Shotgun Proteomic Analysis of Yeast-Elicited California Poppy (Eschscholzia californica) Suspension Cultures Producing Enhanced Levels of Benzophenanthridine Alkaloids

ARTICLE *in* JOURNAL OF PROTEOME RESEARCH · SEPTEMBER 2010

Impact Factor: 4.25 · DOI: 10.1021/pr1000412 · Source: PubMed

6 AUTHORS, INCLUDING:

John T Oldham

4 PUBLICATIONS **17** CITATIONS

SEE PROFILE

Marina Hincapie

Northeastern University

46 PUBLICATIONS **1,147** CITATIONS

SEE PROFILE

Carolyn W. T. Lee-Parsons

Northeastern University

25 PUBLICATIONS **372** CITATIONS

SEE PROFILE

research articles Journal of proteome research

Shotgun Proteomic Analysis of Yeast-Elicited California Poppy (*Eschscholzia californica*) Suspension Cultures Producing Enhanced Levels of Benzophenanthridine Alkaloids

John T. Oldham,[†] Marina Hincapie,[‡] Tomas Rejtar,[‡] P. Kerr Wall,[§] John E. Carlson,[||,#] and Carolyn W. T. Lee-Parsons*[,†]

Department of Chemical Engineering, 342 Snell Engineering Center, 360 Huntington Avenue, Northeastern University, Boston, Massachusetts 02115, Barnett Institute of Chemical and Biological Analysis, Northeastern University, Boston, Massachusetts 02115, Department of Biology and The Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, Pennsylvania 16802, School of Forest Resources and The Huck Institutes of the Life Sciences, 323 Forest Resources Building, Pennsylvania State University, University Park, Pennsylvania 16802, and Department of Bioenergy Science and Technology (WCU), Chonnam National University, 333 Yongbongro, Buk-gu, Gwangju 500-757, Korea

Received January 17, 2010

The California poppy, *Eschscholzia californica*, produces benzophenanthridine alkaloids (BPAs), an important class of biologically active compounds. Cell cultures of *E. californica* were investigated as an alternative and scalable method for producing these valuable compounds; treatment with yeast extract increased production from low levels to 23 mg/g dry weight (DW) of BPAs. A shotgun proteomic analysis of *E. californica* cell cultures was undertaken to explore changes in metabolism associated with enhanced BPA production. We implemented differential centrifugation and then shotgun proteomics based on nanoliquid chromatography/mass spectrometry (nano-LC-MS/MS) for peptide separation and analysis. A unigene database available for *E. californica* was translated and utilized for protein identification. Approximately 646 proteins (3% false discovery rate at the protein level) were identified. Differentially abundant proteins observed with elicitation included enzymes involved in (*S*)-adenosyl methionine (SAM) biosynthesis and BPA biosynthesis. These results demonstrate (1) the identification of proteins from a medicinal plant using shotgun proteomics combined with a well-annotated, translated unigene database and (2) the potential utility of proteomics for exploring changes in metabolism associated with enhanced secondary metabolite production.

Keywords: *Eschscholzia californica* • EST database • LC-MS/MS • secondary metabolism • shotgun proteomics

Introduction

The California poppy, *Eschscholzia californica*, produces benzophenanthridine alkaloids (BPAs) such as the biologically active compounds sanguinarine and chelerythrine.[1–6] Sanguinarine and chelerythrine (Figure 1) show activity against multiple targets associated with inflammation,[3] cell cycle regulation, and apoptosis of cancer cells.[4,6,7] In addition, sanguinarine was previously incorporated as an antiplaque agent in Viadent toothpastes and mouthwashes.[2] Because of BPA's important biological activities, cell cultures of *E. californica* are being investigated as an alternative and scalable method for producing these valuable compounds. The advantage of the cell culture system is that environmental conditions can be controlled and easily manipulated for improving production.

