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Differential expression of 5-*enol*-pyruvyl-shikimate-3-phosphate synthase isoforms in elicitor-treated, cultured maize cells

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Abstract. The expression of two 5-*enol*-pyruvyl-shikimate-3-phosphate synthase (EC 2.5.1.19) isoforms was investigated in *Zea mays* L. suspension-cultured cells following exposure to a fungal elicitor. Activity levels of isozyme II specifically increased soon after treatment, in strict connection with induction of phenylalanine ammonia-lyase (PAL) and attainment of a new free-phenylalanine homeostasis at a higher concentration. However, a few days later, activity of the other enzyme form was also significantly enhanced, concomitant with a sharp rise in overall amino acid content, a further increase in PAL level and a resumption of cell lysis. Besides strengthening the hypothesis that an entire set of genes encoding for shikimate pathway enzymes (whose expression is specifically involved in plant dynamic defence) may exist, a general change in the levels of several amino acids seems to point towards a reprogramming of their metabolism in elicited cells.

Keywords: differential expression of isozymes, elicitors, EPSP synthase, *Fusarium acuminatum*, maize (*Zea mays* L.), oxidative burst, phytoalexin production.

Introduction

Plants react to microbial attack with a number of defence mechanisms, including an oxidative burst associated with hypersensitive cell death (Bolwell 1999) and synthesis of aromatic secondary metabolites (Dixon and Paiva 1995). These responses are triggered by elicitors derived from the cell surface of pathogens (Hahn 1996). Even though recent experimental evidence suggests that H_2O_2 may be neither necessary nor sufficient for programmed cell death (Dorey *et al.* 1999), the generation of superoxides by membrane-bound NAD(P)H oxidases and subsequent H_2O_2 accumu-lation in the apoplast have been implicated in cross-linking of cell wall proteins and lignin precursors, direct destruction of invading pathogens, and the signal transduction pathway that activates the metabolic cascade (Doke *et al.* 1996; Aver'yanov *et al.* 2001). On the other hand, enhanced production of phenolic derivatives may contribute to disease resistance either directly, as phytoalexins, or through incor-poration of phenolic material into structural barriers, such as phenol-conjugated or lignified cell walls.

The first committed step of branched phenylpropanoid metabolism, conversion of ʟ-phenylalanine (Phe) into *trans*-cinnamate, is catalysed by PAL (EC 4.3.1.5). An increase in PAL activity has been shown to represent an early response to attempted penetration by pathogens (Hahlbrock and Scheel 1989), whereas partial suppression of PAL gene expression was found to lead to increased fungal susceptibility (Maher *et al.* 1994). On this basis, pathogen- or elicitor-induced changes in PAL specific activity are usually considered a mark of activation of plant defence responses.

Besides regulation at the PAL level, sustained synthesis of precursors for lignin and other phenolic protectants requires a corresponding increase in carbon flow through the common pre-chorismate branch of aromatic biosynthesis. Consistently, activity of the first enzyme in the shikimate pathway, 3-deoxy-ᴅ-arabino-heptulosonate-7-phosphate (DAHP) synthase (EC 4.1.2.15), was found to be induced in parsley cells treated with a fungal elicitor (McCue and Conn 1989). However, much less information is available on the influence of pathogens and elicitors on regulation of enzymes involved in biosynthesis of Phe relative to those channelling Phe into phenylpropanoid metabolism. Increas-ing experimental evidence suggests that a higher rate of Phe synthesis may be accomplished by expression of specific isoforms of several shikimate pathway enzymes (Weaver and Herrmann 1997). One of two DAHP synthase genes is induced in response to pathogen attack (Keith *et al.* 1991). Two genes, encoding DAHP synthase and chorismate synthase, which show different patterns of tissue expression, are found in tomato (Görlach *et al.* 1993*a*, *b*). In both cases, transcription of only one of these genes is enhanced in elicitor-treated cells (Görlach *et al.* 1995).

The occurrence of multiple forms has also been reported for 5-*enol*-pyruvyl-shikimate-3-phosphate (EPSP) synthase (3-phosphoshikimate-1-carboxyvinyl transferase; EC 2.5.1.19), the enzyme that catalyses the sixth step in the pre-chorismate pathway, the unusual reversible addition of the carboxyvinyl group of phospho*enol*pyruvate (PEP) to shikimate 3-phosphate (S3P; Ream *et al.* 1988; Forlani *et al.* 1992). Two isozymes, both functionally located in the plastid, have been purified to homogeneity from cultured *Z. mays* cells (Forlani *et al.* 1994), but even thorough biochemical characterisation failed to show any difference that could account for a specific role in plant cell metabolism (Forlani 1997). To address the question of whether one of these forms is preferentially expressed during the dynamic defence response, they were resolved and quantified in extracts from cultured cells exposed to mycelial wall fragments purified from *Fusarium acuminatum*, a fungal pathogen of maize.

