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COMPARATIVE BIOCHEMISTRY OF CHALCONE ISOMERASES

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Key Word Index—*Medicago sativa*; *Phaseolus vulgaris*; Leguminosae; *Petunia hybrida*; Solanaceae; plant cell culture; chalcone isomerase.

Abstract—Fungal elicitor-inducible chalcone isomerase activity was present in suspension cultured cells of *Phaseolus vulgaris*, *Glycine max* and *Medicago sativa*, and a non-inducible activity was found in the purple sections of callus cultures of *Petunia hybrida* strain AK-5000. The enzyme from the legume sources catalysed the isomerisation of both 2′,4,4′-trihydroxy-and 2′,4,4′,6′-tetrahydroxychalcones whereas the *Petunia* enzyme was specific for the tetrahydroxychalcone. Apparent differences in the properties of the enzyme from different sources were observed in relation to kinetic parameters, M_r values for holoenzyme and subunits newly synthesised *in vitro* from mRNA, antigenic cross-reactivity and cDNA cross-hybridisation. Our data confirm and extend previous observations suggesting differences and anomalies between the properties of chalcone isomerases from different sources.

INTRODUCTION

Chalcone isomerase (CHI, EC 5.5.1.6) catalyses the stereospecific isomerisation of chalcones to their corresponding (−)flavanones [1]. It is thus involved in the synthesis of flavonoid pigments in the flowers and pollen of many plant species [2–6] and, in legumes, is also involved in the biosynthesis of microbially-induced, antifungal, isoflavonoid phytoalexins [7]. CHI activity is induced in bean hypocotyl tissues infected with the fungal pathogen *Colletotrichum lindemuthianum* [unpublished results], and in bean cell suspension cultures exposed to elicitor polysaccharides from this fungus [8]; this increased activity, which precedes the accumulation of isoflavonoids, results directly from increased synthesis of the enzyme as a consequence of transient induction of mRNA encoding CHI [9]. In addition, fungal elicitor-treatment may also result in the activation of catalytically inactive isomerase enzyme [8, 10].

The availability of specific antisera directed against the CHIs from bean [8] and *Petunia* [5] has facilitated the molecular genetic analysis of the enzyme and mechanism of its induction via the isolation of clones from cDNA expression libraries [1, Van Tunen *et al.*, in preparation]. However, preliminary investigations of *Petunia* CHI(s) using the heterologous anti-(bean CHI) serum suggested that the enzymes from these two sources may exhibit different M_r values and antigenic cross-reactivities. In fact, a survey of previously published literature reveals highly disparate values for the properties of CHIs from different plant sources whereas the earlier phenylpropanoid biosynthetic enzymes phenylalanine ammonia-lyase and chalcone synthase exhibit similar M_rs and antigenic cross-reactivities between plant species. In an attempt to clarify this picture, we have now therefore compared the M_rs, catalytic properties, antigenic cross-reactivities and cDNA hybridisation patterns of CHIs from a number of sources.

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RESULTS AND DISCUSSION

Presence and inducibility of CHI in plant cell cultures

CHI activity was detected in unelicited suspension cultures of bean (*Phaseolus vulgaris* cv Immuna and cv Canadian Wonder), soybean (*Glycine max* cv Harosoy) and alfalfa (*Medicago sativa* cv Europe), and in callus cultures of *Petunia hybrida* strain AK-5000, where it appeared to be primarily localized to the deep purple sections which accumulated anthocyanins (Table 1). The enzyme activity was induced in suspension cultures of the three legumes by treatment with elicitor molecules from the cell walls of the bean pathogen *Colletotrichum lindemuthianum*. No induction of CHI could be demonstrated in white suspension cultures of *Petunia hybrida* strain AK-5000 which do not produce anthocyanin pigments. In *Petunia*, CHI is expressed in the intact plant in a tissue and development-specific manner in petals and pollen; the purple callus (AK-5000) which originated from leaf explants of *Petunia* strain C7158, makes the same anthocyanidins (petunidin and malvidin) as pigmented tissue of the intact plant [12], although it is not understood why anthocyanin synthesis is expressed in these cells. On the basis of the observation of the lack of induction of chalcone synthase (the enzyme preceding CHI in flavonoid synthesis) by elicitor in cultured cells of parsley (which make furanocoumarin phytoalexins) [13], it is perhaps not surprising that CHI is not induced by elicitor in