For example, BPA production is significantly enhanced in cell cultures of *E. californica* and *Papaver somniferum* with elicitation.[8–13] Elicitors activate plant natural defense responses, including increased secondary metabolite production.[14,15] Examples of elicitors include hormones (e.g., jasmonates or salicylic acid), crude biological extracts (e.g., fungal homogenate), and purified biological extracts (e.g., polysaccharides derived from the yeast cell wall).[16,17] In *E. californica* cell cultures, yeast elicitor is believed to increase BPA production by activating a pH-dependent signal transduction pathway.[18,19] Activation of signal transduction pathways triggers gene transcription leading to increased enzyme abundance and secondary metabolite production. For instance, gene transcripts of several BPA biosynthetic enzymes were elevated by elicitation with yeast extract.[20–22] Also, the levels of 6 BPA enzymes were

* To whom correspondence should be addressed. Phone: 617-373-2989. Fax: 617-373-2209. E-mail: clee@coe.neu.edu.
[†] Department of Chemical Engineering, Northeastern University.
[‡] Barnett Institute of Chemical and Biological Analysis, Northeastern University.
[§] Department of Biology and The Huck Institutes of the Life Sciences, Pennsylvania State University.
[||] School of Forest Resources and The Huck Institutes of the Life Sciences, Pennsylvania State University.
[#] Department of Bioenergy Science and Technology (WCU), Chonnam National University.