Materials and methods

Plant cell cultures

Maize (*Zea mays* L. cv. Black Mexican Sweet) cell suspension cultures were grown under dim light (about 30 μmol photons m^{-2} s^{-1}) at $25 \pm 1°C$ on a rotary shaker (120 rpm) in 500-mL Erlenmeyer flasks containing 120 mL of MS medium (Murashige and Skoog 1962) supplemented with 30 g L^{-1} sucrose and 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid. Subcultures were made every 2 weeks by transferring 20-mL aliquots to 100 mL of fresh medium.

Experiments aiming to elucidate the effect of fungal elicitors were performed using suspension cultures maintained in the exponential phase of growth. Aliquots (25 mL) of 7-d-old cultures were used to inoculate 100 mL of fresh medium. After 3 d further incubation, treatments were initiated by adding the appropriate concentration of elicitor. Under these conditions, untreated controls showed a constant growth rate until the end of the trial 5 d later. To evaluate growth, cells were harvested by vacuum filtration, and the dry weight increase was determined on each sample following oven drying at 90°C for 48 h. Each treatment was carried out at least in triplicate. Means ± s.d. over replicates are presented.

Fungal strain and elicitor preparation

A mycelial wall hydrolysate was prepared from a maize pathogen, *Fusarium acuminatum* Ellis & Everhart, isolated from the lower part of the stem of maize plants and kindly provided by Prof. Anna M. Picco (University of Pavia, Italy). The strain was routinely maintained on potato dextrose agar at room temperature. The elicitor fraction was purified as described by Ayers *et al.* (1976), with minor modifications. The fungus was grown in potato dextrose broth in petri dishes at 28°C in the dark for 2 weeks, after which the mycelium was harvested by vacuum filtration on nylon filter (50-μm mesh), washed sequentially with 100 mM potassium phosphate buffer (pH 7.2) and 500 mM potassium phosphate buffer (pH 7.0) and ground in a mortar with liquid nitrogen. The powder was resuspended in 100 mM potassium phosphate buffer (pH 7.2) and the homogenate was filtered as above. Retained material was washed with a 1:1 mixture of methanol and chloroform, and with acetone, and dried under vacuum. Following further homogenisation with liquid nitrogen, the sample was resuspended in distilled water and treated for 3 h at 121°C. Organic matter in this mycelial-wall-fragment suspension was quantified as

chemical oxygen demand, using the Dr Lange cuvette test LCK 014 (Düsseldorf, Germany) according to the manufacturer's instructions.

Enzyme extraction and assay

5-Enol-*pyruvyl-shikimate-3-phosphate synthase*

Maize cultured cells were harvested on a nylon filter (50-μm mesh) by vacuum filtration, resuspended in 2 mL g^{-1} of ice-cold extraction buffer [50 mM Hepes–NaOH (pH 7.4) containing 5% (v/v) glycerol, 2.5 mM reduced glutathione, 0.1 mM EDTA and 10 μM ammonium molybdate], and homogenised on ice in a Teflon-in-glass Potter homogeniser (PBI International, Milan, Italy) with 30 strokes — 10 mg L^{-1} insoluble polyvinylpolypyrrolidone was added to prevent oxidation of phenolics. All subsequent operations were carried out at 0–4°C. The homogenate was centrifuged for 20 min at 12 000 *g*, and solid ammonium sulfate was added to the supernatant to give 70% saturation. Proteins were collected by centrifugation, resuspended in extraction buffer and desalted by passage through a Bio-Gel P6DG column (Bio-Rad, Hercules, CA, USA) equilibrated with the same buffer.

EPSP-synthase isoforms in crude extracts were resolved as described previously (Forlani *et al.* 1994) by anion-exchange fast protein liquid chromatography. Following ammonium sulfate fractionation, pelleted proteins were resuspended in a minimal volume of column buffer [50 mM Tris–HCl (pH 7.4) containing 5% (v/v) glycerol, 1 mM dithiothreitol and 0.1 mM EDTA] and column-desalted as above against the same buffer. Extracts were further centrifuged at 100 000 *g* for 60 min, and 1-mL aliquots of the resulting supernatant were injected onto a Mono-Q 5.5 column (Pharmacia, Uppsala, Sweden) equilibrated at 4°C with column buffer. Proteins were eluted at a flow rate of 0.5 mL min^{-1} using a computer-controlled (Data System 450; Kontron, Munich, Germany) linear gradient from 0–80 mM NaCl (30 mL), while collecting 0.5 mL-fractions.

