

Plant Defense Responses in Opium Poppy Cell Cultures Revealed by Liquid Chromatography-Tandem Mass Spectrometry Proteomics*[§]

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Opium poppy (*Papaver somniferum*) produces a diverse array of bioactive benzyloquinoline alkaloids, including the narcotic analgesic morphine and the antimicrobial agent sanguinarine. In contrast to the plant, cell cultures of opium poppy do not constitutively but produce sanguinarine in response to treatment with certain fungal-derived elicitors. The induction of sanguinarine biosynthesis provides a model platform to characterize the regulation of benzyloquinoline alkaloid pathways and other defense responses. Proteome analysis of elicitor-treated opium poppy cell cultures by two-dimensional denaturing-polyacrylamide gel electrophoresis coupled with liquid chromatography-tandem mass spectrometry facilitated the identification of 219 of 340 protein spots based on peptide fragment fingerprint searches of a combination of databases. Of the 219 hits, 129 were identified through pre-existing plant proteome databases, 63 were identified by matching predicted translation products in opium poppy-expressed sequence tag databases, and the remainder shared evidence from both databases. Metabolic enzymes represented the largest category of proteins and included *S*-adenosylmethionine synthetase, several glycolytic, and a nearly complete set of tricarboxylic acid cycle enzymes, one alkaloid, and several other secondary metabolic enzymes. The abundance of chaperones, heat shock proteins, protein degradation factors, and pathogenesis-related proteins provided a comprehensive proteomics view on the coordination of plant defense responses. Qualitative comparison of protein abundance in control and elicitor-treated cell cultures allowed the separation of induced and constitutive or suppressed proteins. DNA microarrays were used to corroborate increases in protein abundance with a corresponding induction in cognate transcript levels. *Molecular & Cellular Proteomics* 8:86–98, 2009.

Opium poppy (*Papaver somniferum*) is one of our most important medicinal plants as the source of several pharma-

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Received, May 12, 2008, and in revised form, July 21, 2008

Published, MCP Papers in Press, August 5, 2008, DOI 10.1074/mcp.M800211-MCP200

cologically active benzyloquinoline alkaloids, including the analgesic morphine and codeine, the muscle relaxant and vasodilator papaverine, the antineoplastic drug noscapine, and the antimicrobial agent sanguinarine. In opium poppy plants, several benzyloquinoline alkaloids are abundant in cytoplasmic vesicles of specialized cells known as laticifers, which are proximal to sieve elements of the nutrient-conductive phloem. Alkaloid biosynthetic enzymes have been localized to sieve elements (1, 2). In contrast, de-differentiated cell cultures of opium poppy do not constitutively accumulate alkaloids. However, the biosynthesis of sanguinarine is rapidly induced in response to treatment with specific fungal-derived elicitors (3). Elicitor-induced sanguinarine biosynthesis in opium poppy cell cultures provides a platform to characterize the induction of antimicrobial alkaloid and other plant defense pathways under controlled conditions.

Benzyloquinoline alkaloid biosynthesis in opium poppy begins with the condensation of dopamine and 4-hydroxyphenylacetaldehyde by norcoclaurine synthase (4) to yield (*S*)-norcoclaurine (Fig. 1). Dopamine formation involves the decarboxylation of tyrosine and/or dihydroxyphenylalanine by tyrosine/dopa decarboxylase, whereas norcoclaurine 6-*O*-methyltransferase (6OMT)¹ and coclaurine *N*-methyltransferase (CNMT) convert (*S*)-norcoclaurine to (*S*)-*N*-methylcoclaurine. The P450-dependent monooxygenase (*S*)-*N*-methylcoclaurine-3'-hydroxylase (CYP80B3) catalyzes the 3'-hydroxylation of (*S*)-*N*-methylcoclaurine prior to the formation of (*S*)-reticuline by 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase (4'OMT). (*S*)-Reticuline represents the last

¹ The abbreviations used are: 6OMT, norcoclaurine 6-*O*-methyltransferase; CNMT, coclaurine *N*-methyltransferase; CYP80B3, (*S*)-*N*-methylcoclaurine-3'-hydroxylase; 4'OMT, 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase; BBE, berberine bridge enzyme; CYP719A2, stylophine synthase; HPLC, high pressure liquid chromatography; EST, expressed sequence tag; 2-DE, two-dimensional electrophoresis; DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; COR, codeinone reductase; SAM, *S*-adenosylmethionine; TCA, tricarboxylic acid; PR, pathogenesis-related; DIR, dirigent protein; PAL, phenylalanine ammonia lyase; NADP-ME, NADP-malic enzyme; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase/isomerase; HSP70, heat-shock protein 70; BiP, luminal-binding protein; IFR, isoflavone reductase; GDH, glutamate dehydrogenase; GST, glutathione *S*-transferase; LC-MS/MS, liquid chromatography-tandem mass spectrometry.

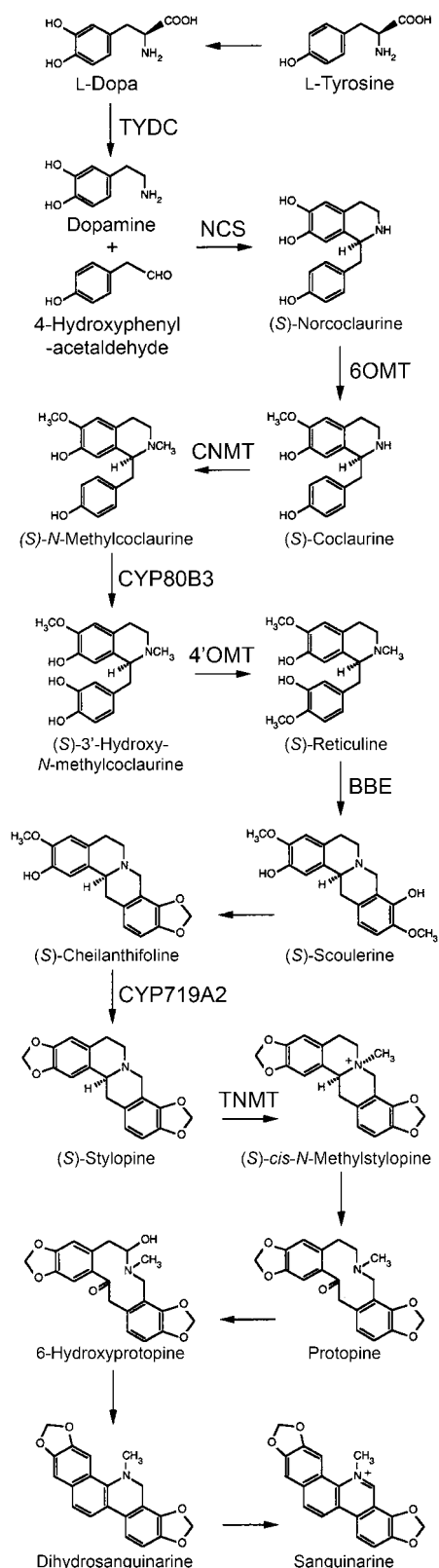


FIG. 1. Sanguinarine biosynthetic pathway showing enzymes for which cognate cDNAs have been isolated. TYDC, tyrosine decarboxylase; NCS, norcoclaurine synthase; TNMT, tetrahydroprotoberberine *cis-N*-methyltransferase.

common intermediate in the biosynthesis of morphine and sanguinarine. Epimerization of (*S*)-reticuline to (*R*)-reticuline is the first step in the formation of morphine. Alternatively, berberine bridge enzyme (BBE) converts (*S*)-reticuline to (*S*)-scoulerine as the first committed step in the sanguinarine pathway (Fig. 1). (*S*)-Scoulerine is converted to (*S*)-stylopine via the formation of two methylenedioxy bridges by the P450-dependent monooxygenases cheilanthifoline synthase and stylopine synthase (CYP719A2). Tetrahydroprotoberberine *cis-N*-methyltransferase converts (*S*)-stylopine to (*S*)-*cis-N*-methylstylopine, which is hydroxylated by the P450-dependent *N*-methylstylopine 14-hydroxylase. The initial reaction product tautomerizes to protopine, which is hydroxylated by protopine 6-hydroxylase to yield dihydrosanguinarine. Subsequent oxidation by dihydrobenzophenanthridine oxidase yields sanguinarine.