10.1021/pr1000412

Published on Web 08/06/2010

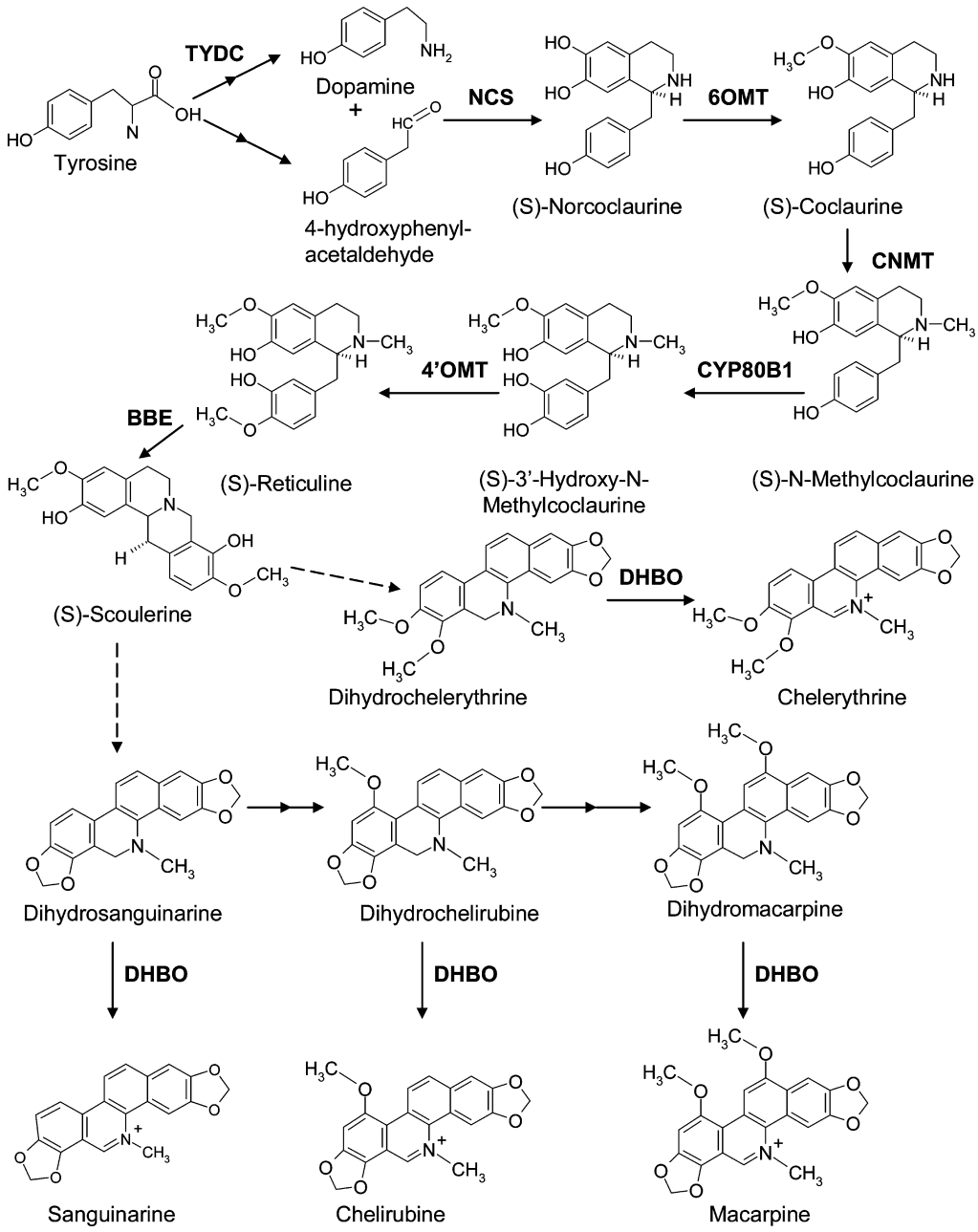

Figure 1. BPA biosynthetic pathway from tyrosine to macarpine adapted from ref 33. Dashed arrows represent multiple reactions. Enzyme abbreviations: TYDC, tyrosine decarboxylase; NCS, norcoclaurine synthase; 6OMT, (*S*)-norcoclaurine-6-*O*-methyltransferase; CNMT, (*S*)-coclaurine *N*-methyltransferase; CYP80B1, (*S*)-methylcoclaurine-3′-hydroxylase; 4′OMT, 3′-hydroxy-(*S*)-*N*-methylcoclaurine-4′-*O*-methyltransferase; BBE, berberine bridge enzyme; DHBO, dihydrobenzophenanthridine oxidase.

increased by elicitation with methyl jasmonate, salicylic acid, and yeast extract.[12,13]

However, less is known about how primary and secondary metabolic pathways are coordinated to support increased secondary metabolite production. Investigating the proteome could provide a more global view of cellular metabolism and its coordination in response to external stimuli. In this paper, changes in the metabolism of *E. californica* cell cultures with enhanced BPA production were explored using a proteomic approach. The proteomic approach has been employed for this purpose in only a few medicinal plants.[23–25]

Proteomic studies in plant systems have primarily been performed in fully sequenced model systems such as *Arabidopsis thaliana*, *Oryza sativa* (rice), *Populus trichocarpa* (black cottonwood), and *Vitis vinifera* (grape vine) since mass spectrometry (MS)-based proteomics requires the availability of a protein database. However, the proteomes of a few medicinal plants with rich but incomplete sequence information have also been explored, including *Catharanthus roseus*,[24] *P. somniferum*,[25,26] *Taxus cuspidata*,[23] ginseng,[27,28] and *Chelidonium majus*.[29] For proteomic analysis of such organisms, one approach utilizes sequence homology to proteins already in a database (e.g., *A. thaliana*). This approach allows identification of highly conserved proteins such as primary metabolic pathways proteins but not necessarily enzymes involved in alkaloid biosynthesis which are specific to individual plant species. Alternatively, an expressed sequence tags (EST) database for a given organism has been used to identify proteins from

tomato[30] and ginseng.[27,28] Importantly, new generation of sequencing methods such as 454 has the potential for generating high quality transcriptomic profiles without the need for full genomic sequence.[31]

For *E. californica*, an EST database has also been constructed.[32] In addition, unlike other alkaloid biosynthetic pathways, the biosynthesis of BPAs such as macarpine (the most oxidized BPA, shown in Figure 1) is completely known and involves 20 enzyme-catalyzed steps and 1 spontaneous step starting from two tyrosine molecules.[33] Furthermore, 10 enzymes in the BPA biosynthetic pathway (7 steps leading up to scoulerine in the Figure 1 and 3 steps after scoulerine, i.e., chelianthifoline synthase, stylopine synthase, and (*S*)-tetrahydroprotoberberine-(*cis*)-*N*-methyltransferase) have been sequenced (http://www.expasy.org), enabling the profiling of a number of genes and enzymes involved in BPA biosynthesis.

Typical plant proteomic experiments utilize two-dimensional gel electrophoresis (2-DE) for protein separation prior to identification.[24–26] Though very powerful and offering a number of advantages, 2-DE yields relatively low number of identified proteins due to inherent limitations of this approach.[34,35] To increase the coverage of the proteome, we implemented an alternative but complementary gel-free method. Recently, shotgun proteomic technologies have been applied to plants such as the *A. thaliana*,[36] *O. sativa*,[37] and *Medicago truncatula*.[38]

In this paper, we implemented the shotgun proteomic method based on nano-LC-MS/MS. Proteins were identified using a database generated by translation of a published *E. californica* uniGene library constructed using ESTs;[32] 15 enzyme sequences from 10 BPA pathway enzymes were also incorporated into this protein database. Shotgun proteomics with nano-LC-MS/MS analysis and identification utilizing a well-annotated, translated unigene database is an approach that has been applied to only a few plant systems where a fully sequenced genome is not available.