EPSP-synthase activity was measured at 35°C by determining release of inorganic phosphate using the malachite-green dye assay method (Forlani 1997). In a final volume of 0.1 mL the reaction mixture contained 50 mM Hepes–NaOH buffer (pH 7.4) 1 mM S3P, 1 mM PEP, 0.5 mM ammonium molybdate and a limiting amount (up to 10 pkat) of enzyme. After incubation for increasing periods up to 30 min, the reaction was stopped by addition of 1 mL of the malachite green–molybdate acid colorimetric solution followed, after exactly 1 min, by 0.1 mL of a 34% (w/v) sodium citrate solution. After 15 min at room temperature, absorption at 660 nm was measured against blanks from which S3P was omitted. Activity was calculated using the linear regression equation of inorganic phosphate production over time. The ammonium salt of S3P was purified from the culture broth of *Klebsiella pneumoniae* strain ATCC 25597 and quantified as previously described (Forlani *et al.* 1992). Protein concentration was determined by the protocol of Bradford (1976), using bovine serum albumin as the standard.

Phenylalanine ammonia-lyase

Cultured cells were harvested and extracted as above, except that Hepes buffer was replaced with 50 mM Tris–HCl buffer (pH 7.4) containing 0.5 mM dithiothreitol. The 0–70% ammonium-sulfate-saturated fraction was resuspended in the same buffer and immediately analysed. PAL activity was measured as production of cinnamic acid (Havir and Hanson 1969). The assay mixture contained 50 mM Tris–HCl buffer (pH 9.0), 5 mM Phe and a limiting amount of enzyme (up to 100 pkat) in a final volume of 1 mL. Samples were incubated at 35°C for 10 min, with continuous monitoring of absorbance at 290 nm against blanks in which Phe was either omitted or replaced with D-Phe. Activity was calculated using the linear regression equation of cinnamate production over time, on the basis of an extinction coefficient estimated with an authentic standard.

Oxidative burst measurement

In vivo induction of H_2O_2 synthesis was measured via oxidation of 3,5-dichloro-2-hydroxybenzenesulfonic acid by exogenous peroxidases, and spectrophotometric detection after reaction with 4-aminoantipyrine (Van Gestelen *et al.* 1998). Cultured cells were harvested by vacuum filtration, resuspended in fresh medium, and allowed to incubate for 20 min at 25°C in order to relieve the effect of mechanical stress from cell manipulation upon production of active oxygen species. The reaction was then started by addition of 1 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 0.1 mM 4-amino-antipyrine and 10 mg L^{-1} horseradish peroxidase (Sigma P8125; St Louis, MO, USA). At increasing periods up to 60 min, culture aliquots were withdrawn and read at 515 nm against time zero blanks. H_2O_2 concentration was extrapolated from a standard curve obtained under the same experimental conditions. Activity was calculated from the linear regression equation of H_2O_2 production over time. Treatments were carried out in triplicate, with s.d. never exceeding 10%.

Amino acid extraction and analysis

Plant material was harvested, resuspended in 1 mL g^{-1} of a 3% (w/v) 5-sulfosalicylic-acid solution and homogenised in a Teflon-in-glass Potter homogeniser with 45 strokes. After centrifugation for 5 min at 8000 *g*, 1-mL aliquots of the supernatant were dried at room temperature in a Speed-Vac Concentrator (Savant Instruments, Hicksville, NY, USA). Samples were reconstituted in 0.1 mL of 2.5 mM NaOH, resulting in a pH value of 10.2 ± 0.2, and immediately analysed as described previously (Forlani *et al.* 2000). Briefly, 10-μL aliquots were mixed with the same volume of an *o*-phthaldialdehyde solution and, after exactly 60 s, derivatized samples were injected onto a 4.6 × 250 mm Zorbax ODS column (Rockland Technologies, Newport, DE, USA). Elution proceeded at a flow rate of 1 mL min^{-1} using a computer-controlled complex gradient from 27–65% (v/v) methanol, monitoring the eluate at 340 nm. The procedure allowed complete resolution of equimolar mixtures of derivatizable amino acids (all protein amino acids but Pro and Cys), with a detection limit of about 0.1 nmol. Peaks were integrated by area, with variation coefficients ranging from 0.8–3.2%. Proline and total amino acid content in the same extracts were quantified by the ninhydrin method (Bates *et al.* 1973). All determinations were carried out in triplicate. Means ± s.d. over replicates are presented.

Results

When the specific activity of EPSP synthase was measured in extracts from *Z. mays* suspension-cultured cells harvested at different times during the culture growth cycle, quite uniform levels were found, ranging from 550–750 pkat mg^{-1} protein (Fig. 1*A*). However, such a constant overall activity resulted from the combination of two different enzyme forms, whose contribution varied greatly with time: EPSP synthase isozyme I was expressed at maximal levels during the stationary phase and declined with the onset of exponential growth, whereas isozyme II showed a complementary pattern, with highest expression in actively-proliferating cells (Fig. 1*B*). On this basis, to avoid variations due to such a time-course, a possible specific role of either form in the dynamic defence response was investigated in suspension cultures maintained in the exponential phase of growth, in which activity levels of both isozymes were stable (399 ± 54 and 207 ± 22 pkat mg^{-1} for isozyme I and II, respectively) for at least 5 d.