Elicitor-induced sanguinarine accumulation in opium poppy cell cultures provides a responsive model system to profile modulations in gene transcripts (5), proteins, and metabolites (5, 6) related to alkaloid biosynthesis and other defense responses. An annotated expressed sequence tag (EST) database was assembled from 10,224 random clones isolated from an elicitor-treated opium poppy cell culture cDNA library. ESTs corresponding to 40 enzymes involved in the conversion of sucrose to sanguinarine were identified. A corresponding DNA microarray probed with RNA from cell cultures collected at various time points after elicitor treatment showed the coordinate induction of diverse transcript populations, with alkaloid biosynthetic enzyme and defense protein transcripts displaying the most rapid and substantial modulations. In addition to all known sanguinarine biosynthetic gene transcripts, transcripts encoding several upstream primary metabolic enzymes were also induced. A combination of Fourier transform-ion cyclotron resonance-mass spectrometry and proton nuclear magnetic resonance (^1H NMR) were used to monitor corresponding changes in metabolite profiles (5, 6). Extensive and rapid changes in pool sizes of primary and secondary metabolites were observed in elicitor-treated cell cultures, but not in controls. A dynamic separation was revealed in the metabolome in response to elicitor treatment. Several alkaloids and other metabolites showed temporal changes in abundance consistent with modulations in the profiles of relevant biosynthetic gene transcripts. A corresponding proteomics analysis has not been performed, but would provide information highly complementary to available transcriptomics and metabolomics datasets.

The use of proteomics to study biological processes in plants has gained momentum, although most applications are focused on model systems that typically do not produce specialized metabolites such as alkaloids (7–11). Proteome reference maps have been produced for a number of plants systems including cell cultures (12) and root (13) of the model legume *Medicago truncatula*, BY-2 suspension cultures of tobacco (14), mature pollen of *Arabidopsis thaliana* (15), veg-

etative tissues in pea (16), and maize endosperm (17). Recently, plant proteomics has been used to investigate organ development, biotic and abiotic stress, post-translational modification, and the protein interactome (10). Advanced proteomics methods have been used to study the binding of calmodulin-related proteins to specific targets (18) and to identify the ubiquitinated proteome in *A. thaliana* cell cultures (19).

Proteomics investigations on non-model plants include the application of MALDI-TOF-MS to identify tissue-specific proteins in the leaves, flowers and glandular trichomes of *Cannabis sativa* (20). Although over 800 spots were resolved by two-dimensional gel electrophoresis (2-DE), protein identity scores were poor due to the lack of specific genomic resources for *C. sativa*. Similarly, proteins that increased in abundance concomitantly with the accumulation of monoterpene indole alkaloids in *Catharanthus roseus* cell cultures were subjected to MALDI-based MS/MS (21). Again, the restricted availability of *C. roseus* sequence data limited the reliable identification of proteins.

Few proteomics efforts have targeted plants that produce benzylisoquinoline alkaloids. Two-dimensional electrophoresis coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS) identified 21 proteins in the latex of *Chelidonium majus*, which is a member of the Papaveraceae family related to opium poppy, mainly involved in plant defense, signal transduction, the tricarboxylic acid cycle, and nucleic acid binding (22). Soluble proteins in the cytosolic and vesicle fractions of opium poppy latex were subjected to 2-DE and were identified by microsequencing and MALDI-TOF-MS (23). 75 and 23 proteins were microsequenced from the cytosolic and vesicle fractions, respectively. Primary metabolic enzymes were found in both fractions, whereas chaperonins, signaling components, and major latex proteins were more abundant in the cytosol. However, codeinone reductase (23) and reticuline 7-*O*-methyltransferase (24), which catalyze the penultimate step in morphine biosynthesis and the formation of laudanine, respectively, were the only alkaloid biosynthetic enzymes identified in opium poppy latex using this approach.

In this study, proteomic analysis of opium poppy cell cultures by 2-DE coupled with LC-MS/MS identified several proteins involved in primary and secondary metabolism and defense response networks activated in response to elicitor treatment. Almost a third of the identified proteins resulted from the availability of a tissue- and treatment-specific EST database for opium poppy. The qualitative induction of many proteins was accompanied by an increase in the accumulation of corresponding gene transcripts determined by DNA microarray analysis.

EXPERIMENTAL PROCEDURES

Cell Cultures and Elicitor Treatment—Opium poppy (*P. somniferum* cv Marianne) cell suspension cultures were maintained in the light at 23 °C on Gamborg 1B5C medium consisting of B5 salts and vitamins,

100 mg/liter *myo*-inositol, 1 g/liter hydrolyzed casein, 20 g/liter sucrose, and 1 mg/liter 2,4-dichlorophenoxyacetic acid. Cells were sub-cultured every 6 days using a 1:3 dilution of inoculum to fresh medium. Sections (1 cm²) of *Botrytis cinerea* mycelia grown on potato dextrose agar were used to inoculate 50 ml of 1B5C medium including supplements, but lacking 2,4-dichlorophenoxyacetic acid. Fungal cultures were grown at 120 rpm on a gyratory shaker at 22 °C in the dark for 6 days. Mycelia and medium were homogenized using a Polytron (Brinkman instruments, Westbury, NY) and autoclaved at 121 °C for 20 min. One milliliter of the fungal homogenate was added to 50 ml of cultured cells in rapid growth phase (2–3 days after subculture). Cells were collected by vacuum filtration and stored at –80 °C.

Protein Extraction—Cells (1 g) were ground to a fine powder under liquid nitrogen and extracted in 0.5 M Tris-HCl, pH 7.5; 50 mM EDTA; 1% (w/v) SDS; 2% (w/v) 2-mercaptoethanol. The slurry was centrifuged at 15,000 × *g* to pellet debris, and an equal volume of phenol was added to the supernatant, which was subsequently centrifuged at 15,000 × *g* to separate the phases. The aqueous phase was discarded, an equal volume of extraction buffer was added to the phenol phase, the solution was centrifuged again, and the phenol phase was recovered. Five volumes of methanol containing 0.1 M ammonium acetate and 0.068% (v/v) 2-mercaptoethanol were added and incubated overnight at –20 °C. Precipitated proteins were collected by centrifugation at 15,000 × *g*, and the pellet was washed twice with the methanol solution. The pellet was dried and dissolved in rehydration buffer (7 M urea, 2 M thiourea, 56 mM dithiothreitol (DTT), and 2.5% (v/v) 3-[[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid (CHAPS)). Protein concentration was determined using the RC DC protein assay (Bio-Rad).