Using the combined approach described above, we performed a proteomic investigation of *E. californica* cell cultures elicited with purified yeast extract (PYE). Elicited cultures produced up to 23 mg/g dry weight (DW) of BPAs corresponding to a 20-fold increase over untreated cultures. Nearly 646 proteins from primary and secondary metabolism and other functional protein classes were identified. Furthermore, differential abundance of proteins from several pathways was observed. Hence, in this paper, we demonstrate the application of the shotgun proteomics method utilizing a well-annotated, translated unigene database to (1) identify proteins from a medicinal plant (*E. californica*) and (2) explore changes in global metabolism associated with enhanced alkaloid production.

Materials and Methods

Maintenance of Cell Cultures. *E. californica* suspension cultures were a gift from Dr. Sung-Yong Yoon (formerly at Pohang University of Science and Technology; currently at Exelixis Plant Sciences). The suspension cultures were initiated from leaf sections of seedlings germinated on agar-containing medium, as described by Park et al.[39] Maintenance conditions were provided by Drs. Hwa-Young Cho (Pohang University of Science and Technology) and Sung-Yong Yoon and were modified from previously published reports.[12,13,40]

Cultures were maintained on Linsmaier and Skoog's medium (Caisson Laboratories, North Logan, UT),[41] supplemented with 30 g/L sucrose (Sigma), 0.37 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma), 0.11 mg/L α-napthaleneacetic acid (Sigma), and adjusted to pH of 5.5 with 1 N NaOH. Cells were subcultured every 14 days by transferring 20 mL of culture (containing 6 mL of packed cell volume) to 80 mL of fresh medium in 250 mL Erlenmeyer flasks. Cultures were maintained at 22 °C with 16 h light per day, at 120 rpm in a Forma Scientific incubator shaker (Marietta, OH). Sterile water was added weekly to compensate for evaporation.

Elicitation of Cell Cultures. Purified yeast extract (PYE), containing polysaccharides associated with the yeast cell wall, was prepared based on the method of Hahn and Albersheim.[17] Hahn and Albersheim[17] isolated the active component of the yeast cell wall (i.e., a glycan) which elicited the production of the phytoalexin, glyceollin, from soybeans.

Yeast extract (50 g, Becton-Dickinson, Sparks, MD) was solubilized in 200 mL of water; ethanol (Fisher-Scientific) was added to a final concentration of 80% (v/v), and then the mixture was stored at 4 °C for 4 days. The supernatant was discarded and the precipitate was redissolved in 200 mL of water and precipitated again with ethanol (80% v/v) at 4 °C for 4 days. The final precipitate was then resuspended in 200 mL of water, lyophilized, and stored at −20 °C.

For proteomic analysis, cells were transferred from growth medium on day 14 to fresh medium without 2,4-D in 250 mL Erlenmeyer flasks (50 mL of culture); these cells were cultured in 2,4-D-free medium for 7 days and then elicited with 40 mg/g fresh cell weight (FW) PYE on day 7 (midexponential phase). Cells were harvested for alkaloid analysis after 48 and 96 h by vacuum filtration, frozen, lyophilized, and stored at −20 °C. For proteomic analysis, cells were harvested after 48 h by vacuum filtration, flash frozen in liquid nitrogen, and stored at −80 °C.

Alkaloid Analysis. The alkaloid extraction and HPLC protocols were provided by Drs. Hwa-Young Cho and Sung-Yong Yoon and were modified from previously published reports.[12,13] Freeze-dried cells (10 mg) were extracted in 1 mL of 0.2% HCl in methanol. Extracts were sonicated for 1 h, vortexed for 30 min, and then centrifuged for 20 min at 13 200*g* and 4 °C. The supernatant was filtered through a Millex-FH 0.45 μm syringe filter (Millipore, Billerica, MA) prior to HPLC analysis.

Alkaloids were separated and quantified by HPLC using a Waters Alliance 2695 separations module, 996 photodiode array detector (Waters, Milford, MA), and a Phenomenex C_{18} reversed phase column (4.60 mm i.d. × 150 mm, 5 μm) with a C_{18} precolumn (Phenomenex, Torrance, CA). The mobile phases used for separation were (A) 0.032% tetrabutylammonium hydroxide (Sigma) in water, pH 3.0; (B) acetonitrile (Fisher, HPLC grade); and (C) methanol (Fisher, HPLC grade). The HPLC protocol was provided by Drs. Hwa-Young Cho and Sung-Yong Yoon and was adapted from Cho et al.;[13] the protocol consisted of gradients at 1.0 mL/min from (1) 80% A, 10% B, 10% C to 75% A, 10% B, 15% C in 5 min; (2) to 60% A, 15% B, 25% C in 5 min; (3) to 40% A, 25% B, 35% C in 10 min; (4) to 1% A, 10% B, 89% C in 15 min; (5) to 80% A, 10% B, 10% C in 10 min; and (6) isocratically at 80% A, 10% B, and 10% C for 15 min; 30 μL of the cell extract was injected into the HPLC.