Table 1. Presence and inducibility of chalcone isomerase in plant cell cultures

Source (S = suspension, C = callus)		Specific activity (mkat/kg protein)* Uninduced	+ Elicitor† 8 hr	+ Elicitor† 24 hr
1. *Phaseolus vulgaris*, cv Canadian Wonder	(S)	2.00	3.85	5.12
2. *Phaseolus vulgaris*, cv Immuna	(S)	0.65	1.50	1.75
3. *Glycine max*, cv Harosoy	(S)	3.59	7.30	11.26
4. *Medicago sativa*, cv Europe	(S)	4.73	7.92	11.71
5. *Petunia hybrida*, strain AK-5000 (white)	(S)	0.00	0.00	0.00
6. *Petunia hybrida*, strain AK-5000:	(C)			
Purple sections		1.75	ND	ND
White sections		0.19	ND	ND

*The substrate in assays of the enzyme from sources 1–4 was 2',4,4'-trihydroxychalcone and in sources 5 and 6 2',4,4',6'-tetrahydroxychalcone.

†Suspensions were treated with *Colletotrichum lindemuthianum* cell wall elicitor to a final concentration of 60 μg glucose equivalents ml^{-1}, and cells harvested after a further 8 hr or 24 hr of incubation.

‡ND = Not determined.

Petunia which, as a member of the Solanaceae, would be expected to make terpenoid, not isoflavonoid, phytoalexins.

Size and kinetic properties of CHI from different sources

CHI was partially purified from cultures of bean, alfalfa, soybean and *Petunia hybrida* strain AK-5000 by ammonium sulphate precipitation and gel filtration through Sephadex G-100. Holoenzyme M_rs were calculated from G-100 elution profiles (Fig. 1 and Table 2), and kinetic parameters for the G-100-purified enzymes measured. Table 2 also includes compiled data on CHI from other sources, taken from previously published papers. Apart from a higher K_m value for 2',4,4',6'-tetrahydroxychalcone, the alfalfa enzyme appeared similar to that from bean, whose properties have been described in detail elsewhere [14]. The enzyme from soybean was typical of the other legume CHIs in its ability to catalyse the isomerisation of 6'-hydroxy-and 6'-deoxychalcones, and its significantly lower holoenzyme M_r value [15] was confirmed (Table 2). In contrast, the enzyme from *Petunia* could not catalyse the isomerisation of 2',4,4'-trihydroxychalcone, a property in common with the isomerases from other plants which do not appear to synthesise 6'-deoxy chalcone-derived flavonoid compounds (refs given in Table 2). Extensive purification of the bean [14, 8] and soybean [15] isomerases indicates that a single enzyme exhibits the dual substrate specificity.

The M_rs for CHI holoenzyme from *Petunia* corollas (determined by HPLC) and the M_r for the denatured enzyme from the same source (determined by SDS-PAGE) are closely comparable (30 000 and 29 000 respectively, [5]). Similarly, M_rs of 30 000 and 25 000 are observed for the intact and denatured enzyme from *Petunia* pollen. However, the CHI holoenzyme from purple *Petunia* callus appeared to be of higher M_r (45 000) than the enzyme from bean (27 500) (Fig. 1). The above data (in addition to *in vitro* translation and further Western blotting studies described below) fail to confirm an M_r value of 62 500 for the enzyme from *Petunia* on the basis of gel filtration experiments [6]. This previous report draws attention to a problem in working with pigmented *Petunia* extracts, namely the very high levels of phenolics which may bind to enzymes and alter, for example, their apparent pI value and possibly M_rs. This may explain the high M_r of the *Petunia* callus enzyme in Fig. 1; although Dowex was used to bind phenolics in the extract, the ammonium sulphate pellet was still blue. It is not clear whether such problems were also responsible for the multiple charge forms of CHI reported in earlier work (summarized in Table 2), or even whether peroxidases were being measured in addition to CHI due to the lack of inclusion of sufficient KCN in the assay mixtures. *In vitro* translation clearly circumvents any potential problems of abberant gel filtration/electrophoretic mobility as a result of binding of phenolics (see below).