Activation of defence mechanisms in cultured cells was triggered by contact with a mycelial wall hydrolysate purified from a maize pathogen, *F. acuminatum*. Addition to the culture medium of the elicitor at concentrations ranging from 1–100 mg L^{-1} immediately increased generation of H_2O_2, with an evident quantitative relationship (Fig. 2*B*). Twenty-four h after addition, maximal effect was achieved at doses exceeding 10 mg L^{-1}, with a 5–6-fold stimulation of the basal rate (2.3 ± 0.4 nmol s^{-1} g^{-1} dry weight). A dramatic increase in PAL activity in treated cells was also evident, even though the maximal effect (a 120-fold stimulation of specific activity levels, with a similar dose–effect relationship) was found 4 d after elicitation (Fig. 2*A*). Within 2 d, maize cells turned from pale yellow to brown, suggesting production of aromatic secondary metabolites (Islam *et al.* 1998). Collapse and cell death then occurred, as indicated by the sharp decrease in yield of soluble cell proteins, which
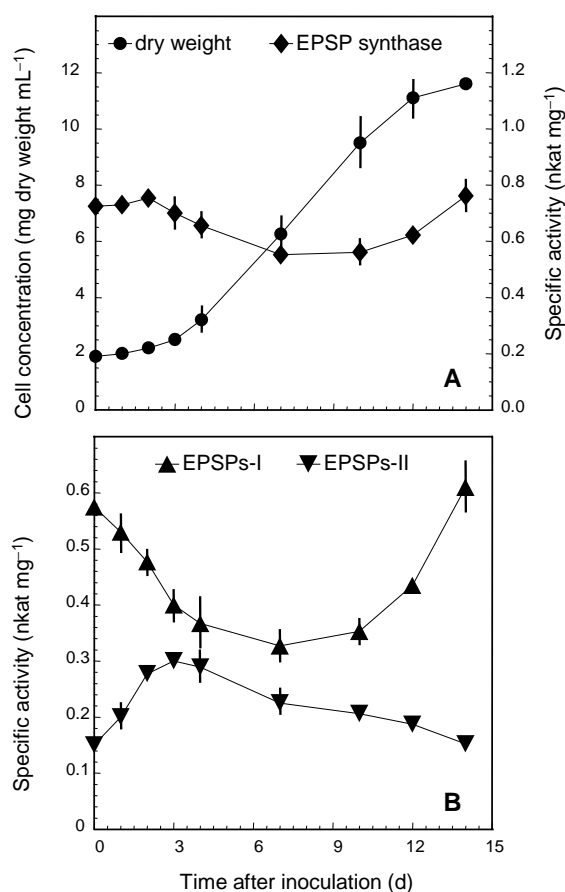

Fig. 1. Time-course of activity of isoforms of maize EPSP synthase. Total enzyme activity was measured in desalted extracts from suspension-cultured cells harvested at different times during the culture growth cycle. Cell growth was evaluated as dry weight increase (*A*). Following fractionation of the same extracts by fast protein liquid chromatography, specific activity of each isozyme (named EPSP synthase I and II from their order of elution from a DEAE-Sephacel column; Forlani *et al.* 1992) was quantified (*B*). Results are mean ± s.d. of three replications.

was proportional to elicitor addition (Fig. 2*C*). Similar results were obtained with a cell-death assay based upon uptake of Evans blue (Delledonne *et al.* 1998; data not shown). Therefore, from this point onward, a concentration of 20 mg L^{-1} was used to ensure elicitor effectiveness while slowing cell lysis.
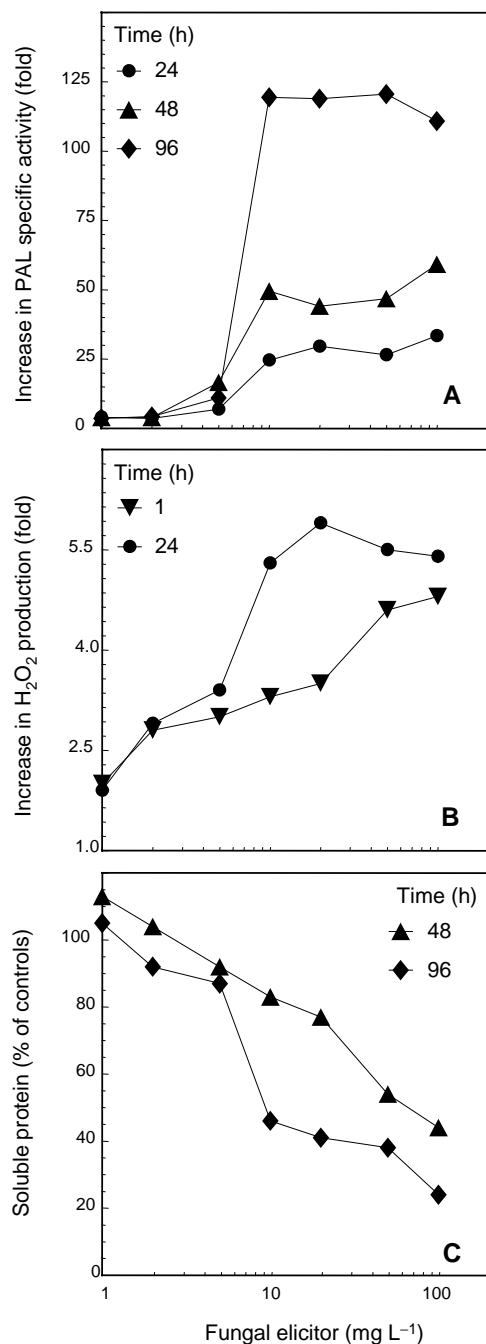