Two-dimensional Gel Electrophoresis—After 1-h incubation in rehydration buffer, 150 mM iodoacetamide was added, and the protein extracts were incubated at 20 °C for an additional 1.5 h to alkylate sulfhydryl groups. Aliquots containing 300 μg of protein were adjusted to a final volume of 300 μl with a solution of 8 M urea, 4% (v/v) CHAPS, 0.2% (v/v) carrier ampholites (pH 3–10), 50 mM DTT, and 1 μl of bromophenol blue. After centrifugation at 15,000 × *g* to remove insoluble material, the sample was subjected to isoelectric focusing using a 17-cm immobilized pH gradient strip (pH 4–7; Bio-Rad). Active rehydration of the isoelectric focusing strip was completed overnight at 50 V. Isoelectric focusing was performed at 20 °C using linear voltage ramping under the following conditions: 250 V for 15 min, 4000 V for 2 h, and 4000 V for 20,000 V/h. Gel strips were subsequently equilibrated for 10 min in equilibration buffer (6 M urea, 2% (v/v) SDS, 0.05 M Tris-HCl, pH 8.8, 20% (v/v) glycerol; 2% (w/v) DTT) followed by 15 min in equilibration buffer containing 2.5% (w/v) iodoacetamide rather than DTT. Second-dimension SDS-PAGE was performed at 180 V for 6 h using a Protean XL cell and 10% (w/v) acrylamide.

Sypro Ruby Staining, Imaging, and Spot Selection—Immediately following 2-DE, gels were fixed in 50% (v/v) methanol and 7% (v/v) acetic acid for 30 min, which was repeated once with fresh solution. The fixation solution was replaced with 400 ml of Sypro Ruby (Bio-Rad). Gels were incubated overnight on a rotary shaker, subsequently washed in 10% methanol (v/v) and 7% acetic acid (v/v) for 30 min, and rinsed twice for 10 min with HPLC-grade water. Gels were scanned at a resolution of 50 μm on a Molecular Imager PharoFX and imaged using Quantity One software (Bio-Rad). Proteins were visualized under UV at 280 nm, and 340 spots were manually excised using glass capillaries. Gel plugs were stored separately at 4 °C.

In-gel Destaining and Digestion—Gel plugs were rinsed once with 200-μl HPLC-grade water, twice with 200-μl 25 mM ammonium bicarbonate in 50% (v/v) acetonitrile, and finally incubated in 50-μl 10 mM DTT in 100 mM ammonium bicarbonate, pH 8.0, for 1 h at 56 °C. Excess reagent was removed, 50-μl 55 mM iodoacetamide in 100 mM

ammonium bicarbonate, pH 8.0, was added, and samples were incubated in the dark at 20 °C for 30 min. Excess reagent was removed, and samples were rinsed twice with 200 μ l of 100 mM ammonium bicarbonate and once with acetonitrile to dehydrate the gel plugs, which were then lyophilized. The dry gel plugs were rehydrated in 5–7 μ l of 25 mM ammonium bicarbonate, pH 8.0, containing 12.5 ng/ μ l trypsin. After rehydration, an additional 20 μ l of 25 mM ammonium bicarbonate was added, and the gel plugs were incubated overnight at 37 °C. Peptides were extracted from gel plugs by sonication in 50 μ l of 1% (v/v) formic acid followed by 50% (v/v) acetonitrile. The pooled extracts were reduced to dryness and reconstituted in mobile phase buffer A for liquid chromatography.

Mass Spectrometry and Data Analysis—Digests were analyzed using an integrated Agilent 1100 LC-Ion-Trap-XCT-Ultra system (Agilent Technologies, Santa Clara, CA), fitted with an integrated fluidic cartridge for peptide capture, separation, and nanospraying (HPLC Chip). Injected samples were trapped and desalted on a pre-column channel (40-nl volume; Zorbax 300 SB-C₁₈) for 5 min with 0.2% (v/v) formic acid delivered by an auxiliary pump at 4 μ l/min. The peptides were then reverse-eluted from the trapping column and separated on the analytical channel (43-mm channel length; Zorbax 300SB-C₁₈) at 0.3 μ l/min. Peptides were eluted using a 5–70% (v/v) acetonitrile gradient in 0.2% (v/v) formic acid over 10 min. MS/MS spectra were collected by data-dependent acquisition, with parent ion scans of 8100 Th/s over m/z 400–2,000 and MS/MS scans at the same rate over m/z 100–2200. Peak-list data were extracted from these files by DataAnalysis software for the 6300 series ion trap, v3.4 (build 175). Mascot v2.1 (Matrix Science, Boston, MA) was used to search the MS/MS data using the following parameters: 2 Da precursor ion mass tolerance, 0.8 Da fragment ion mass tolerance, 1 potential missed cleavage, carbamidomethyl modification of Cys, and variable oxidation of Met. NCBI nr 2007.06.01 (4,988,250 sequences) was searched, with a restriction to *Viridiplantae* (green plants, 381,872 sequences). An EST database containing 10,148 sequences from elicitor-treated cell cultures (5), 7947 from stems, and 3941 EST from roots, were deposited in Mascot and similarly searched in all potential open reading frames. Results were indexed with the aid of a prior clustering and annotation exercise. In all cases, trypsin and human keratins were included in the searches to avoid contaminant-based erroneous assignment of the data.

Protein hits were scored based on the quality and abundance of the underlying peptide MS/MS data and their scores. A cut-off score ($p = 0.05$) of 48 was used for all peptides identified through matches with the public databases, and a cut-off score ($p = 0.05$) of 40 for all peptides identified through matches to the EST databases. These criteria were preserved for all proteins identified with two or more peptides. For proteins identified by a single peptide hit above these scores, greater stringency was applied to reduce false positive identifications. An expectation value of $e = 0.01$ was applied in all cases, corresponding to cut-off scores of 56 (public) and 47 (EST). The resulting MS/MS spectra were manually assessed for consistency with the proposed sequence and distance from the next highest scoring peptide(s). Proteins identified in this manner were associated with the protein spot of origin and considered a separate entry provided that spot resolution was obvious. Protein names associated with the hits were determined by considering the highest scoring entry and the most common name representing the dataset. In most instances, subunit delineation was removed. Where more than one database entry was returned in a search, these were reported as unique proteins only if the entries utilized unique sets of MS/MS data and did not represent related proteins from different taxa or EST databases.

Microarray Hybridization and Analysis—RNA was isolated according to Logemann *et al.* (25) and purified using RNeasy Maxi columns (Qiagen Inc., Valencia, CA). High quality RNA (100 μ g) was reverse

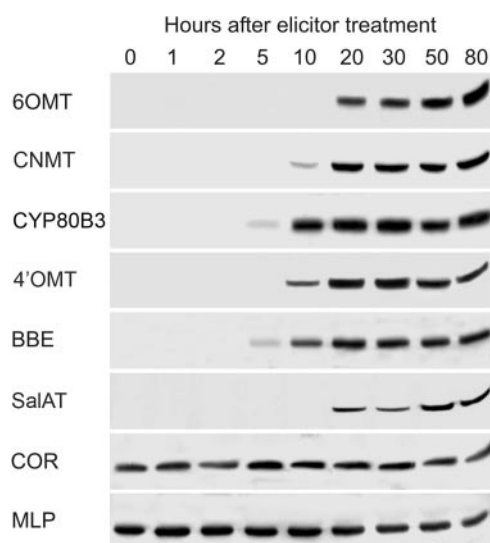


FIG. 2. Sanguinarine biosynthetic enzymes are induced in opium poppy cell cultures in response to elicitor treatment. Immunoblots show the relative abundance of 6OMT, CNMT, CYP80B3, 4'OMT, BBE, salutaridine reductase (SalAT), codeinone reductase (COR), and major latex protein (MLP) in crude protein extracts from opium poppy cell cultures at various time points after elicitor treatment. Western blots were probed with protein-specific polyclonal antibodies. The data are representative of three independent experiments.

transcribed using BD PowerScript reverse transcriptase (BD Biosciences) and labeled with Cy3- or Cy5-dCTP fluorescent dyes (Amersham Biosciences). Microarray slides were rehydrated, cross-linked with 65 mJ of 254 nm UV light using a UVC-508 Ultraviolet Crosslinker (Ultra-Lum, Claremont, CA) and blocked for 1 h using 1X Block-it buffer (Telechem, Sunnyvale, CA).

