was ^{14}C-labelled, in order to trace the metabolic fate of the isoflavonoids decreased by elicitation. To the cell cultures which had been pretreated with [^{14}C]-daidzein (3.6×10^5 dpm flask^{-1}) for 12 hr, YE was added for elicitation. Freshly harvested cells were fractionated as described in the Experimental. Radioactivity (1.38×10^5 dpm in control flask) in the methanol extract decreased rapidly to reach 50% within 4 hr of elicitation; it was not recovered for 48 hr (Table 2). A possibility that the radioactivity flowed out into the liquid medium could be excluded because only 5% of the radioactivity lost from the methanol extract was detected in the medium. In addition, only 3–7% of the elicitor-induced loss of radioactivity from the methanol extract was detected in solubilized cell wall fractions (pectins, hemicelluloses, etc.), suggesting that nearly 90% of the lost portion of radioactivity was incorporated into non-extractable lignocellulose fractions of the cell wall (Table 2). To investigate whether the aglycone moiety or the glycosylated malonic acid residue of the isoflavonoid conjugate is essential for the elicitor-induced incorporation into insoluble lignocellulose, we prepared and fed daidzein malonyl glucoside in which the malonyl residue is ^{14}C-labelled. But no significant change in the radioactivity of the methanol extract was observed for 24 hr in elicited cells (data not shown). In addition to out previous findings that elicitation results exclusively in rapid decrease of the malonyl conjugates of *O*-glucosylated isoflavonoids, e.g. daidzein and genistin, but not in that of *C*-

Table 2. Incorporation of radioactivity into non-extractable lignocellulose fraction of elicited *P. lobata* cells treated with [^{14}C]-daidzein

Time after elicitation (hr)	Extractable frs ($\times 10^3$ dpm) Methanol extract	Medium	Solubilized cell-wall fr.*	Total	Non-extractable lignocellulose fr. ($\times 10^3$ dpm)†	Incorporation ratio (%)‡
0	138.0	—	—	138.0	—	—
4	78.3	2.9	4.0	85.2	52.8	88.4
24	70.1	4.7	3.3	78.1	59.9	88.2
48	65.3	0.2	2.3	67.8	70.2	96.5

Results are the means of fractionations of duplicate cell treatments.

*For preparation of solubilized cell-wall fractions see Experimental.

†Radioactivities of non-extractable fraction were calculated by subtracting the total extractable radioactivities of elicited cells from that of the methanol extract of 0 hr-elicited cells.

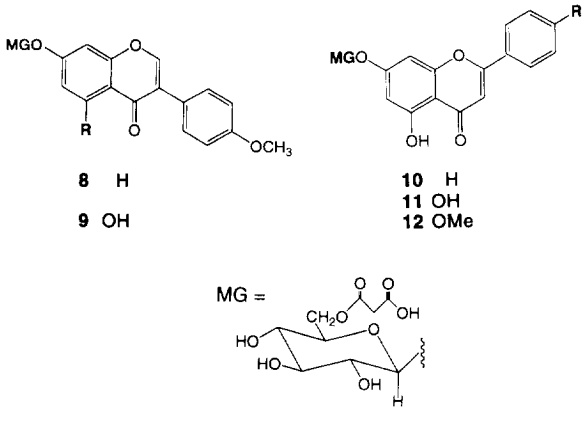
‡$\dfrac{\text{dpm of lignocellulose fr. (at each time)}}{\text{dpm of methanol extract (0 hr)} - \text{dpm of methanol extract (at each time)}} \times 1100\ (\%)$.

glucosylated ones, e.g. puerarin, it may be concluded that the aglycone moiety of isoflavone malonyl glucosides is essential for the elicitor-induced metabolism.

Biotransformation of exogenous compounds

Since most of the constitutive isoflavonoids exist as their malonyl glucosides in *P. lobata* cells, we can expect that both glycosyltransferase(s) and malonyltransferase(s) are constitutively expressed in the cells without elicitation. In our preliminary experiment with simple phenolics, e.g. resacetophenone and paeonol, the cells converted both phenolics to their respective malonyl glucosides within three days (data not shown). This observation enabled us to compare the difference between elicitor-induced metabolism of isoflavonoids and other phenolics.