Fig. 2. Effects of increasing concentrations of fungal elicitor upon cultured maize cells. Mycelial wall fragments purified from the maize pathogen *Fusarium acuminatum* were added to actively-proliferating cultures, and PAL specific activity (*A*), H$_2$O$_2$ production (*B*) and soluble protein content (*C*) were measured at the indicated times, as described in 'Materials and methods'. Values are expressed with respect to those obtained from untreated controls. Data are means of at least three replicates with s.d. never exceeding 10%.
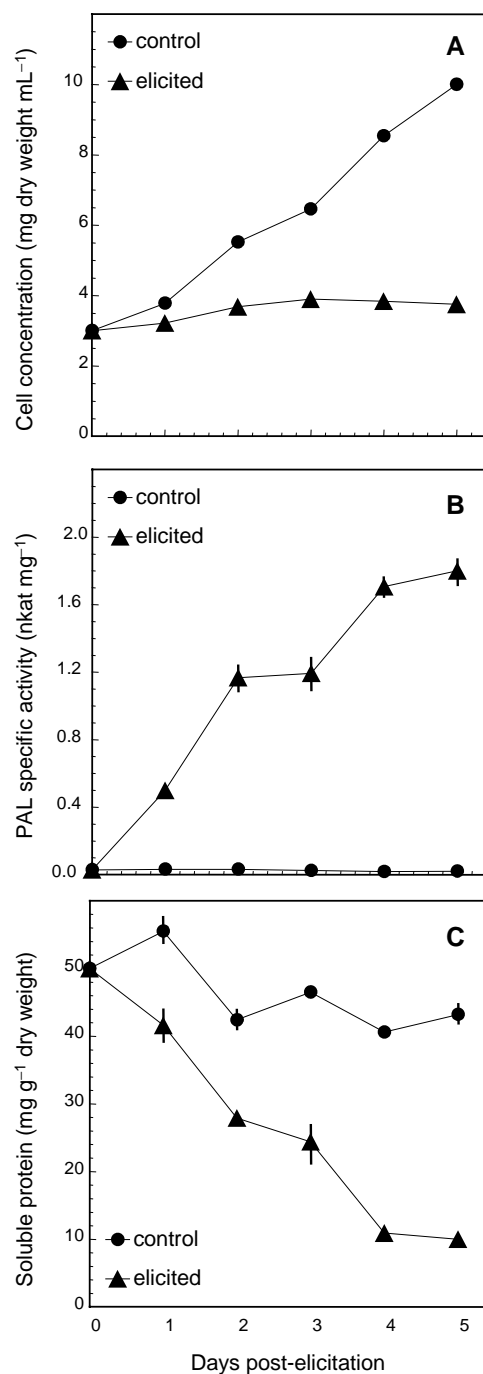

Fig. 3. Variations over time in cell growth (*A*), PAL specific activity (*B*) and soluble protein yield (*C*) of maize cells following addition of 20 mg L^{-1} mycelial wall hydrolysate to culture medium. Data were obtained as described in 'Materials and methods', and are means ± s.d. of three replications. Corresponding values for untreated controls are also reported.

Contact with the mycelial wall hydrolysate gave rise to an immediate block of cell proliferation (Fig. 3*A*). PAL activity levels increased during the first 2 d to a specific activity of about 1.2 nkat mg^{-1} protein, which was maintained for approximately 24 h. However, a further increase was evident afterward, leading to maximal expression of around 2 nkat mg^{-1} protein (Fig. 3*B*). The same pattern was evident in all three independent experiments that were performed. Similar behaviour was also found for cell lysis, evaluated as a reduction in soluble protein yield (Fig. 3*C*).

Soon after contact with fungal hydrolysate, the pool of free Phe significantly increased (from 3.7–8.1% of total amino acid content 24 h after elicitation; Table 1), while those of tryptophan and tyrosine were unaffected. A concomitant decrease in free glutamate (from 25.0–16.7%) was also evident. This may be consistent with *de novo* Phe synthesis in which chorismate is converted to phenylpyruvate and Phe is synthesised by transamination of phenylpyruvate with glutamate, with no energy required. On the contrary, 3 d later a dramatic increase in absolute values was evident for all amino acids. In percentage, free Phe was only slightly higher than in untreated controls (4.7 *vs* 3.1%), while significant variations were found for other amino acids, mainly glycine, threonine and alanine (Table 1). When the time-course of free amino acid levels was evaluated, these differences became more appreciable. Total, as well as free tyrosine, content initially increased slowly, but 3–4 d following addition of the elicitor a sharp rise was observed

(Figs 4*A* and *C*, respectively). A strikingly different profile was obtained for Phe — it increased greatly during the first hours up to a steady-state level that was maintained for 48 h. Then, a further increase occurred in connection with the general increase in free amino acids (Fig. 4*B*).