Alkaloids were detected by UV absorbance at 283 nm and quantified by peak areas using sanguinarine as a standard (Sigma). Calibration curves for chelerythrine, chelirubine, dihydrochelerythrine, dihydrochelirubine, and dihydrosangui-

narine were estimated using the calibration curve for sanguinarine and published extinction coefficients.[42]

Protein Extraction and Fractionation by Differential Centrifugation. Freeze-dried cells were lysed in a BioSpec bead beater (BioSpec, Bartlesville, OK) with protein extraction buffer as modified from Cho et al.[12] The protein extraction buffer consisted of 7 M urea (Fisher), 2 M thiourea (Sigma), 50 mM tris-HCl (Fisher) pH 8.0, 5 mM dithiothreitol (DTT, Sigma), ROCHE protease inhibitor cocktail, and benzonase nuclease (Novagen, Madison, WI). Vials containing cells (500 mg FW), extraction buffer (500 μL), and glass beads (BioSpec) were shaken for 30 s at 5000 rpm, cooled on ice, and then the cycle was repeated 3 more times. Vials were centrifuged at 5000*g* for 15 min at 4 °C; the supernatant was saved while the pellet was then resuspended with fresh protein extraction buffer; the extraction procedure was repeated 2 more times.

The supernatant fractions from the 3 extraction procedures were combined and centrifuged for 20 min at 16 000*g* at 4 °C. The resulting supernatant was centrifuged for 45 min at 100 000*g* at 4 °C in a Beckman-Coulter TI-100 ultracentrifuge. The supernatant enriched in soluble proteins was transferred to Eppendorf tubes. The remaining pellet was thoroughly washed with phosphate-buffered saline (PBS) to obtain maximum recovery of soluble proteins and then the remaining pellet was saved and stored at −80 °C. The protein concentration of the resulting fractions was then determined by the Bradford assay[43] using BSA (Pierce, Rockford, IL) as a standard.

Using the protein extraction and differential centrifugation methods above, two biological replicates for each cell culture condition (e.g., 2 unelicited cultures and 2 elicited cultures) were prepared. In addition, 2 technical replicates were obtained from each biological replicate by separately digesting 2 aliquots from the 100 000*g* supernatant fraction, as described below. A summary of the biological and technical replicates prepared is shown in Supplementary Table 1.

In-Solution Trypsin Digestion of Protein Fraction. Only the supernatant from the 100 000*g* ultracentrifugation step was analyzed. Briefly, for each sample, 100 μg of protein was reduced in 5 mM tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (Pierce, Rockford, IL), alkylated with 15 mM iodoacetamide (Sigma), and quenched with 5 mM DTT. Samples were digested overnight with trypsin (Promega, Madison, WI, 1:40 w/w ratio) and the digestion reaction was stopped by adjusting the pH to less than 4 with 3% formic acid.

Peptides resulting from protein digestion were desalted and separated from any undigested or partially digested proteins by reversed phase liquid chromatography using a Discovery BIO C18 cartridge (3 μm, 4.6 mm ×30 mm; Sigma) and a Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD). The mobile phases used were (A) 0.1% trifluoroacetic acid (TFA, Pierce, Rockford, IL) in water and (B) 0.1% TFA in acetonitrile. The HPLC method consisted of three isocratic steps: (1) 2% B for 3 min at 2.0 mL/min to remove salt; (2) 30% B for 6 min at 0.6 mL/min to elute tryptic digested peptides; and (3) 90% B for 1.5 min at 2.0 mL/min to elute very hydrophobic peptides, large protein fragments, or non-digested protein. The cartridge was re-equilibrated to initial conditions (2% B) for 1.5 min at 2.0 mL/min. Peptides eluted with 30% organic solvent were concentrated under vacuum using a CentriVap concentrator (Labconco, Kansas City, MO) to a volume of approximately 10−15 μL and then adjusted to 20 μL with 0.1% formic acid (Pierce, Rockford, IL) in water.