Some exogenous isoflavones and flavones which are not produced in *P. lobata* were individually fed to the cells. As the transformed products, **8** and **9** were detected in the extract of the cells fed with formononetin and biochanin A, respectively. Chrysin-, apigenin- and acacetin-fed cells afforded **10**–**12**, respectively. All the transformed products (**8**–**12**) showed relatively high polarity compared with the respective mother compounds, as checked by TLC, suggesting they might be glycosylated and further malonylated. ^{1}H NMR spectra revealed one anomeric proton signal near δ5.1 (1H, *d*, J = 7–8 Hz) indicating that their component sugar is one molar β-glucopyranose. ^{13}C NMR spectra clearly showed an additional three signals assignable to a malonyl residue. By comparison of the ^{13}C NMR data of each compound with those reported for the corresponding glucosides [10], the carbon signals due to C-6 of the glucopyranosyl moiety shifted to lower field, suggesting that malonic acid is linked to C-6 of the glucose moiety. The acylation shift values (Δ δ3–5 ppm) were similar to those observed in endogenous isoflavone malonyl glucosides [6], and all the compounds were converted to their 7-*O*-glucosides by mild alkaline hydrolysis (1 N NaOH at room temperature) and to their aglycones by acid hydrolysis (1 N HCl, reflux), which was confirmed by TLC and HPLC using authentic samples. Consequently the biotransformation products were established as **8**: formononetin-7-*O*-glucoside-6″-*O*-malonyl ester, **9**: biochanin A-7-*O*-glucoside-6″-*O*-malonyl ester, **10**: chrysin-7-*O*-glucoside-6″-*O*-malonyl ester, **11**: apigenin-7-*O*-glucoside-6″-*O*-malonyl ester and **12**: acacetin-7-*O*-glucoside-6″-*O*-malonyl ester. Compounds **8** and **9** have been identified as major isoflavonoids of chickpea [11] and alfalfa [9]. Compound **11** was reported as a vacuolar pigment in parsley [12]. Compounds **10** and **12** are the first examples as natural products.



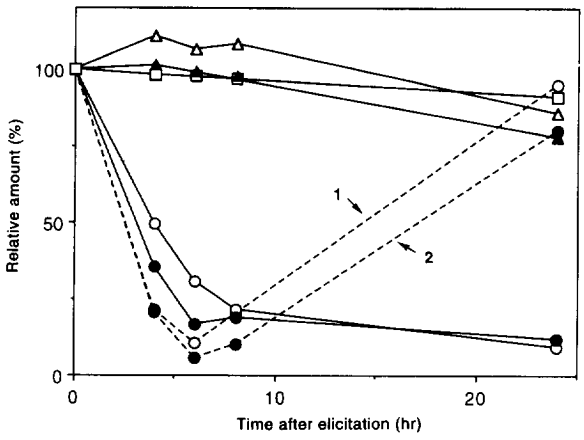


Fig. 5. Elicitor-induced changes in the amount of biotransformation products in suspension-cultured cells of *P. lobata*. For elicitation, YE (1 mg ml^{-1}) was applied to the cells which had been pretreated for three days with exogenous compounds. ○—○, Formononetin malonyl glucoside (**8**); ●—●, biochanin A malonyl glucoside (**9**); △—△, chrysin malonyl glucoside (**10**); ▲—▲, apigenin malonyl glucoside (**11**); □—□, acacetin malonyl glucoside (**12**). Arrows indicate endogenous isoflavone malonyl glucosides.