EPSP-synthase specific activities were then measured in control and elicitor-treated cells. A trend almost identical to that obtained for PAL was obtained for overall activity, even though only a 6-fold increase was evident (Fig. 5*A*). In this case also, the progress with time seems to suggest attainment of a first steady-state level a couple of days after elicitation, and a second increase after a further 24 h. When the two EPSP-synthase isoforms were resolved and quantified, strikingly different results were observed, which may account for such behaviour. Soon after addition of the elicitor, the specific activity of isozyme II increased sharply up to a 12-fold higher level that was maintained thereafter (Fig. 5*B*). On the contrary, activity levels of isozyme I were indistinguishable from those of untreated controls (Fig. 5*C*). However, 4 d after contact with fungal hydrolysate, the level of isozyme I had also increased 5-fold, concomitant with the overall increase in free amino acid content (Fig. 4*A*), resumption of cell lysis (Fig. 3*C*) and a further increase of PAL expression (Fig. 3*B*).

Discussion

Plant cell cultures have been widely employed to unravel fine details of host–pathogen interactions. Calli derived from

Table 1. Free amino acid content of maize cells at increasing time after elicitation
Fungal elicitor was added to the culture medium at 20 mg L^{-1}. Cys was not detectable under the experimental conditions employed. Individual amino acids are expressed as a percentage of total content (nmol mg^{-1} soluble protein). Data are means ± s.d. of three replications. *, $P < 0.10$; **, $P < 0.05$; ***, $P < 0.01$

Amino acid	Control (0 h)	Control (24 h)	Treated (24 h)	Control (96 h)	Treated (96 h)
Total	1879 ± 13	1734 ± 56	2342 ± 56***	1647 ± 56	9552 ± 297***
Asp	18.1 ± 0.6	17.1 ± 1.0	16.5 ± 1.1	17.0 ± 0.7	16.5 ± 2.4
Glu	25.2 ± 2.6	25.0 ± 4.0	16.7 ± 1.3**	12.9 ± 2.0	13.1 ± 0.6
Asn	1.1 ± 0.3	1.2 ± 0.2	1.4 ± 0.2	2.8 ± 0.1	3.0 ± 0.4
Ser	6.6 ± 1.1	6.2 ± 0.2	6.5 ± 0.3	5.2 ± 0.4	5.2 ± 0.8
Gln	9.5 ± 0.2	11.0 ± 1.6	11.7 ± 0.4	12.1 ± 0.1	14.3 ± 1.0
His	1.0 ± 0.0	1.1 ± 0.0	0.9 ± 0.3	1.7 ± 0.4	1.5 ± 0.3
Arg	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 0.2	1.0 ± 1.0
Gly	1.0 ± 0.0	1.2 ± 0.1	2.0 ± 0.1***	14.4 ± 0.7	1.8 ± 0.9***
Thr	4.0 ± 0.2	3.6 ± 0.4	5.1 ± 0.0***	2.1 ± 0.6	7.1 ± 1.0***
Ala	18.3 ± 2.5	15.5 ± 1.1	13.4 ± 1.2*	6.8 ± 0.3	11.6 ± 0.8***
Tyr	**3.3 ± 0.9**	**4.6 ± 0.3**	**4.9 ± 0.6**	**6.8 ± 0.7**	**3.9 ± 0.2*****
Trp	**0.3 ± 0.0**	**0.3 ± 0.1**	**0.3 ± 0.0**	**0.5 ± 0.2**	**0.5 ± 0.1**
Met	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
Val	4.1 ± 0.2	4.3 ± 0.5	5.2 ± 0.1**	5.1 ± 0.4	7.1 ± 0.4***
Phe	**2.7 ± 0.3**	**3.7 ± 0.3**	**8.1 ± 0.4*****	**3.1 ± 0.5**	**4.7 ± 0.2*****
Ile	0.8 ± 0.0	0.8 ± 0.1	1.3 ± 0.2**	1.6 ± 0.0	1.7 ± 0.1
Leu	1.2 ± 0.0	1.2 ± 0.1	2.1 ± 0.2***	1.8 ± 0.0	2.6 ± 0.2***
Lys	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
Pro	2.4 ± 0.1	2.8 ± 0.1	3.2 ± 0.1***	4.7 ± 0.1	3.8 ± 0.5**

plants of resistant cultivars usually retain tolerance, thus allowing selection of resistant clones at the undifferentiated tissue level (Daub 1986). In incompatible combinations, the