Nano-LC-ESI-MS/MS Analysis. The complex peptide mixture was separated using a 2D nano Eksigent HPLC system (Dublin, CA) on a reversed phase C18 capillary (175 mm ×0.075 mm i.d.) column in-house packed with Magic C18 media (particle size 5 μm, pore size 300 Å, Michrom Bioresources, Auburn, CA) and directly connected to a linear ion trap (LTQ) instrument (Thermo Fisher, Waltham, MA). The electrospray conditions were the following: temperature of the transfer tube, 245 °C; spray voltage, 2.0 kV; normalized collision energy, 35%. The mass spectrometer was operated in the data-dependent mode and switched automatically between MS and MS/MS using MS acquisition software (Xcalibur 2.0, Thermo Fisher, Waltham, MA). Each MS full scan (mass range of m/z 400 to m/z 1600) was followed by MS/MS scans of the 7 most intense peaks.

Peptides (5 μL/injection) were loaded at a higher flow rate (1 μL/min) and concentrated on a peptide Captrap cartridge (Michrom Bioresources, Auburn, CA). The peptides were then eluted onto the capillary column and chromatographed at 250 nL/min. The mobile phases used for peptide separation were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The method consisted of an isocratic segment at 5% B for 5 min, followed by a linear gradient to 35% B in 115 min, gradient to 80% B in 20 min, isocratic at 80% B for 10 min, gradient to 5% B in 1 min, and then isocratic at 5% B for 4 min. The capillary column was equilibrated for 30 min at 250 nL/min before injection of the next sample.

Protein Identification and Quantitation. The *E. californica* unigene sequences (build 5) were downloaded from http://pgn.cornell.edu and run through several iterations of SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) to trim contaminants, including vector, low quality, and low-complexity sequences. Each cleaned unigene was run through the program ESTScan[44] to predict coding sequence, using the *Arabidopsis* hidden Markov model. To annotate each unigene sequence, each of the four sequenced angiosperm genomes was downloaded, which included 31 921 gene models from *A. thaliana*,[45] 45 555 from *P. trichocarpa*,[46] 66 710 from *O. sativa*,[47] and 30 434 from *V. vinifera*.[48] The predicted proteins for all four species were compared in an all-against-all BLASTP (e-value < e−10) using the NCBI BLAST package.[49] The program orthoMCL[50] was then used to identify all ortholog/co-ortholog gene sets. Co-orthologs are defined as two or more genes within a species that share orthology with one or more genes in another species due to duplication events after the two species diverged. Each orthoMCL group was annotated with a representative *Arabidopsis* gene. The poppy unigene sequences were searched against the entire protein data set using BLASTX and best hits were recorded. The best hit, description, and orthoMCL annotation of the best hit are provided in Supplementary Table 2.

MS/MS spectra were processed by the CPAS system[51] using the Sequest search against the *E. californica* EST database translated into protein sequences (4700 protein sequences) combined with 15 protein sequences from 10 BPA pathway enzymes (http://www.expasy.org). This protein database was appended with a random protein database created by reversing the protein sequences in order to facilitate estimation of the false discovery rate.[52] The database search was performed with trypsin specified as the digestion enzyme, up to 2 missed cleavages and carbamidomethylation as a fixed modification of cysteines. Mass tolerance was set to 1.6 Da for the precursor ion while 1 Da was used for MS/MS fragment ions. The peptide

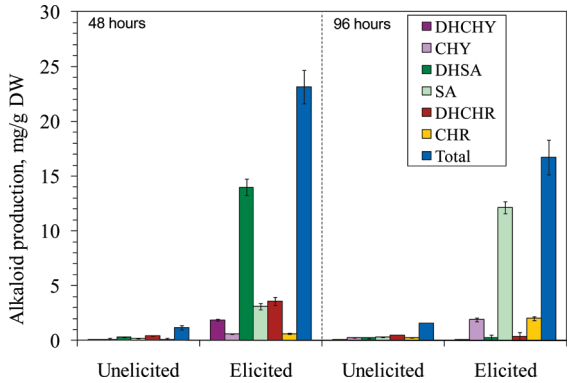

Figure 2. Alkaloid production was enhanced by elicitation with purified yeast extract (PYE). PYE (40 mg/g FW) was added on day 7 to *E. californica* cultures and cells were harvested after 48 and 96 h. Error bars represent the standard deviation of triplicate cultures. Abbreviations: DHCHY, dihydrochelerythrine; CHY, chelerythrine; DHSA, dihydrosanguinarine; SA, sanguinarine; DHCHR, dihydrochelirubine; CHR, chelirubine.

matches were filtered using PeptideProphet probability >0.9 and Sequest Xcorr values greater than 1.9 for 1+, 2.2 for 2+, and 3.8 for 3+ ions. Then, the protein list was generated by the Protein-Prophet tool with the protein probability cutoff set to 0.9 for protein groups. These filtering criteria led to approximately 3% false discovery rate at the protein level. Special care was taken to remove protein redundancy and only protein groups with a least one peptide that is not shared by any other group were retained for further evaluation (Supplementary Table 2).