Elicitor-induced metabolism of biotransformation products

Cells which had been previously fed with exogenous isoflavones (formononetin and biochanin A) and flavones (chrysin, apigenin and acacetin) for three days were elicited with YE to elucidate the substrate-selectivity of the elicitor-induced metabolism. Elicitor-prompted change in the levels of biotransformation products together with endogenous isoflavonoids was monitored by HPLC. The level of 7-*O*-glucoside-6″-*O*-malonyl esters of flavones, i.e. chrysin, apigenin and acacetin, remained unchanged during 24 hr after elicitation, whereas exogenous isoflavone-7-*O*-glucoside-6″-*O*-malonyl esters, e.g. formononetin and biochanin A, rapidly disappeared from the methanol extract in a similar time-course response as endogenous isoflavonoids (Fig. 5). These results indicate that elicitation may induce rapid metabolism which is selective for isoflavonoids, but not for other secondary metabolites in *P. lobata* cells.

DISCUSSION

Phytoalexins are generally regarded as being produced by way of *de novo* biosynthesis. In chickpea cell suspen-

sion cultures treated with elicitors, however, the constitutive isoflavone malonyl glucosides served as precursors for pterocarpan phytoalexins in the presence of a PAL inhibitor [8], suggesting that the isoflavonoid conjugates are not metabolically inert end products under certain physiological conditions. Based on the findings in chickpea, cell suspension cultures of *P. lobata* and elicitors were used to investigate whether the metabolic relationship between the constitutive isoflavonoid conjugates and elicitor-induced isoflavones may be common to legumes. Upon treatment with YE as an elicitor, the constitutive isoflavonoid conjugates (7-*O*-glucoside-6″-*O*-malonyl esters) showed a rapid decline in their contents within the first 4 hr and after 8 hr they began to reaccumulate along with the accumulation of their aglycones and daidzen-dimers (Figs 1 and 2). A similar result was obtained from cells treated with a low concentration (40 $\mu g\,ml^{-1}$) of a fungal elicitor, *Pmg* glycoprotein (Table 1). Simultaneous treatment of the cells with a potent translation inhibitor, cycloheximide, and YE resulted in almost complete inhibition of both elicitor-inducible reaccumulation of the conjugates and formation of isoflavones, indicating that a *de novo* biosynthetic pathway is involved in the elicitor-induced production of the conjugates and isoflavones. In contrast, the elicitor-induced decrease of the constitutive isoflavonoids was not significantly influenced by the inhibitor treatment. Since no alternative turnover product could be detected in methanol extracts within the first 4 hr after elicitation, the isoflavonoids removed by elicitation might be converted to insoluble product(s) or incorporated into insoluble fraction. Exogenously fed ^{14}C-labelled isoflavones readily flowed into the pool of their respective malonyl glucosides. YE-elicitation of cells which had been fed with ^{14}C-labelled isoflavones resulted in a rapid decline of the radioactivities in methanol extracts within 4 hr. Fractionation of cell walls revealed that nearly 90% of the lost portion of the radioactivity was incorporated into a non-extractable lignocellulosic fraction. Moreover, no recovery of the lost radioactivity was observed for 48 hr in the methanol-soluble fraction, supporting our previous finding that reaccumulation of isoflavonoid conjugates should be regulated by *de novo* biosynthetic pathway, but not by re-utilization of lost isoflavonoids.

To investigate the substrate-selectivity of the elicitor-induced metabolism, we took advantage of the biotransforming capability of *P. lobata* cells. Some exogenous isoflavones and flavones were converted into their respective malonyl glucosides within three days. Elicitor-induced changes in the cellular contents of the conjugates were monitored during 24 hr after elicitation. Strikingly, only isoflavonoids (the malonyl glucosides of formononetin and biochanin A), together with endogenous isoflavonoids (the malonyl esters of *O*-glucosides) showed a rapid decline in their contents, whereas flavonoids (the malonyl glucosides of chrysin, apigenin and acacetin) did not. This implies that the isoflavone skeleton with an *O*-glycosidic linkage is essential for the elicitor-induced metabolism in *P. lobata* cells.