resulting oxidative burst is more pronounced than in compatible interactions (Baker and Orlandi 1995; Aver'yanov *et al.* 2001). The use of partially-purified elicitors instead of
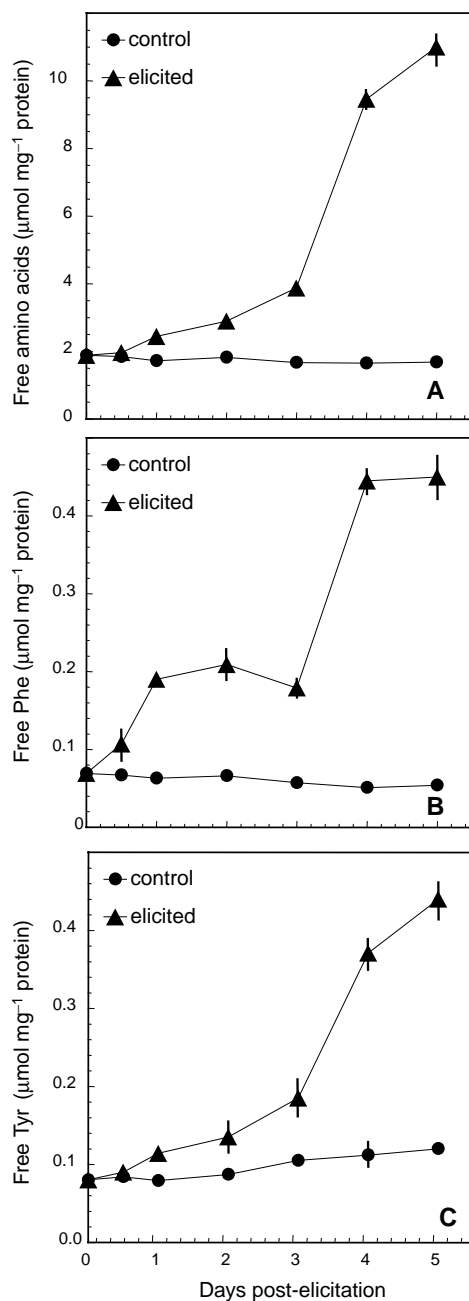

Fig. 4. Total (*A*) and aromatic [phenylalanine (*B*) and tyrosine (*C*)] free amino acid content in control and elicitor-treated maize cells. Extracts were prepared from cultures at increasing periods after addition of 20 mg L^{-1} mycelial wall hydrolysate to culture medium. Total amino acid content was estimated colorimetrically by the ninhydrin method, whereas single amino acids were quantified by reverse phase-HPLC following derivatization with *o*-phthaldialdehyde. Values are expressed with respect to soluble protein content in the same samples, and are means ± s.d. from three independent replications.
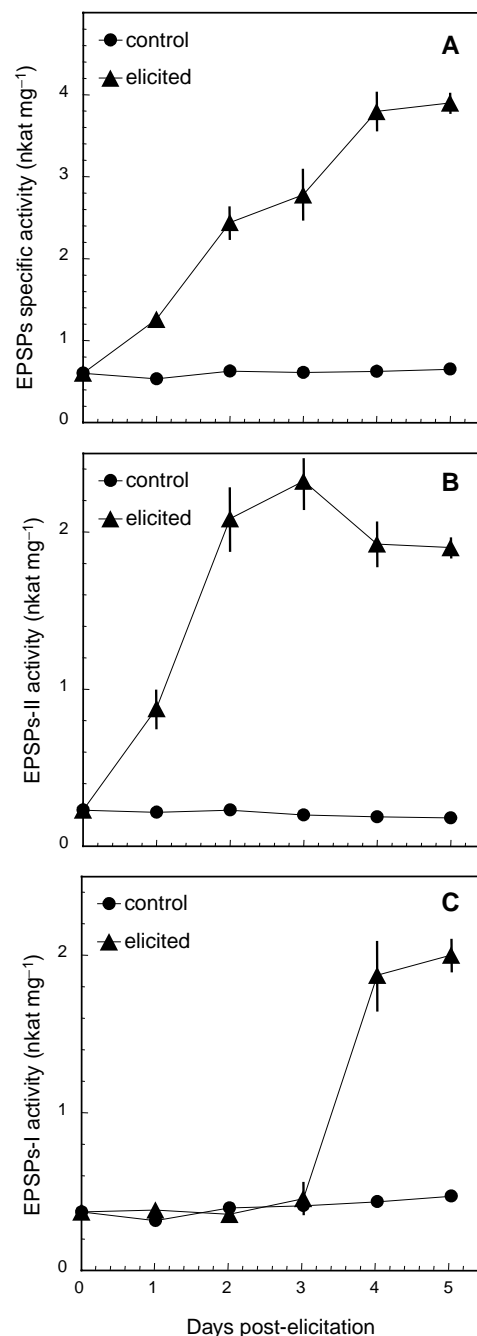
Fig. 5. Differential expression of EPSP-synthase isoforms in control and elicitor-treated maize cells. At increasing periods following addition of 20 mg L^{-1} mycelial wall hydrolysate, total enzyme activity (*A*) was measured in desalted crude extracts. Protein fractionation by anion-exchange fast protein liquid chromatography allowed resolution of isozymes, whose specific activity levels (*B, C*) were calculated from recovery in column eluate. Means ± s.d. of three independent replications are presented.

pathogen infection may further simplify the experimental system. In most cases, models in which plants and pathogens are replaced with cultured cells and elicitors, respectively, can adequately simulate *in vivo* infections (Lamb *et al.* 1989), thus providing a reliable tool to resolve the biochemical cascade of the plant dynamic response.