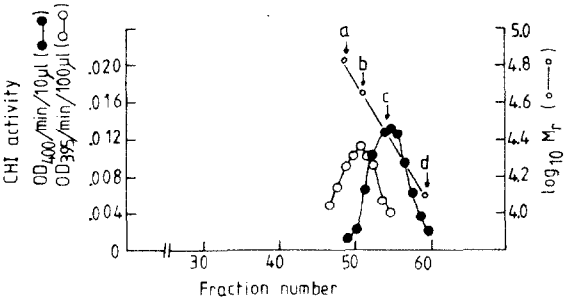

Fig. 1. Chromatography of chalcone isomerases on Sephadex G-100 (150 × 1.5 cm). See text for details. Arrows indicate the elution volumes of the M_r markers bovine serum albumin (a), ovalbumin (b), carbonic anhydrase (c) and cytochrome *c* (d). ○—○ = *Petunia* CHI activity (OD_{395}/min/100 μl, ●—● = bean CHI activity (OD_{400}/min/10 μl). Note that cell extracts were stirred with Dowex-1 (PO_4^{2-} form) to remove phenolics prior to $(NH_4)_2SO_4$ precipitation.

Table 2. Properties of chalcone isomerase from various plant sources

Species	Holoenzyme M_r	Number of charge isoforms		pH optimum	K_m (μM) 2',4,4'-trihydroxychalcone	K_m (μM) 2',4,4',6'-tetrahydroxychalcone	Reference
Cicer arietinum	ND*	2–3		8.5	16	44 (enzyme 2)	[26]
Glycine max	15 600	ND		7.6	4	21	[15]
Glycine max	18 500	ND		7.7	7	11	This paper
Medicago sativa	28 000	ND		8.0	9.5	100	This paper
Phaseolus aureus	ND	2	Form-I	7.5	57	18	[26]
			Form-II	8.4	14	44	
Phaseolus vulgaris	27 500	1		8.0	8.9	13	[8, 14]
Petroselinum hortense	50 000	up to 5		8.4	No activity	16	[17, 26]
Petunia hybrida	30 000 Corolla‡	2–3		8.0–8.6	No activity	1–2†	
	30 000 Pollen‡	1		7.5	No activity		[5]
Callistephus chinensis	ND	ND		8.6	No activity	ND	[4]
Dianthus caryophyllus	ND	ND		8.6	No activity	ND	[2]

*Not determined.

†An approximate value calculated from $1/v$ vs $1/S$ plots for crude preparations of the enzyme (data from ref. 6).

‡In contrast to other published data, see text for details.

Immunological cross-reactivity between chalcone isomerases

The production and characterisation of antisera against highly purified native bean CHI and SDS-denatured *Petunia* CHI have been described elsewhere [8, 5]. An apparently weak antigenic cross-reactivity between the isomerases from bean and *Petunia* is demonstrated by the effects of the anti-(bean) and anti-(*Petunia*) CHI sera on the enzyme activities from these two sources (Table 3). Such cross-reactivity is only noted if the mixture is treated with protein A-Sepharose, thus removing CHI-anti (CHI) IgG complexes; under such conditions, the *Petunia* enzyme activity may effectively be removed in the presence of excess anti-(bean CHI) serum [16]. In separate experiments, it was observed that part of the CHI activity in soybean extracts was removed by anti-(bean CHI) serum and protein A Sepharose, but was totally unaffected by antiserum directed against the *Petunia* enzyme. The enzyme from alfalfa was removed by neither anti-(bean CHI) serum nor anti-(*Petunia*) CHI serum in the presence of protein A Sepharose (Table 3).