Isoflavonoids from several leguminous plants have also been reported to occur as their malonyl conjugates [13, 14]. A number of studies have suggested that isoflavone malonyl glucosides are possibly a storage form of biosynthetic precursors of pterocarpan phytoalexins in *Glycine max* [7], *C. arietinum* [8] and *M. sativa* [9]. From the above observations, isoflavone malonyl glucosides must be referred to as metabolically active compounds, but not inert end products any more. Our finding that elicitation stimulates rapid association of the constitutive isoflavonoid conjugates with the lignocellulose of the cell wall is the first example of a plant cell–elicitor interaction. Considering that both the reaccumulation of isoflavone malonyl glucosides and the accumulation of isoflavones (daidzein and its dimers, etc.) can be observed only after 6 hr of elicitation, the rapid metabolism of pre-formed isoflavonoids into cell walls could be regarded as an early defence response of *P. lobata* cells in a plant–pathogen interaction.

EXPERIMENTAL

General. Yeast extract was purchased from Bacto Difco Lab, cycloheximide and biochanin A from Sigma Chemical Co, L-[U-^{14}C] phenylalanine (17.6 $GBq\,mmol^{-1}$) from Amersham and all flavones from Extrasynthese, France. All other isoflavonoids were from our collections. ^1H and ^{13}C NMR were measured at 400 and 100 MHz, respectively, and chemical shifts are given in δ rel. to TMS.

Cell cultures. Cell suspension cultures of *P. lobata* were established from callus cultures which had been induced from stems as reported in ref. [15] and maintained in MS liquid medium containing 2,4,-D (2 ppm), kinetin (0.1 ppm) and sucrose (3%).

Preparation of Pmg *glycoprotein.* Two-week cultured *Phytophthora megasperma* f. sp. *glycinea* in asparagine-sucrose medium [16] was harvested by filtering through a sintered glass filter. Mycelia were washed with distilled H_2O, lyophilized, ground in a mortar and then extracted with 0.1 M NaOH with ultrasonication. The extract was centrifuged at 3000 rpm for 10 min and washed with 0.1 M NaOH ($\times 3$). The combined supernatants were neutralized, dialysed and lyophilized to give a glycoprotein consisting of 40% protein and 60% carbohydrate. Protein concns were determined according to ref. [17] using γ-globulin as ref. Carbohydrates were measured by the anthrone–H_2SO_4 method [18] as glucose equivalents.

Elicitation. Five-day-old cells at growth phase (150 ml in 500 ml flask) were combined in an autoclaved 2 l flask just before elicitation and the cells were subdivided into 150 ml in a 500 ml flask in order to increase cell homogeneity. YE (150 mg), dissolved in 2 ml distilled H_2O and then autoclaved, was aseptically added to the cells for elicitation. Control cells were supplied with an equal vol. of sterile H_2O instead of YE. *Pmg* glycoprotein (40 μg), suspended in 1 ml distilled H_2O and autoclaved, was applied to 150 ml cell cultures.

Application of cycloheximide. Cycloheximide (30 μmol), dissolved in 50 μl EtOH and filter-sterilized, was applied to cell cultures (150 ml in 500 ml flask) on day 5 of the growth cycle. YE (1 mg ml^{-1}) was simultaneously added to the cells for elicitation. Control cells were supplied with an equal vol. of sterile H_2O or YE (1 mg ml^{-1}) instead of cycloheximide.