To address the question of whether either of the EPSP-synthase isoforms purified (Forlani *et al.* 1994) and characterised (Forlani 1997) from maize suspension-cultured cells play a specific role during the defence response, the hypersensitive reaction was triggered by addition of a cell wall hydrolysate purified from a maize pathogen, *F. acuminatum*. This strain was selected because it is the most effective of six different fungal isolates obtained from either the leaf or lower part of the stem of maize plants (G. Forlani, E. Nielsen and A. M. Picco, unpublished results). Because enhancement of phenylpropanoid synthesis and an oxidative burst represent two of the earliest reactions to microbial infection (Dixon and Paiva 1995; Lamb and Dixon 1997), the actual induction of plant defences was verified by measuring both H_2O_2 production in the apoplast and PAL specific activity. Evaluation of PAL levels was also used to link the time-course of EPSP-synthase isoforms with that of secondary aromatic metabolism.

Measurement of free amino acid content in elicited cultures showed specific alteration of Phe homeostasis soon after addition of fungal hydrolysate. In order to avoid possible interference due to progressive cell lysis, amino acid levels were expressed with respect to soluble protein content rather than to cell dry mass (Fig. 4) — otherwise, the effect would be underestimated because of the higher percentage of unviable material relative to untreated controls. In any case, a specific increase of Phe was evident even when values were expressed as percentage of total free amino acids (Table 1). In a previous study with tomato cell cultures, an increase in Phe content was detectable only in the presence of PAL inhibitors (Görlach *et al.* 1995). Attainment of higher steady-state levels in maize may therefore suggest elicitor-induced expression in the Phe branch of enzyme forms less sensitive to feedback inhibition. In fact, two isoforms of chorismate mutase have been reported in *Arabidopsis thaliana* — a plastidic isozyme is activated by tryptophan and inhibited by Phe and tyrosine, whereas a cytosolic form is insensitive. However, only the gene encoding the plastidic and feedback-regulated isozyme was shown to be pathogen-inducible (Eberhard *et al.* 1996).

The possibility that an entire set of genes encoding shikimate pathway enzymes exists, whose expression may ensure availability of the high levels of Phe required for synthesis of aromatic defence compounds, has been hypothesised (Weaver and Herrmann 1997). Representative members of the pre-chorismate branch (DAHP synthase, shikimate kinase and chorismate synthase) have been studied in tomato at the transcript level. When two different genes encoding for isozymes were available, only one was induced in cultured cells exposed to fungal elicitors. With the sole exception of shikimate kinase, transcript accumulation occurred more rapidly for PAL than for shikimate pathway enzymes (Görlach *et al.* 1995). The results described herein seem to strengthen this overall picture. In strict connection with a change in Phe homeostasis and induction of PAL, only activity of EPSP synthase isozyme II was found to increase in elicited cells, whereas levels of the other form were indistinguishable from those in untreated controls (Fig. 5). A specific role of isozyme II in the plant defence response may be consistent with its properties. A higher catalytic efficiency and stability (Forlani 1997) might derive from a higher selective pressure, most likely exerted under conditions of high demand for aromatic amino acids, such as those that follow pathogen infiltration.

Interestingly, 3–4 d after contact with the fungal elicitor, isozyme I was also strongly induced (Fig. 5). At the same time, PAL activity levels were further enhanced, overall free amino acid content showed a sharp rise, and a decrease of soluble protein yield suggested the occurrence of further cell lysis (Figs 3, 4). Although only one gene coding for PAL has been cloned in maize to date (Rosler *et al.* 1997), there are at least five genes in tomato and four in parsley (Logemann *et al.* 1995 and references therein) that show different patterns of expression. Thus, the possibility exists that the further increase in PAL activity depends upon expression of a second isozyme. Overall, these elements may be consistent with the occurrence of a second step in the plant defence response, in which an increase of carbon flow in the shikimate pathway may be required to sustain enhanced production of aromatic secondary metabolites. The increase in free amino acids over the time-course could be simply explained by a block in protein synthesis just before cell lysis. However, changes in their relative proportions (Table 1) are suggestive of a general reprogramming of amino acid metabolism, in which EPSP synthase isozyme I may be involved. Work is currently in progress to obtain further data on this aspect, and to address the question of how this might contribute to the plant reaction against pathogens.

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