Hybridizations were performed overnight at 65 °C in a humidified chamber. Microarrays were washed with 2 \times SSC and 0.5% SDS at 55 °C twice for 5 min each, and 0.5 \times SSC and 0.05 \times SSC for 5 min each, and dried by centrifugation at 1,000 \times g . Fluorescence signatures were captured using a ChipReader (Virttek, Waterloo, ON) and analyzed using the TIGR TM4 suite of microarray tools (26). Poor quality, low intensity, or missing spots were flagged and excluded from further analysis. Fluorescence signals were normalized for each slide using iterative \log_2 mean centering, and triplicate spots were merged using in-slide replicate analysis. Two independent biological replicates and two dye-flip technical replicates were performed for each time point. All replicates were manually averaged, and the entire dataset normalized using iterative \log_2 mean centering.

RESULTS

Induction of Alkaloid Biosynthetic Enzymes in Response to Elicitor Treatment—Treatment of opium poppy cell cultures with the fungal-derived elicitor resulted in the induction of six tested alkaloid biosynthetic enzymes for which polyclonal antibodies were available (Fig. 2) (1, 2). None of these proteins were detected in control cultures but were apparent between 5 and 20 h post-elicitor treatment and reached maximum abundance at 50 h. The enzymes 6OMT, CNMT, CYP80B3, and 4'OMT catalyze the first four steps in the biosynthesis of the central branch point intermediate (S)-reticuline, and BBE converts this alkaloid to (S)-scoulerine as the first committed

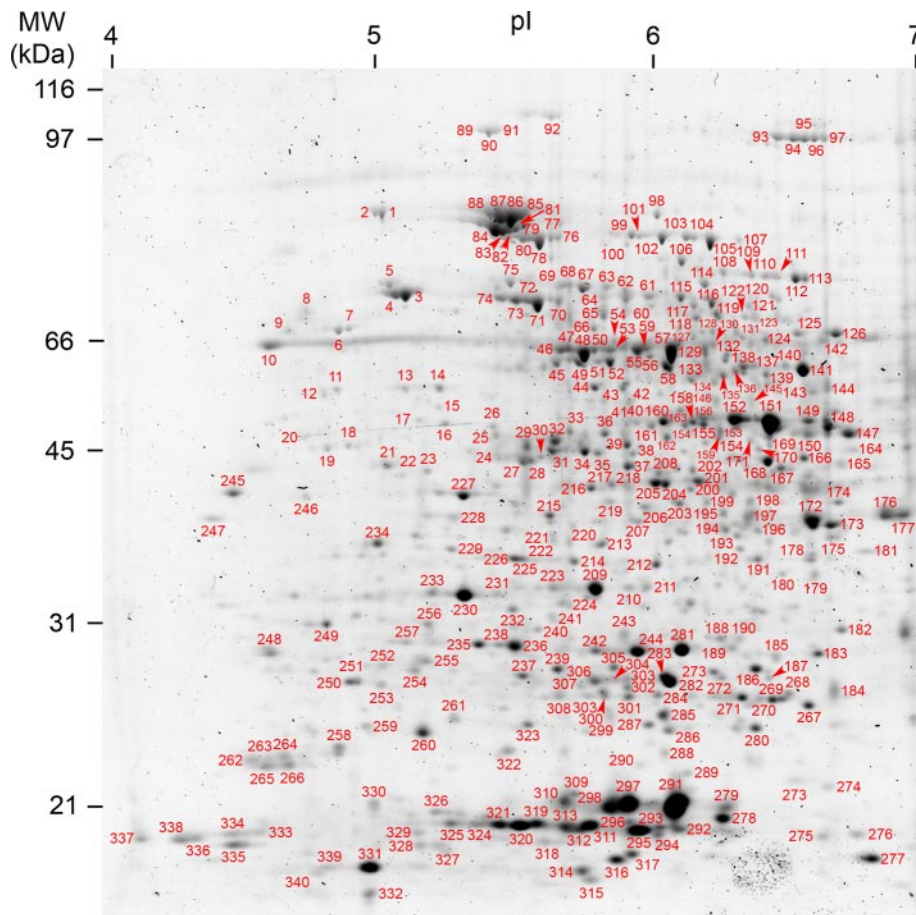


FIG. 3. Two-dimensional electrophoresis of a total protein extract from opium poppy cell cultures collected 50 h after elicitor treatment. A total of 340 individual protein spots were excised from this reference gel for LC-MS/MS analysis.

step in sanguinarine biosynthesis. In contrast, salutaridine acetyltransferase and codeinone reductase, which are constitutive in the cultured opium poppy cells, are specifically involved in the morphinan alkaloid branch pathway; however morphine is not produced in dedifferentiated opium poppy cells. The major latex proteins were also constitutive in cultured opium poppy cells and served as a loading control. The experiment was repeated on independent, triplicate biological samples.

Identification of Proteins in Elicitor-treated Opium Poppy Cell Cultures—Three hundred and forty spots were manually excised from a Sypro Ruby-stained gel prepared by 2-DE of protein extracts from opium poppy cell cultures collected 50 h after elicitor treatment (Fig. 3), which were subsequently digested with trypsin and subjected to LC-MS/MS analysis. Peptide fragment spectra were used to search both the NCBI non-redundant green plant protein database and specific opium poppy EST databases consisting of 10,148 sequences from elicitor-treated cell cultures, 7947 from stems, and 3941 ESTs from roots. Of the 340 spots interrogated by LC-MS/MS, 120 proteins represented by two or more peptides were identified. These are displayed in Supplemental Table I. Initially, 214 single peptide hits were obtained, but after application of a more stringent cut-off as described, this list reduced to 129. After removing peptide redundancies between

EST and public databases that tracked to the same protein spots, this list was further reduced to 109 peptides. When grouped by 2-DE gel spot, peptide hits deriving from the same protein across different species in the public database were combined for a single protein hit. Similarly, peptide hits deriving from both the public and EST databases or just the EST databases were grouped as a single protein hit if they shared the same annotation. This process resulted in 99 protein hits from 84 spots (Supplemental Table II), for a total of 219 protein identifications from the original 340 spots. Implementing the EST databases, particularly the larger cell culture database, added significant identification power. Of the 219 hits, 63 (29%) were identified by matching to predicted translation products in opium poppy EST databases, 129 (59%) were identified through pre-existing plant proteome databases, and 27 (12%) shared evidence from both classes of database. Overall, the identified protein lists (Supplemental Tables I and II) contain a degree of apparent redundancy likely arising from the separation of highly similar isoforms and post-translationally modified polypeptides. This is emphasized by the inequalities between the empirical and theoretical pI values. Extrapolating the percent apparent redundancy observed in Supplemental Table I (54%) to the entire set, we conservatively estimate the identification of 119 unique proteins.