Prepn of ^{14}C-labelled isoflavones. Cell suspension cultures (150 ml in 500 ml flask) on day 7 after subculture were elicited with YE for 4 hr. To the cultures 40 μCi of L-[^{14}C]-phenylalanine was aspetically supplied and the cells were cultured for an additional 18 hr. Cells were harvested and extracted with a total of 200 ml MeOH (× 3) under reflux. The extract was filtered off, concd *in vacuo* and then hydrolysed with 2 N HCl containing 20% MeOH at 80° for 3 hr. The hydrolysate was concd to remove MeOH and then extracted with a total of 200 ml EtOAc (× 2) and the combined organic phases applied to a silica gel (Merck Kieselgel G60) column and eluted with $CHCl_3$–MeOH (9:1–4:1). Frs containing the corresponding isoflavones were collected for further purification by HPLC. Purification by semiprep. HPLC was conducted as follows: column, ODS-80TM (7.8 i.d. × 300 mm); eluent, 68% MeOH for daidzein or 72% MeOH for genistein; flow rate, 1.2 ml min^{-1}; detection, UV 254 nm. Consequently, 4.5 μmol (3.8×10^6 dpm) of [^{14}C]-daidzein (sp. act. 8.44×10^8 dpm mmol^{-1}) and 0.4 μmol (3.2×10^5 dpm) of [^{14}C]-genistein (sp. act. 8.01×10^8 dpm mmol^{-1}) were obtained.

Feeding experiments and elicitation. [^{14}C]-Daidzein (3.6×10^5 dpm) or [^{14}C]-genistein (3.0×10^4 dpm) was individually fed to suspension cultures on day 5 of the growth cycle. At 12 hr after application of the isoflavones, cells were harvested and extracted with MeOH. An aliquot (50 μl), passed through ODS-PAK (Tosoh Co., Japan), was analysed by HPLC for distribution of the radioactivity. HPLC conditions were the same as described previously [6]. The radioactivity of each fr. was directly measured by liquid scintillation counting. Cell cultures (150 ml) which had been treated for 12 hr with [^{14}C]-daidzein of [^{14}C]-genistein were aseptically harvested and transferred to an equal vol. of conditioned medium. Control cells were prepd by adding 2 ml of sterile H_2O instead of YE. At appropriate times after elicitation, the radioactivity of the cells was counted.

Cell wall fractionation. Cells which had been pretreated with [^{14}C]-daidzein (3.6×10^5 dpm) for 12 hr and then elicited with YE were aseptically harvested, washed with sterile H_2O and transferred to an equal vol. of conditioned medium in order to remove residual radioactivity from the initial medium. For a control, an equal vol. of sterile H_2O was added instead of YE to cell cultures. Freshly harvested cells were lyophilized and 1 g (dry wt) cells was exhaustively extracted with hexane, 80% EtOH and MeOH successively, to remove lipophilic materials. Further fractionation of cell walls was carried out according to ref. [19].

Feeding of exogenous compounds and isolation of biotransformation products. Each compound (20 mg) dissolved in DMSO (400 μl) and filter-sterilized, was individually fed to suspension cultures. After 3 days of incubation, the cells were harvested, extracted with MeOH (× 2) with ultrasonication and then filtered off. The combined filtrates were evapd *in vacuo* below 40° and the aq. phase washed with EtOAc. The resulting aq. phase was extracted with BuOH and the organic phase was evapd *in vacuo*. The extracts dissolved in MeOH (2 ml), were applied to prep. TLC (Merck Kieselgel G60) and developed in $CHCl_3$–MeOH–H_2O (40:16:3). The bands corresponding to the products were scraped off, extracted with MeOH with ultrasonication and then filtered off. The filtrates were further purified by prep. HPLC as follows: column, ODS-80TM (21.5 i.d. × 300 mm); eluent, 65–72% MeOH in 1% HOAc; flow rate, 7.5 ml min^{-1}; detection, UV 254 nm.