Western blots of proteins separated from bean, *Petunia*, soybean and alfalfa cell culture extracts by SDS-polyacrylamide gel electrophoresis were developed using anti-(*Petunia* CHI) serum (Fig. 2). Extracts from flower buds of *Petunia* R27 (the source of the antigen against which *Petunia* antiserum was directed) were included as a standard. The anti-(*Petunia* CHI) serum recognized polypeptide subunits of M_r *ca* 29 000 from *Petunia* R27 flower buds, 29 000 and 30 000 from purple *Petunia* callus, 28 500 from alfalfa (faintly visible) and 25 500 from bean (Fig. 2). The loadings and short development time used for the blot in Fig. 2 result only in detection of major recognised polypeptides. In a second series of blots (Fig. 3), higher loadings were used, and individual tracks on the nitrocellulose cut longitudinally and developed for longer time periods with anti-(bean CHI) serum and anti-(*Petunia* CHI) serum, both separately and together. These conditions result in the appearance of a number of extra bands, presumably the result of non-specific binding. It is clear, however, that the bean polypeptide of M_r 25 000 recognised by anti-(*Petunia* CHI) serum is not the band recognised by the anti-(bean CHI) serum, (M_r 27 500 and previously identified as CHI [8]). Likewise, the anti-(bean CHI) serum, although recognising a polypeptide of M_r *ca* 20 000 in blots of both bean and *Petunia* extracts, does not recognise the M_r 30 000 *Petunia* CHI band. Anti-(bean CHI) serum failed to detect any polypeptides on blots of alfalfa and soybean extracts in spite of high loadings (data not shown). In another experiment (data not shown), the anti-(*Petunia* CHI) serum failed to recognise any polypeptides on Western blots of extracts from parsley (*Petroselinum hortense*). The enzyme from this source has been reported to possess an M_r of 50 000 [17].

In vitro *translation analysis*

The anti-(bean)- and anti-(*Petunia*) CHI sera recognized polypeptides of M_r 27 000 and 29 000 respectively on immunoprecipitation of the *in vitro* translation products of mRNA from homologous sources (Fig. 4). The anti-(bean CHI) serum, which does not immunoprecipitate *Petunia* CHI from solution in the absence of protein A Sepharose [8], did not, however, recognise any *in vitro* translation products from *Petunia*. The fact that newly synthesized, *in vitro* translated bean and *Petunia* CHIs are of the same M_r as the subunits revealed on Western blotting indicates that these enzymes are not significantly modified post-translationally, and, by comparison with holoenzyme M_r values, are both monomers. This correlation does, however, throw even more doubt on the accuracy of M_r determinations by gel filtration for the *Petunia* enzyme. In contrast, holoenzyme M_r values

Table 3. Effects of anti-(bean) and anti-(*Petunia*) CHI sera on the activities of chalcone isomerase in extracts from bean, soybean, alfalfa and *Petunia* callus cultures

Enzyme extract*	Antibody	Protein A-Sepharose†	Enzyme activity‡ (ΔOD_{400}/min)	% inhibition
Bean	None	–	0.060	
	Anti-(bean CHI)	–	0.010	83
	Anti-(Petunia CHI)	–	0.063	0
	Anti-(Petunia CHI)	+	0.052	14
	Pre-immune serum	+	0.060	0
Alfalfa	None	–	0.036	
	Anti-(bean CHI)	–	0.035	0§
	Anti-(bean CHI)	+	0.034	0
	Anti-(Petunia CHI)	–	0.033	0
	Anti-(Petunia CHI)	+	0.034	0
Soybean	None	–	0.042	
	Anti-(bean CHI)	–	0.042	0
	Anti-(bean CHI)	+	0.032	24
	Anti-(Petunia CHI)	–	0.042	0
	Anti-(Petunia CHI)	+	0.044	0
Petunia (purple callus)	None	–	0.020	
	Anti-(Petunia CHI)	–	0.002	90
	Anti-(bean CHI)	–	0.020	0
	Anti-(bean CHI)	+	0.013	35

*Cells were homogenised in two volumes of 50 mM KH_2PO_4, pH 8.0, containing 1.4 mM 2-mercapthoethanol and 0.15 M NaCl. Extracts were centrifuged at 4°C. Supernatants were used directly for assay (bean and *Petunia*) or concentrated by 50–75% $(NH_4)_2SO_4$ fractionation and Amicon ultrafiltration (alfalfa and soybean). Extracts were incubated with 50 μl antisera per 400 μl enzyme extract, and samples incubated for 1 hr prior to assay.

†Protein A-Sepharose (5 mg per 400 μl enzyme extract) was added to samples incubated on an end-over-end mixer for 1 hr prior to centrifugation and assay of supernatants.