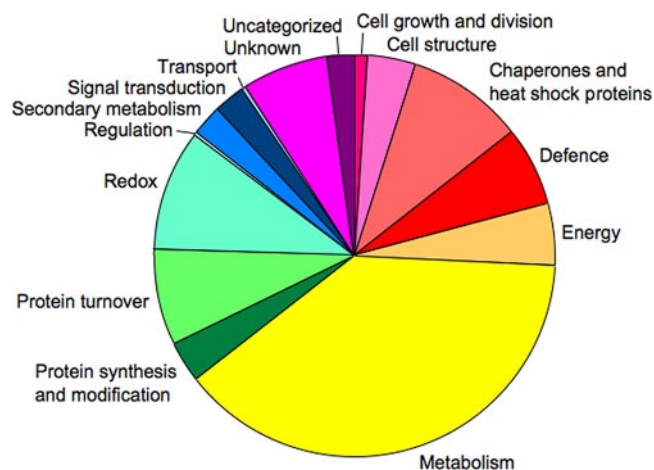


FIG. 4. **Functional classification of proteins identified by LC-MS/MS.** Proteins were identified using the NCBI non-redundant protein database for green plants or an opium poppy EST database. A total of 219 peptides were assigned a functional classification.

Identified proteins were grouped into 14 functional categories based on putative roles in eukaryotic cellular processes (Supplemental Tables I and II, Fig. 4). Putative functional classification could be assigned to 97% of proteins, although 3% could not be categorized. The most abundant group, representing 38.4% of all identified proteins, included enzymes involved in primary metabolism. Within this category, 10% were identified as *S*-adenosylmethionine (SAM) synthetase, which was also one of the most abundant protein spots (Fig. 3; spot 151). Central metabolic pathways such as glycolysis and the tricarboxylic acid (TCA) cycle were highly represented (Supplemental Tables I and II). Identified glycolytic enzymes included fructose biphosphate aldolase (3 spots), trisphosphate isomerase (7 spots), glyceraldehyde 3-phosphate dehydrogenase (6 spots), phosphoglycerate mutase (5 spots), and enolase (9 spots). A nearly complete TCA cycle was also identified including pyruvate dehydrogenase (3 spots), aconitase (3 spots), isocitrate dehydrogenase (2 spots), 2-oxoglutarate dehydrogenase (1 spot), succinyl CoA synthase (2 spots), and malate dehydrogenase (11 spots). A substantial number of proteins (11%) were functionally associated with cellular redox processes, including components of oxidative stress protection and several uncharacterized oxidoreductases. Chaperones and heat shock proteins (10%), protein degradation factors (8%), and defense related proteins (6%) were also well represented. Of the latter, 43% were annotated as pathogenesis-related (PR) proteins. A PR protein is the most abundant spot on the reference gel (Fig. 3, spot 291), which is in agreement with DNA microarray data showing gene transcripts encoding PR proteins as the second most abundant defense-related ESTs in the opium poppy database (5). Other cellular processes represented among detected proteins included energy transfer (5%), cell structure (4%), and protein synthesis and modification (3%). Eight proteins (2%) were functionally associated with sec-

ondary metabolic pathways and included a catechol *O*-methyltransferase, phenylalanine ammonia lyase (PAL), an *N*-methyltransferase similar to caffeine/theobromine synthase, three proteins with homology to dirigent (DIR) proteins, and 6OMT, which converts (*S*)-norcoclaurine to (*S*)-coclaurine in benzylisoquinoline alkaloid biosynthesis. Signal transduction was represented by 2% of the proteins, most of which were involved in calcium signaling. One putative transporter and one predicted transcription factor were also identified. Proteins with no known cellular function were classified as unknown (7%).

Qualitative Comparison of Protein Abundances in Control and Elicitor-treated Samples—Alignment of the reference 2-DE gel representing soluble proteins from a 50 h elicitor-treated opium poppy cell culture with a corresponding gel containing 0 h control sample proteins allowed a qualitative comparison of modulations in the abundance of specific protein in response to elicitor treatment (Fig. 5). A list of proteins substantially more abundant in 50 h elicitor-treated cell cultures compared with controls is shown in Supplemental Table III. Some spot numbers are associated with two or more identifications due to protein co-migration. In these cases, the specific identity of the protein increasing in abundance cannot be confirmed based solely on gel comparisons. The increase in abundance of heat shock proteins was considerable in elicitor-treated cells (Fig. 5; *panel A*, spots 81–89). Several primary metabolic enzymes increased in abundance (Fig. 5; *panels B* and *C*), including SAM synthetase (spots 151, 152, and 160), and NADP-malic enzyme (NADP-ME; spots 112 and 113), and glutamate dehydrogenase (spot 166). PAL (spot 168) and 6OMT (spots 194 and 196) also increased in abundance (Fig. 5; *panel B*). Also increasing in abundance in response to elicitor treatment were several GST isoforms (spots 249, 267, 270, 271, and 302), isoflavone reductase (spots 225 and 226), and pyridine nucleotide cytochrome reductase (spot 182). Two proteins (spots 209 and 230) substantially more abundant in the elicitor-treated extract correspond to a catalytic/coenzyme-binding protein with two conserved components, β -hydroxysteroid dehydrogenase/isomerase (β -HSD) and NmrA. Three DIR proteins (spots 184, 270, and 288) and several early tobacco anther proteins (spots 262, 263, 265, 266, and 327) also increased substantially in abundance (Fig. 6, *panel D*). In the lowest molecular weight range (Fig. 6, *panel E*), four PR proteins, calmodulin (spot 336 and 337), a calcium-binding protein (spot 337), a non-symbiotic hemoglobin (spots 320 and 324), ATP synthase (spot 309), and an uncharacterized methyltransferase (spot 312) showed much higher abundance in elicitor-treated cells. Several proteins could not be identified. The experiment was repeated on independent, triplicate biological samples.

Comparison of Protein Abundances in Control and Elicitor-treated Samples—DNA microarray analysis was used to characterize modulations in the abundance of transcripts corresponding to proteins induced in response to elicitor