Formononetin-7-O-glucoside-6″-O-malonyl ester (**8**). Yield 12 mg from HPLC (70% MeOH in 1% HOAc) as a powder. ^{1}H NMR (DMSO-d_6): δ8.42 (1H, *s*, H-2), 8.09 (1H, *d*, *J* = 8.8 Hz, H-5), 7.55 (2H, *d*, *J* = 8.8 Hz, H-2′, 6′), 7.25 (1H, *d*, *J* = 2.0 Hz, H-8), 7.17 (1H, *dd*, *J* = 8.8, 2.0 Hz, H-6), 7.02 (2H, *d*, *J* = 8.8 Hz, H-3′,5′), 3.81 (3H, *s*, OMe), 5.16 (1H, *d*, *J* = 6.8 Hz, glucose H-1). ^{13}C NMR: δ176.3 (*d*, C-4), 169.7 (*s*, $-CO_2H$), 169.0 (*s*, $-CO_2-$), 162.8 (*s*, C-7), 160.6 (*s*, C-9), 158.6 (*s*, C-4′), 155.2 (*d*, C-2), 131.7 (*d*, C-2′, C-6′), 128.7 (*d*, C-5), 125.6 (*s*, C-3), 125.0 (*s*, C-1′), 120.2 (*s*, C-10), 117.0 (*d*, C-6), 115.3 (*d*, C-3′, C-5′), 105.2 (*d*, C-8), 101.4 (*d*, C-1″), 77.8 (*d*, C-3″), 75.5 (*d*, C-5″), 74.7 (*d*, C-2″), 71.3 (*d*, C-4″), 65.5 (*t*, C-6″), 56.8 (*q*, OMe), 43.8 (*t*, $-CH_2-$).

Biochanin A-7-O-glucoside-6″-O-malonyl ester (**9**). Yield 15 mg from HPLC (70% MeOH in 1% HOAc) as a powder. ^{1}H NMR (DMSO-d_6): δ8.46 (1H, *s*, H-2), 7.55 (2H, *d*, *J* = 8.8 Hz, H-2′, 6′), 7.04 (2H, *d*, *J* = 8.8 Hz, H-3′,5′), 6.74 (1H, *d*, *J* = 2.0 Hz, H-8), 6.51 (1H, *d*, *J* = 2.0 Hz, H-6), 3.82 (3H, *s*, OMe), 5.14 (1H, *d*, *J* = 7.2 Hz, glucose H-1). ^{13}C NMR: δ182.0 (*s*, C-4), 169.5 (*s*, $-CO_2H$), 168.5 (*s*, $-CO_2-$), 164.3 (*s*, C-7), 163.2 (*s*, C-5), 160.8 (*s*, C-9), 158.8 (*s*, C-4′), 156.4 (*d*, C-2), 131.8 (*d*, C2′, C-6′), 124.3 (*s*, C-3), 123.8 (*s*, C-1′), 115.3 (*d*, C-3′, C-5′), 107.8 (*s*, C-10), 101.1 (*d*, C-6, C-1″), 96.2 (*d*, C-8), 77.8 (*d*, C-3″), 75.4 (*d*, C-5″), 74.6 (*d*, C-2″), 71.2 (*d*, C-4″), 65.6 (*t*, C-6″), 56.8 (*q*, OMe), 43.1 (*t*, $-CH_2-$).

Chrysin-7-O-glucoside-6″-O-malonyl ester (**10**). Yield 6 mg from HPLC (68% MeOH in 1% HOAc) as a brown powder. ^{1}H NMR (DMSO-d_6): δ8.09 (2H, *dd*, *J* = 8.4, 2.0 Hz, H-2′,6′), 7.60 (3H, *m*, H-3′,4′,5′), 7.02 (1H, *s*, H-3), 6.84 (1H, *d*, *J* = 2.0 Hz, H-8), 6.48 (1H, *d*, *J* = 2.0 Hz, H-6), 5.10 (1H, *d*, *J* = 7.6 Hz, glucose H-1). ^{13}C NMR: δ182.1 (*s*, C-4), 168.2 (*s*, $-CO_2H$), 167.7 (*s*, $-CO_2-$), 163.7 (*s*, C-1), 163.1 (*s*, C-7), 161.3 (*s*, C-5), 157.3 (*s*, C-9), 132.2 (*s*, C-1′), 130.8 (*d*, C-4′), 129.3 (*d*, C-3′, C-5′), 126.6 (*d*, C-2′, C-6′), 105.7 (*s*, C-10), 105.1 (*d*, C-3), 99.9 (*d*, C-6, C-1″), 95.0 (*d*, C-8), 76.4 (*d*, C-3″), 74.1 (*d*, C-5″), 73.2 (*d*, C-2″), 69.8 (*d*, C-4″), 63.9 (*t*, C-6″), 42.8 (*t*, $-CH_2-$).