‡The enzyme activity values represent the activity of the volume of extract treated with 5 μl of antiserum.

§Inhibitions of less than 10% are not considered significant

determined by gel filtration for the enzymes from bean and alfalfa correlate well with immunologically determined subunit M_rs in the 30000 range. The above situation for CHI contrasts with that for chalcone synthase, the enzyme preceding CHI in flavonoid biosynthesis, whose experimentally determined M_r value is similar in all tissues so far studied; in addition, the antiserum against parsley chalcone synthase cross-reacts with the *in vitro* translated enzyme from unrelated plants such as bean [18] and soybean [19].

cDNA hybridisation studies

Total RNA from flower buds of *Petunia* R27, and polysomal RNA from elicitor-induced bean cell cultures, were separated on 1.4% agarose gels and Northern blotted to PALL Biodyne nylon membranes. These were then probed with a less than full length, 0.865 kb cDNA complementary to bean chalcone isomerase [11] and a 0.750 kb cDNA complementary to *Petunia* chalcone isomerase (Van Tunen *et al.*, unpublished). The bean cDNA recognised an elicitor inducible, *ca* 1 kb transcript, corresponding to bean CHI. The induction of this mRNA preceded the increase in CHI enzyme activity in the elicitor-treated bean cells (Fig. 5). When the same blots were hybridized with *Petunia* CHI cDNA, no RNA transcripts bound the probe. The *Petunia* cDNA strongly hybridized to electrophoresed cDNA insert from *Petunia* (i.e. to itself), but not to bean CHI cDNA insert. Note that in Fig. 5 the same blot was probed first with the *Petunia* cDNA, then with the bean. The apparent weak hybridization of the bean cDNA to the immobilised *Petunia* insert probably reflects residual hybridized *Petunia* probe from the previous hybridization. Since the bean and *Petunia* cDNAs show no cross hybridization the bean cDNA probe did not hybridize to mRNA transcripts from *Petunia* flower buds, although a CHI transcript was readily detected with the *Petunia* probe.

These data provide further confirmation of the lack of relatedness between the CHIs from the two sources, although it is not yet known whether CHIs more related to the *Petunia* enzyme may be expressed in bean during floral development, or whether 'bean CHI-like' sequences are ever expressed in *Petunia*.

Taken together, the data in this paper indicate that chalcone isomerase activity, although present in a number of plant species, does not appear to reside in closely related polypeptides. The reasons for this apparent divergence are not yet clear. They do not solely relate to species differences or differences in catalytic type, e.g. legume activity vs tri-and tetrahydroxychalcones and the other activities vs tetrahydroxylchalcone alone. Although some of the major differences reported to date depend on

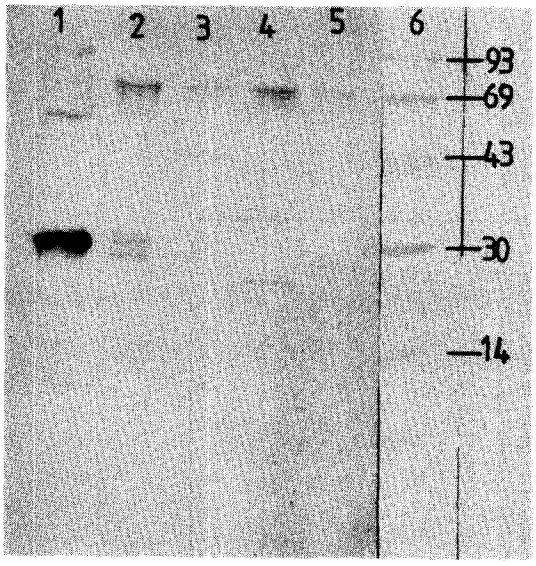

Fig. 2. Western blot of proteins from *Petunia* R27 flower buds (Track 1), *Petunia* purple callus (Track 2), soybean (Track 3), bean (Track 4) and alfalfa (Track 5) extracts separated by SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose, antigens were detected with anti-(*Petunia* CHI) serum and goat anti-(rabbit Ig) alkaline phosphatase conjugate [5]. M_r markers (Track 6) were detected by staining with amido black dye (values in RH column are $M_r \times 10^{-3}$). The loadings of CHI activity (pkat) per track are: *Petunia* R27, 580; purple *Petunia* callus, 220; soybean, 280; bean, 110 and alfalfa, 160. The blot was developed with alkaline phosphatase substrate for 2 min.