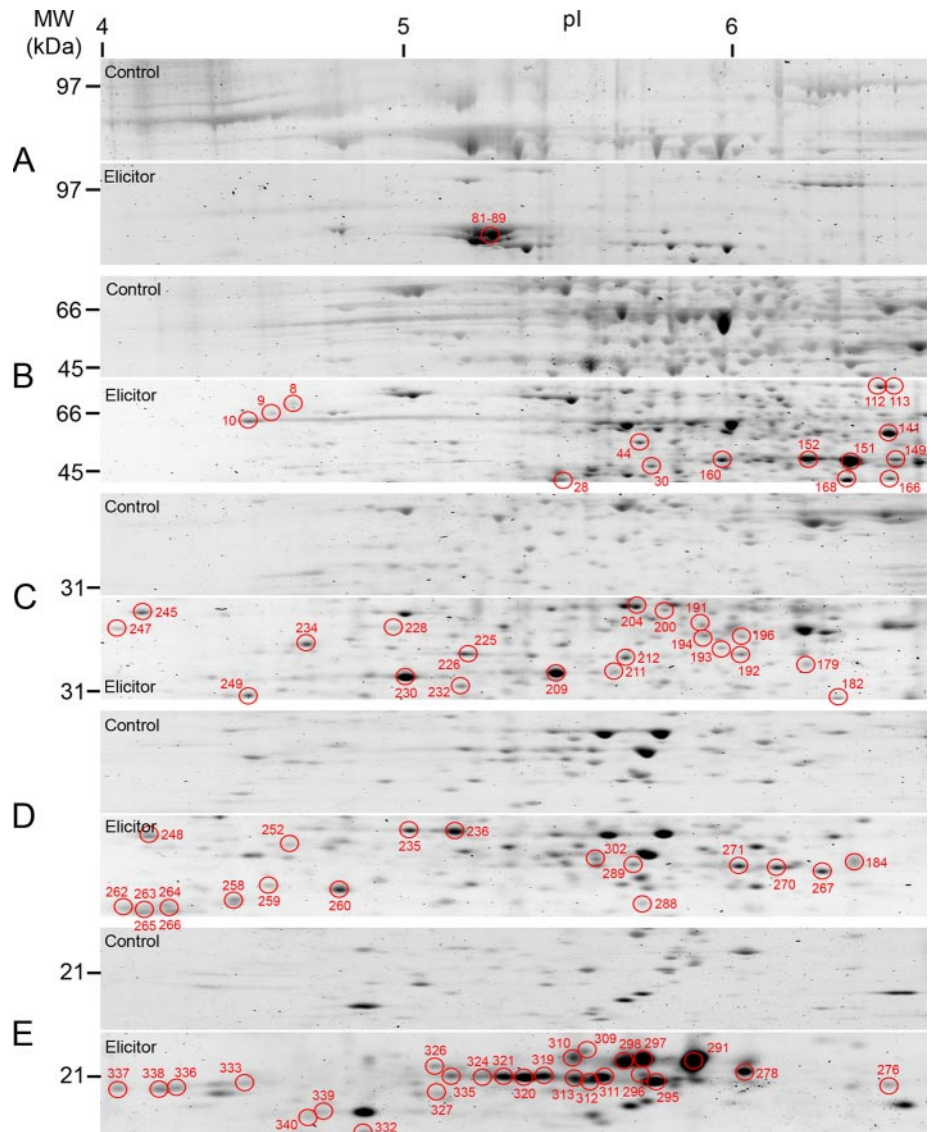


FIG. 5. **Comparative abundance of corresponding proteins in control and elicitor-treated cell cultures.** Two-dimensional electrophoresis gels were divided into five molecular weight regions to facilitate direct comparisons of protein abundance. Proteins that showed a substantial elicitor-induced increase in abundance are circled and numbered according to the reference gel shown in Fig. 3. This gel is representative of three independent experiments.

treatment and identified from opium poppy EST databases (Fig. 6; Supplemental Table III). A strong correlation between the induction of gene transcripts and cognate proteins was generally revealed. Some of those that did not exhibit a corresponding transcriptional induction represented proteins that co-migrated with enzymes expected to show increased abundance in response to stress (Fig. 6; spots 168, 194, and 196). Notable proteins that showed a corresponding induction of cognate transcripts included SAM synthetase, PAL, DIR proteins, 6OMT, isoflavone reductase, fructokinase, GST, PR proteins, non-symbiotic hemoglobin, and profiling. Many of these proteins are involved in known defense-related processes. Calmodulin, malate dehydrogenase, and the early tobacco anther protein were notable exceptions that did not show correlations between the relative abundance of protein and cognate transcripts in response to elicitor treatment.

DISCUSSION

LC-MS/MS Using Public and Opium Poppy-specific Databases—The use of LC-MS/MS has several advantages over MALDI-TOF-MS in the analysis of proteins from a non-model plant, such as opium poppy. Samples containing predominantly one protein excised from a 2-DE gel require shorter chromatography gradients, and relatively low abundance proteins can be more easily analyzed without interference from abundant proteins, which increases the dynamic identification range. LC-MS/MS also provides structural information related to the peptide sequence, which increases identification confidence. The sensitivity of LC-MS/MS further facilitates the identification of low abundance proteins.

Opium poppy genomics resources were important for achieving a high rate of successful protein identification by LC-MS/MS. In addition to the 29% of proteins uniquely iden-

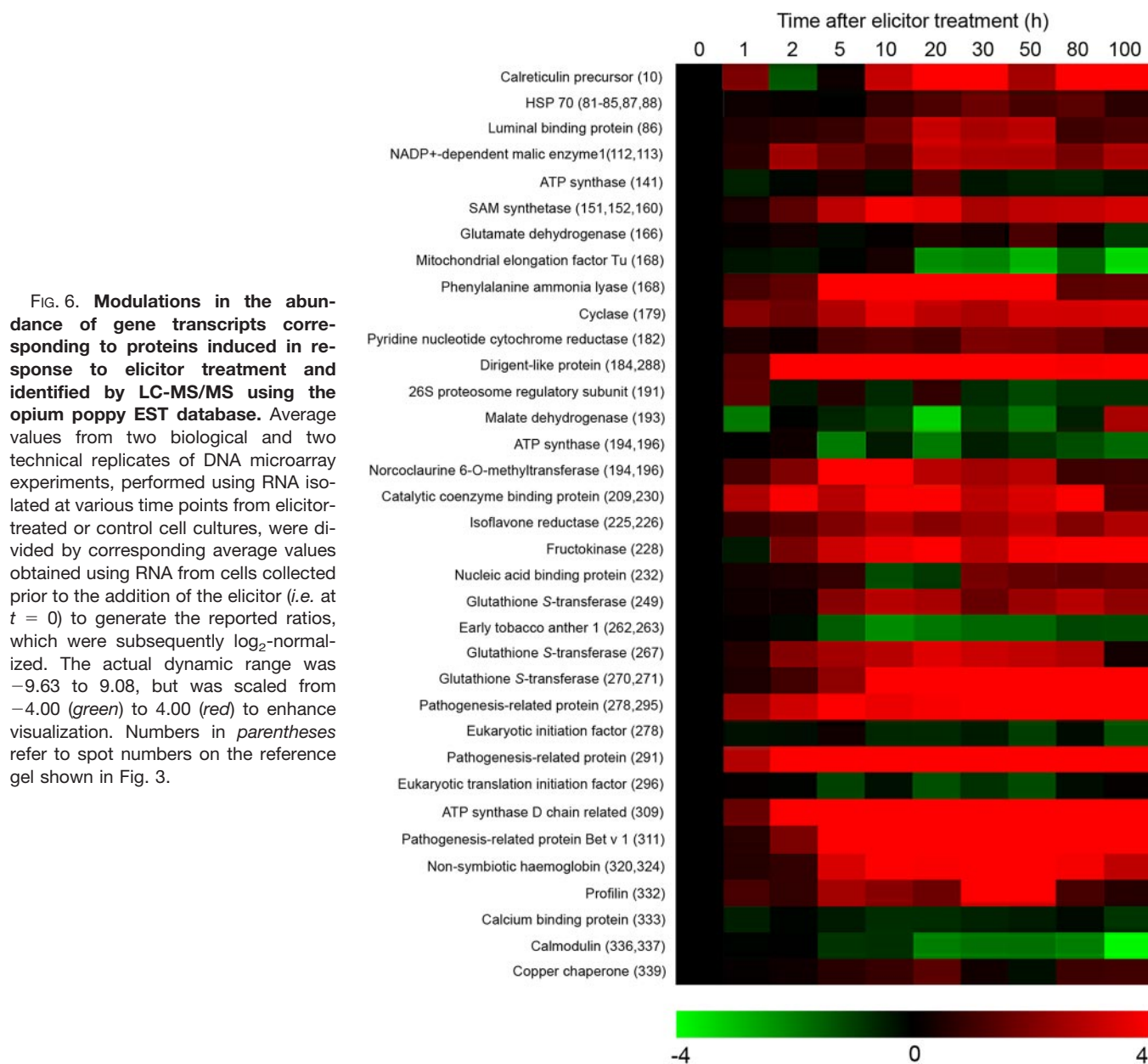


FIG. 6. **Modulations in the abundance of gene transcripts corresponding to proteins induced in response to elicitor treatment and identified by LC-MS/MS using the opium poppy EST database.** Average values from two biological and two technical replicates of DNA microarray experiments, performed using RNA isolated at various time points from elicitor-treated or control cell cultures, were divided by corresponding average values obtained using RNA from cells collected prior to the addition of the elicitor (*i.e.* at $t = 0$) to generate the reported ratios, which were subsequently \log_2 -normalized. The actual dynamic range was -9.63 to 9.08 , but was scaled from -4.00 (*green*) to 4.00 (*red*) to enhance visualization. Numbers in *parentheses* refer to spot numbers on the reference gel shown in Fig. 3.

tified through the EST databases, searching the data against both public and EST databases provided the opportunity to corroborate hits from other plants. Approximately 12% of the identifications could be identified from both approaches, and in most cases unique sets of peptides were used. These findings support the utility of EST databases for proteomic profiling in plants and provides a strong argument for their use. We note that most of the hits derived from the cell culture EST database, likely a result of its better transcript coverage.