Semiquantitative analysis was performed using spectral counting; the relative protein abundance was expressed as the total number of peptides identified for a particular protein in elicited or control sample.[53,54] In addition, spectral index[55] and permutation analysis were used to determine the level of significance for differentially abundant proteins. On the basis of the permutation analysis, proteins with spectral index values greater than 0.55 or less than −0.60 were considered differentially abundant with 95% confidence. Finally, all differentially abundant proteins, 95% confidence, and at least 3-fold change in the spectral counts were also manually validated (MS/MS spectra shown in Supplementary Figures).

Results

Elicitation of Alkaloid Production. Benzophenanthridine alkaloid (BPA) production was elicited in *E. californica* suspension cultures using purified yeast extract (PYE). Elicitation conditions (i.e., 40 mg of PYE/g FW added on day 7, midexponential phase) were chosen to optimize total BPA production.[12] After 48 h, elicited cultures accumulated 23.1 mg of BPAs/g DW, a 20-fold increase over unelicited cultures (Figure 2). After 96 h, the total alkaloid production did not increase in the elicited cultures (i.e., 16.7 mg/g DW), but the profile of alkaloids changed. For example, sanguinarine and chelerythrine accounted for 13.4% and 2.5%, respectively, of the total BPA production in elicited cultures after 48 h compared to 72.5% and 11.3% after 96 h. The protein profile of the cultures under low (i.e., untreated) and high BPA-producing (i.e., PYE-elicited) conditions were then compared.

Overview of Proteomic Method. Soluble protein extracts from untreated and PYE-elicited cells were prepared by ultracentrifugation (see Protein Extraction and Fractionation by Differential Centrifugation of the Materials and Methods). Extracts enriched in soluble proteins were then digested with trypsin (see In-Solution Trypsin Digestion of Protein Fraction); the resulting peptides were separated by capillary reversed phase chromatography and analyzed by MS/MS (see Nano-LC-ESI-MS/MS Analysis). MS/MS spectra were searched using the Sequest algorithm against the *E. californica* protein database. For untreated and PYE-elicited cultures at 48 h, 2 biological replicates with 2 technical replicates (samples digested in parallel) each were analyzed.

Using the constructed *E. californica* protein database (see Protein Identification and Quantitation of the Materials and Methods), approximately 646 proteins were identified with a false discovery rate of 3% at the protein level (Supplementary Table 2). The classes of identified proteins included structural proteins (e.g., ribosomal proteins, actin, tubulin), protein synthesis (e.g., elongation factor 1A), chaperone proteins (e.g., heat shock proteins, peptidyl *cis*−*trans* isomerase), calcium binding proteins (e.g., calreticulin), ubiquitin-associated proteins, defense response proteins (glutathione-*S*-transferase), and enzymes involved in nucleic acid biosynthesis and binding, amino acid biosynthesis, energy metabolism, and secondary metabolism.

Table 1. Selected Differentially Abundant Proteins Identified with Spectral Index Values Greater Than 0.55 or Less Than −0.6[a]

protein	name	average spectral counts: Elicited	average spectral counts: Unelicited	spectral index
MS	Methionine synthase[b]	17.50	3.80	0.64
SAMS	*S*-adenosyl methionine synthase	9.00	1.20	0.84
SAHH	*S*-adenosylhomocysteine hydrolase	3.00	1.00	0.60
4′OMT	3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase[c]	14.50	0.60	0.94
PFK	Phosphofructokinase	2.00	0.60	0.63
Aldolase	Fructose bisphosphate aldolase	2.50	0.80	0.56
PEPC	Phosphoenolpyruvate carboxylase	0.00	2.20	−1.00
GS	Glutamate synthase	3.25	0.00	1.00
GST	Glutathione-*S*-transferase	5.00	1.40	0.56

[a] Relative protein abundance is expressed by the average spectral counts from elicited samples (from 2 technical replicates for each of the 2 biological replicates, 4 samples total) and the average spectral counts from unelicited samples (from 2 technical replicates for each of the 2 biological replicates including duplicate injections of a single technical replicate, 5 samples total). The spectral index was calculated according to Fu et al.[55] [b] Three isoforms for methionine synthase were detected using unique peptides from each isoform. The high sequence homology prevented exact determination of spectral counts for each isoform. [c] Sequence showed nearly 100% identity with 4′OMT.