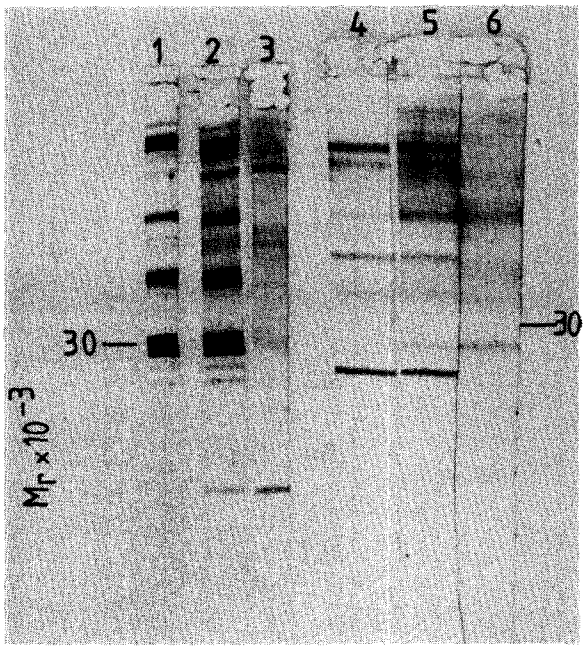

Fig. 3. Western blots of proteins from *Petunia* R27 flower buds (Tracks 1–3) and cell cultures of bean (Tracks 4–6), separated by SDS polyacrylamide gel electrophoresis. After transfer to nitrocellulose, antigens were detected with anti-(*Petunia* CHI) serum (Tracks 1 and 4), anti-(bean CHI) serum (Tracks 3 and 6) or a mixture of both antisera (Tracks 2 and 5). Loadings were *ca* 2 times higher than in Fig. 2, and development of all blots with alkaline phosphatase substrate was for 10 min. The R27 flower bud extract had been partially purified by chromatography on Blue Sepharose CL 6-B [5].

chromatographic determination of M_r which may be prone to artefacts (e.g. binding of polyphenols), the lack of relatedness between bean and *Petunia* CHIs at the molecular level is clearly demonstrated by the immunological and DNA hybridization studies reported here. Further work is now in progress to determine whether sequences similar to *Petunia* CHI are found in the genomes of bean and other legumes.

EXPERIMENTAL

Chemicals. 2′,4′4′-Trihydroxychalcone was synthesized from *p*-hydroxybenzaldehyde and resacetophenone as described in ref. [20]. 2′,4,4′,6′-Tetrahydroxychalcone was prepared by the method of ref. [21].

Plant material. Callus cultures of bean, soybean and alfalfa were initiated from excised radical tips on modified Schenk and Hildebrandt medium [22]. The same medium was used for the growth (under constant illumination) of callus cultures of *Petunia hybrida* strain AK-5000 which contained purple, pigmented sections [12]. Growth conditions for all suspension cultures were as described [8]. Flower buds of *Petunia* line R27 were from plants grown under greenhouse conditions at the Free University, Amsterdam.

Preparation and assay of CHI. For analysis of CHI M_r values by gel filtration, cell samples (up to 50 g) were homogenized in 2 vols of 100 mM KH_2PO_4, pH 8.0, containing 1.4 mM 2-mercaptoethanol. After centrifugation, 50–75% (35–75% in the case of *P. hybrida*) $(NH_4)_2SO_4$ fractions were prepared from the supernatants, and the samples analysed on a column of Sephadex G-100 previously calibrated with standard proteins of known M_r. CHI activity was determined, using tri-and/or tetra-hydroxychalcones as substrate, in the presence of 50 mM KCN as described [14].

Immune blotting. The preparations and characterizations of anti-(bean CHI) and anti-(*Petunia* CHI) sera were as described [8, 5]. Enzyme extracts for blotting were prepared by 50–75% $(NH_4)_2SO_4$ fractionation and concn on Amicon 10 000 M_r cut-off membrane centrifuge filters. Immune blotting of CHI preparations, with detection by goat anti-(rabbit IgG) alkaline phosphatase conjugate, was as described [5].