Comparing the abundance of gene transcripts corresponding to proteins identified by LC-MS/MS allowed the confirmation of elicitor-induced responses at two levels of regulation. Most of the proteins that increased in abundance in elicitor treated cells also showed transcriptional induction (Fig. 6).

Some transcripts were repressed in response to elicitor treatment despite an increase in the abundance of the cognate proteins, suggesting post-transcriptional regulation.

Defense-related Proteins—A group of 16 spots on the 2-DE reference gel annotated as heat-shock proteins (Fig. 3; Supplemental Tables I and II). Spots 76, 78, 79, and 80 were identified as heat-shock protein 70 (HSP70)/chaperone DnaK. HSP70s are a major class of chaperones involved in protein-folding and organelle transport and play an important role in biotic and abiotic stress responses. These proteins localize to the nucleus and nucleolus when plants are subjected to stress and have been implicated in pre-ribosome assembly (27). Genes encoding HSP70s are also induced upon virus infection in plants as a generalized response to cytosolic protein

accumulation similar to the unfolded protein response (28), which occurs as a response to the stress-induced accumulation of unfolded proteins. In contrast, HSP70s levels did not substantially increase in elicitor-treated opium poppy cell cultures (Fig. 5), suggesting that the unfolded protein response does not occur or does not activate HSP70s.

Spots 81, 83, 84, 87, and 88 annotated as a single HSP70/luminal-binding protein (BiP) isoform, however, spot 86 matched a different member of the HSP70/BiP family. BiP is an endoplasmic reticulum resident chaperone induced in response to pathogen attack (29) and is encoded by a multigene family in tobacco (30). A novel signal transduction pathway triggers the rapid synthesis of BiP specifically to support an increase in PR protein biosynthesis in the rough endoplasmic reticulum (29). HSP70/BiP and PR proteins increase in abundance in response to elicitor treatment (Fig. 5, Supplemental Tables I and II), suggesting that these proteins are part of a coordinated defense response in opium poppy cells. Gene transcripts corresponding to proteins spots 81, 83, 84, and 85 were moderately induced by elicitor treatment (Fig. 6). In contrast, the gene transcript corresponding to protein spot 86 was more substantially induced (Fig. 6), suggesting independent regulation mechanisms for different members of the HSP70/BiP family.

PR-10 proteins were among the most abundant protein spots in elicitor-treated cells (Fig. 3), but were absent from the control gel (Fig. 5). PR-10 proteins were also induced in response to elicitor treatment (Fig. 6), and cognate gene transcripts were highly represented in the elicitor-treated opium poppy cell culture EST database (5). In general, PR-10 proteins are induced by pathogen challenge (31) and exhibit antibacterial and antifungal activities potentially via ribonuclease activity (32). Phosphorylation has been suggested to play a role in the ribonuclease activity of PR-10 proteins (33), which might have contributed to the differential pI of PR-10 isoforms in opium poppy (Fig. 3).

Two non-symbiotic hemoglobin isoforms and their cognate gene transcripts also increased in abundance in response to elicitor treatment (Figs. 5 and 6). Plant hemoglobin detoxifies nitric oxide (NO), a highly reactive signaling molecule produced as part of the defense response (34). The two hemoglobin isoforms suggest post-translational modification, which is in agreement with the tyrosine nitration and reversible cysteine S-nitrosylation of NO (34). The induction of four different GSTs (Fig. 5; Supplemental Tables I and II) is consistent with the role of these enzymes in the conjugation of toxic electrophilic compounds to glutathione (35). Although the abundance of all identified opium poppy GSTs increased (Fig. 5), others represented in the opium poppy EST databases were not induced at the transcriptional level suggesting a defensive role for specific members. GSTs typically display differential regulation in response to various biotic and abiotic stresses (36, 37).

Redox-related Proteins—Several proteins involved in oxidative stress protection, including ascorbate peroxidase, thiore-

doxin reductase, and peroxiredoxin were identified, but did not increase in abundance in response to elicitor treatment (Fig. 5, Supplemental Tables I and II). Redox regulation and reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical (OH^-), and singlet oxygen (1O_2) play central roles in cell signaling and defense (38, 39). Reactive oxygen species are produced in a biphasic oxidative burst and initiate the hypersensitive response resulting in programmed cell death (40). Cultured *Eschscholtzia californica* cells accumulate benzylisoquinoline alkaloids in response to elicitor treatment via a pH signaling pathway mediated by phospholipase A_2 , which is independent of the oxidative burst associated with the hypersensitive response (41, 42). This might explain the lack of change in ascorbate peroxidase and other enzymes involved in oxidative stress protection.

Enzymes Involved in Secondary Metabolism—Several secondary metabolic enzymes were identified. 6OMT, which converts (S)-norcoclaurine to (S)-coclaurine, was the only benzylisoquinoline alkaloid biosynthetic enzyme detected (Supplemental Tables I and II). 6OMT was suggested as a rate-limiting step in the pathway (43–46), and overexpression of 6OMT increased benzylisoquinoline alkaloid accumulation in transgenic *E. californica* cell cultures (43). The induction and relative abundance of 6OMT in elicitor-treated opium poppy cell cultures (Fig. 5) is consistent with a role for this enzyme in flux determination. Although 6OMT co-migrated with other proteins, the levels of transcripts encoding mitochondrial elongation factor Tu, malate dehydrogenase, and ATP synthase were unaffected by elicitor treatment, whereas 6OMT transcript abundance increased in agreement with the induction of the protein (Fig. 5, spots 194 and 196; Fig. 6).

Similarly, PAL co-migrated with a mitochondrial elongation factor (Supplemental Tables I and II). However, transcript levels for the elongation factor were unaffected by elicitor treatment, whereas PAL transcripts were induced (Fig. 6). PAL is the first committed step in the phenylpropanoid pathway that leads to several important defense-related secondary metabolites, such as flavonoids (47). Four isoflavone reductase (IFR) homologues were identified, two of which increased in abundance in response to elicitor treatment (Fig. 5, Supplemental Tables I and II). Although isoflavonoids are generally restricted to legumes (48), IFR belongs to a larger family of proteins that have been isolated from a variety of plants. An IFR homologue from grapefruit was induced by ultraviolet light irradiation, wounding, and pathogen attack (49). However, the biochemical functions of these IFR homologue proteins are not known. A protein with similarity to isoflavone synthase (Fig. 3, spot 307), the first step in isoflavone biosynthesis in legumes (50), was also identified. A catechol O-methyltransferase was also identified that was identical to a known opium poppy enzyme (Fig. 3, spot 206).

A SAM-dependent carboxyl methyltransferase with similarity to caffeine/theobromine synthase was identified. Carboxyl

methyltransferases act on a variety of substrates including salicylic acid, jasmonic acid, and 7-methylxanthine. Although opium poppy does not produce theobromine or caffeine, methyl transfer is an important feature of benzyloquinoline alkaloid metabolism, and the enzyme might function in an uncharacterized branch pathway. However, a carboxyl methyltransferase could also be involved in the production of volatile methyl esters of the defense signaling molecules, jasmonic acid and salicylic acid (51).