Apigenin-7-O-glucoside-6″-O-malonyl ester (**11**). Yield 4 mg from HPLC (65% MeOH in 1% HOAc) as a yellow powder. ^{1}H NMR (DMSO-d_6): δ8.00 (2H, *d*, *J* = 8.8 Hz, H-2′,6′), 6.97 (2H, *d*, *J* = 8.8 Hz, H-3′,5′), 6.89 (1H, *s*, H-3), 6.83 (1H, *d*, *J* = 2.0 Hz, H-8), 6.46 (1H, *d*, *J* = 2.0 Hz, H-

6), 5.13 (1H, *d*, $J = 7.2$ Hz, glucose H-1). ^{13}C NMR: δ181.7 (*s*, C-4), 169.3 (*s*, $-CO_2H$), 168.4 (*s*, $-CO_2-$), 165.9 (*s*, C-2), 164.2 (*s*, C-7), 162.9 (*s*, C-5), 162.7 (*s*, C-4'), 162.6 (*s*, C-9), 1302 (*d*, C-2', C-6'), 122.6 (*s*, C-1'), 117.5 (*d*, C-3', C-5'), 107.0 (*s*, C-10), 104.7 (*s*, C-3), 101.1 (*d*, C-6, C-1''), 96.3 (*d*, C-8), 77.7 (*d*, C-3''), 75.4 (*d*, C-5''), 74.6 (*d*, C-2''), 71.2 (*d*, C-4''), 65.7 (*t*, C-6''), 43.2 (*t*, $-CH_2-$).

Acacetin-7-O-glucoside-6''-O-malonyl ester (**12**). Yield 9 mg from HPLC (72% MeOH in 1%HOAc) as yellow powder. ^{1}H NMR (DMSO-d_6): δ8.06 (2H, *d*, $J = 8.8$ Hz, H-2',6'), 7.13 (2H, *d*, $J = 8.8$ Hz, H-3',5'), 6.94 (1H, *s*, H-3), 6.83 (1H, *d*, $J = 2.0$ Hz, H-8), 6.45 (1H, *d*, $J = 2.0$ Hz, H-6), 5.11 (1H, *d*, $J = 7.6$ Hz, glucose H-1). ^{13}C NMR: δ182.1 (*s*, C-4), 167.9 (*s*, $-CO_2H$), 167.2 (*s*, $-CO_2-$), 164.0 (*s*, C-7), 162.9 (*s*, C-2), 162.6 (*s*, C-4'), 161.2 (*s*, C-5), 157.2 (*s*, C-9), 128.5 (*d*, C-2', C-6'), 122.9 (*s*, C-1'), 114.8 (*d*, C-3', C-5'), 105.7 (*s*, C-10), 104.0 (*d*, C-3), 99.8 (*d*, C-6, C-1''), 94.9 (*d*, C-8), 76.3 (*d*, C-3''), 74.0 (*d*, C-5''), 73.1 (*d*, C-2''), 69.7 (*d*, C-4''), 64.1 (*t*, C-6''), 55.7 (*q*, OMe), 41.9 (*t*, $-CH_2-$).

Elicitation of cells fed with exogenous compounds. Each isoflavone or flavone (5 mg) dissolved in 20 μl DMSO and filter-sterilized, was fed to suspension cultures (150 ml in 500 ml flask) on day 5 after subculture. Compounds were converted to their 7-*O*-glucoside-6''-*O*-malonyl esters in the cells after 3 days incubation, as monitored by HPLC. Cells were aseptically sepd from medium by filtration and then transferred to an equal vol. of conditioned medium to remove any residual aglycone. Then YE (1 mg ml^{-1}) was added to the cultures for elicitation and the cells incubated for an additional time. At the appropriate time, cells were harvested and extracted with MeOH for quantitation of phenolics by HPLC as described previously [6].

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