Labelling of CHI subunits in vitro. Preparation and *in vitro* translation of polysomal mRNA, indirect immunoprecipitation of CHI subunits and detection of subunits by fluorography were as previously described [8, 23]. Poly A+mRNA from *Petunia* flower buds was prepared by chromatography on oligo-(dT) cellulose or mAP paper (Amersham).

Northern blotting. Polysomal mRNA was glyoxalated, electrophoresed in a 1.4% agarose gel and blotted to PALL Biodyne nylon membranes using standard procedures [24]. Blots were hybridized with labelled cDNA probes according to the membrane manufacturer's instructions and subjected to autoradiography. cDNA probes complementary to bean CHI [11] and *Petunia* CHI were labelled with α-^{32}P dATP by the random oligonucleotide priming procedure [25].

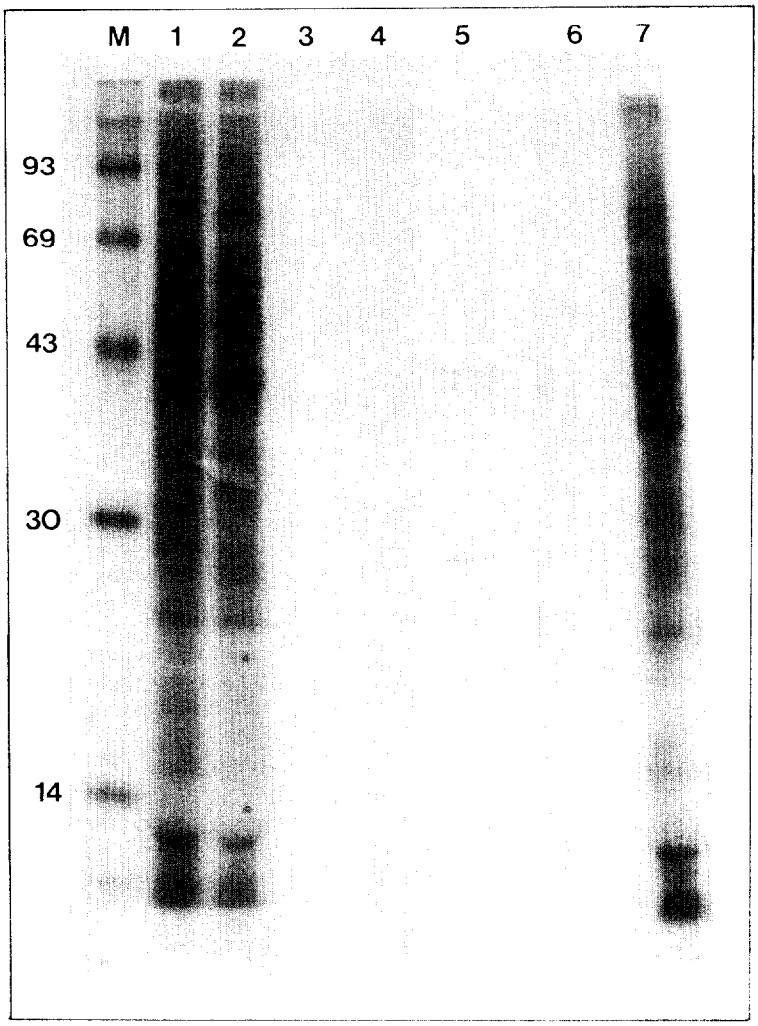

Fig. 4. Fluorographs of polypeptides newly synthesized *in vitro* from bean polysomal mRNA or *Petunia* R27 flower bud polyA + mRNA in rabbit reticulocyte lysate cell-free translation systems. Track M = ^{14}C M_r markers (values in LH column are $M_r \times 10^{-3}$); Tracks 1 and 2 = total polypeptides synthesized *in vitro* from polysomal mRNA isolated from unelicited (Track 1) and elicited (Track 2) bean cell cultures; Tracks 3 and 4 = subunits immunoprecipitated by anti-(bean CHI) serum and protein A-Sepharose after *in vitro* translation of polysomal mRNA from unelicited (Track 3) and elicited (Track 4) bean bell cultures; Track 5 = subunits immunoprecipitated by anti-(*Petunia* CHI) serum and protein A Sepharose after *in vitro* translation of polyA + mRNA from flower buds of *Petunia* R27; Track 6 = as Track 5, but using anti-(bean CHI) serum; Track 7 = total *in vitro* translation products from *Petunia* mRNA.