Three unique DIR-like proteins were identified, all of which were induced at the transcript and protein levels in response to elicitor treatment (Figs. 5 and 6). DIR proteins are present in all major plant taxa and were initially shown to direct stereoselective bimolecular phenoxy radical coupling in lignin biosynthesis (52, 53). DIR proteins might also be involved in the formation of viniferin, an antifungal derivative of resveratrol in grape (*Vitis vinifera*) (54), and are induced in spruce (*Picea spp.*) upon feeding by stem-boring weevils or mechanical wounding. The molecular functions of most DIR-like proteins are not known, but have been suggested to participate in the production of phenolic metabolites associated with the defense response (55).

Enzymes Involved in Primary Metabolism—The largest group (12 spots) of identified proteins representing a single metabolic enzyme was SAM synthetase. The abundance of SAM synthetase is consistent with the prevalence of cognate in elicitor-treated opium poppy cell cultures transcripts (5) and the role of the enzyme as a methyl donor in alkaloid and phenylpropanoid metabolism. Other highly represented enzymes included those involved in glycolysis, the TCA cycle, and amino acid metabolism/degradation pathways. Three proteins representing fructose biphosphate aldolase matched different entries in the public and EST databases, suggesting different isoforms. Most triosphosphate isomerase isoforms only matched sequences from the EST databases, suggesting relatively low sequence conservation of triosphosphate isomerase between opium poppy and other plants. The large number of enolase spots is consistent with the occurrence of several isoforms in plants (56). Enolase has also been shown to exist in several processed forms (57).

NADP-ME protein and cognate transcripts increased in abundance in response to elicitor treatment (Fig. 5, Supplemental Tables I and II). NADP-ME converts malate to pyruvate in gluconeogenesis and might participate in defense-induced lignin and flavonoid biosynthesis by supplying NADPH (58). A non-photosynthetic NADP-ME from maize was induced at the transcript and protein levels in response to jasmonic acid, fungal elicitors, and cellulase (59). The induction of NADP-ME in elicitor-treated opium poppy cell cultures might be required to provide reducing power for flavonoid biosynthesis and/or to sustain the supply for phosphoenol pyruvate as a precursor to the shikimate pathway.

Glutamate dehydrogenase (GDH) was induced at the transcript and protein levels (Figs. 5 and 6). GDH, which deami-

nates glutamate to 2-oxoglutarate in the GDH shunt (60), is transcriptionally induced by pathogen challenge, elicitor treatment, and phytohormone application (61, 62). The catabolic activity of GDH might also replenish primary metabolic pathways, such as the TCA cycle, when carbon is in high demand (63); thus, increased GDH could funnel carbon back to the TCA cycle to sustain secondary metabolite pathways.

One of two components of the cysteine synthase complex [O-acetylserine (thiol)lyase], which catalyzes the last step in cysteine biosynthesis, increased in abundance in response to elicitor treatment in opium poppy cells (Fig. 5, Supplemental Tables SI and SII). Cysteine is the main product of sulfur assimilation and is incorporated into defense metabolites, such as glutathione. Such compounds are induced in response to biotic and abiotic stress through jasmonic acid and other signaling pathways (64). An induction of cysteine levels in elicitor-treated opium poppy cell cultures might support the production of glutathione as a substrate for several induced GSTs (Figs. 5 and 6; Supplemental Tables SI and SII).

Signal Transduction—Most proteins identified as signal transduction components belong to calcium signaling pathways and include a calreticulin precursor, a calcium binding protein, and calmodulin, all of which increased in abundance in response to elicitor treatment (Fig. 5, Supplemental Tables I and II). Calcium flux is important for the elicitor-induced accumulation of secondary metabolites (65), and calmodulin is a ubiquitous calcium sensor. Calmodulin has been implicated in the salicylic acid-independent defense response in tobacco (66). A calmodulin inhibitor was shown to block the fungal elicitor-induced accumulation of sanguinarine in opium poppy cell cultures, suggesting calcium/calmodulin-mediated processes are involved in the induction of some benzyloquinoline alkaloid biosynthetic genes (3). Calmodulin has also been reported to activate phospholipase A₂ (50), which is purportedly involved in the activation of benzyloquinoline alkaloid pathways (41, 42). Unexpectedly, calmodulin transcript levels were suppressed in opium poppy cell cultures beginning 20 h after elicitor treatment (Fig. 6). However, calmodulin transcripts were rapidly induced in fungal elicitor-treated soybean cell cultures between 0.5 and 3 h, and subsequently returned to basal levels (66). The transcriptional induction of calmodulin in opium poppy cells might occur too rapidly for detection given the selected time points. Alternatively, calmodulin could be post-transcriptionally regulated.

The abundance of a calreticulin precursor increased at the protein and transcript levels in response to elicitor treatment (Figs. 5 and 6). Calreticulin is a calcium sequestering protein found in the lumen of the endoplasmic reticulum and has been implicated in the regulation of intercellular calcium homeostasis, signaling and chaperone activity (67). Calreticulin has also been shown to have chaperone activity and interact with BiP (68), which also increased in abundance in response to elicitor treatment. In opium poppy, calreticulin might play a role in stress-induced chaperone-mediated protein-folding.

Jasmonic acid is a critical signaling molecule in plant defense responses (65). 12-Oxophytodienoate reductase is involved in jasmonic acid biosynthesis and was identified in this study (Supplemental Tables I and II) and in a proteomics survey of a *C. roseus* cell culture producing monoterpenoid indole alkaloids (21).

Other Induced Proteins—The abundance of two catalytic/coenzyme-binding proteins increased in response to elicitor treatment (Fig. 5, Supplemental Tables I and II). This protein contains 3 β -HSD and NmrA components. 3 β -HSD has been implicated in the biosynthesis of the steroid cardenolide in foxglove (*Digitalis lanata*) (69) and has been detected in potato and tobacco, suggesting a role in general steroid metabolism (70). In a proteomics survey of *Panax ginseng*, the abundance of 3 β -HSD increased in response to light and was linked to the biosynthesis of steroidal saponins (71). 3 β -HSD was also increased in abundance in rice challenged with a fungal pathogen suggesting an involvement in the biosynthesis of defense-associated steroids (72). NmrA is a negative transcriptional regulator involved in nitrogen metabolism and potentially in redox sensing (73). Four induced proteins were similar to early tobacco anther proteins (Fig. 5, Supplemental Tables I and II), and one was annotated as a type III membrane protein. Neither of these proteins has been functionally characterized.

The identification of proteins reported in this study generally corroborates and provides a valuable link between previous transcriptomics (5) and metabolomics (5, 6) surveys to characterize the induction of benzyloquinoline alkaloid metabolism and other defense responses in elicitor-treated opium poppy cell cultures. The availability of complementary transcript, protein, and metabolite datasets already provides an opportunity to examine plant defense responses from a systems biological perspective. Although the identification capacity of 2-DE coupled with LC-MS/MS and species-specific informatics resources allowed the identification of 219 proteins from a non-model plant, greater sensitivity is required to penetrate further into the proteome to detect other key proteins. In particular, the sensitivity limit of the approach described in this study appeared to just reach the abundance level of benzyloquinoline alkaloid biosynthetic enzymes in opium poppy. The use of gel-free methods such as two-dimensional liquid chromatography and multidimensional protein identification technology (74) will facilitate the further characterization of the proteome of this important medicinal plant.

* This work was supported by funds provided by Natural Sciences and Engineering Research Council of Canada Discovery and Strategic Grants (to P. J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The amino acid sequences of these proteins can be accessed through NCBI Protein Database under NCBI accession numbers FE964053–FE968617 and FG598405–FG614008.

§ The on-line version of this article (available at <http://www.mcp.org>) contains Supplemental Fig. S1 and Supplemental Tables SI–SIII.

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