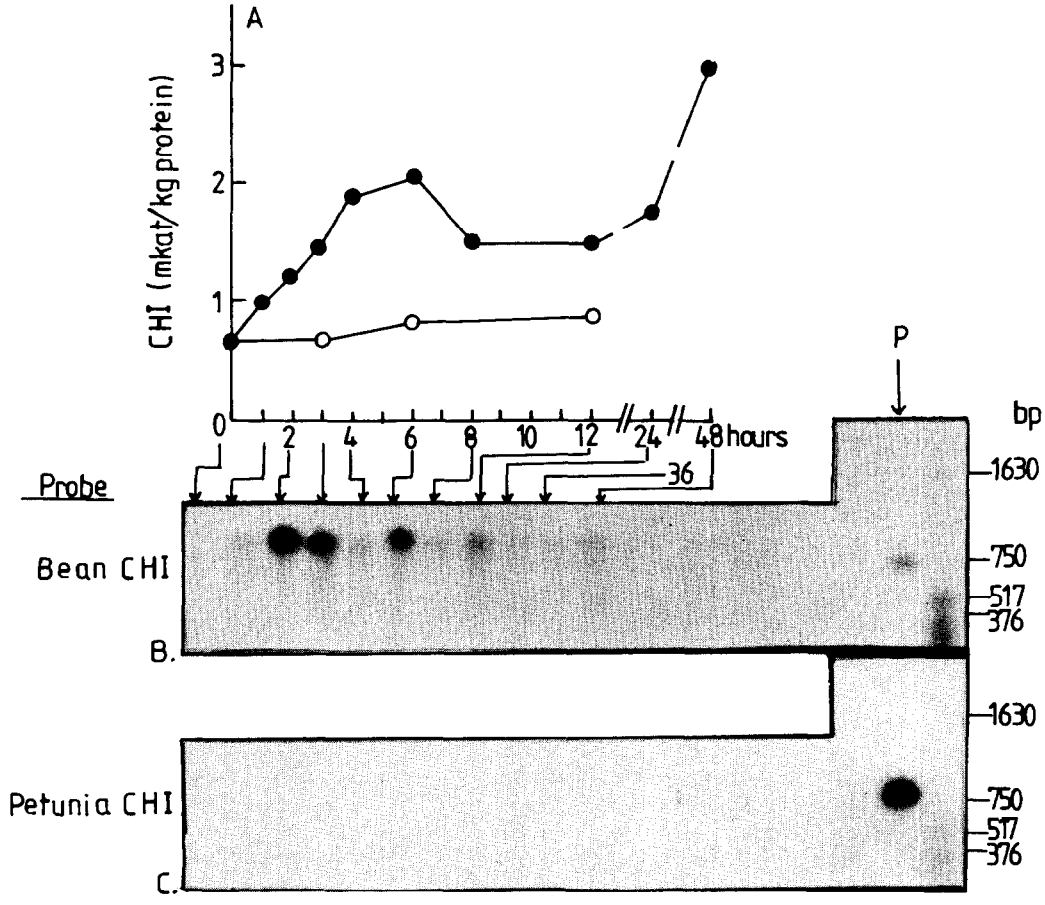

Fig. 5. Detection of chalcone isomerase mRNA transcripts from elicited bean cell suspension cultures. Cells were exposed to elicitor from *Colletotrichum lindemuthianum* (60 μg glucose equivalents, ml^{-1}) and harvested thereafter at the times shown. A. CHI enzyme activities in extracts of elicited (●——●) and control cells. B. and C. Northern blots of total polysomal mRNA (25 μg/track) isolated at the times shown. The same filter was hybridized first with a ^{32}P-labelled *Petunia* CHI cDNA and autoradiographed (C). The blot was then washed at high stringency and re-probed with ^{32}P-labelled bean CHI cDNA (B). The blots include a track containing purified *Petunia* cDNA (P).

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