Differentially Abundant Proteins. The spectral index[55] was calculated to determine the level of significance for the differentially abundant proteins. Proteins with spectral index values greater than 0.55 or less than −0.60 were considered differentially abundant with 95% confidence. The differentially abundant proteins are summarized in Table 1.

The increased abundance of several enzymes within a specific pathway suggests a coordinated effort by the cell to increase flux through this pathway. For instance, the levels of three enzymes involved in the biosynthesis of methionine and (*S*)-adenosylmethionine (SAM) were increased in the high alkaloid-producing cultures (Figure 3, Table 1): cobalamin-independent methionine synthase (MS), SAM synthase (SAMS), and (*S*)-adenosyl homocysteine hydrolase (SAHH). In addition to primary metabolic enzymes, one enzyme from the BPA

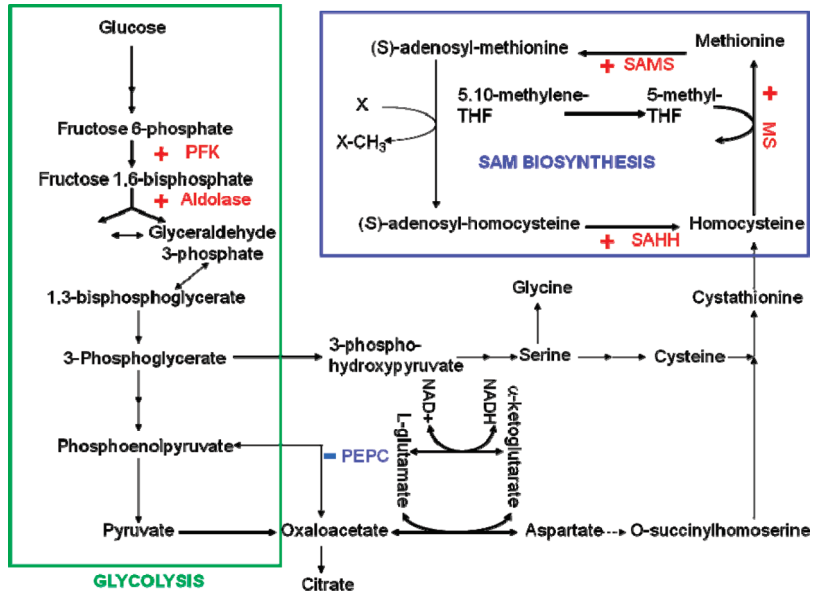

Figure 3. Metabolic pathways leading to (*S*)-adenosyl methionine (SAM) production from glycolysis. See Table 1 for abbreviations.

biosynthetic pathway (4′OMT) was identified in the high-producing cultures. Importantly, increased flux in the BPA pathway is consistent with a higher abundance of 4′OMT. As shown in Figure 1, the biosynthesis of BPAs begins with the conversion of two molecules of tyrosine in several steps to form (*S*)-norcoclaurine. Then, (*S*)-norcoclaurine is converted to (*S*)-reticuline by three methylation reactions, one of which is catalyzed by 4′OMT, and finally one hydroxylation reaction (i.e., CYP80B1).

Other proteins that exhibited a significant difference based on spectral index values above the 95% cutoff included glycolytic enzymes (e.g., phosphofructokinase, fructose bisphosphate aldose, phosphoenolpyruvate carboxylase, see Figure 3), stress response proteins (e.g., glutathione-*S*-transferase), and structural proteins (e.g., ribosomal subunits and histones).

Discussion

Relatively few studies have applied proteomics for investigating secondary metabolism of medicinal plants[23–25] and in particular have focused on applying proteomics for discovering new enzymes involved in secondary metabolism.[56,57] In Ounaroon et al,[57] a proteomic analysis of latex from the opium poppy using 2-D gel electrophoresis led to the isolation and identification of two methyltransferase enzymes involved in benzylisoquinoline alkaloid biosynthesis. In Jacobs et al,[24] a proteomic analysis of *C. roseus* cell cultures at different production stages using 2-D gels was pursued to identify novel proteins associated with terpenoid indole alkaloid biosynthesis. While unique sequences were discovered, the analysis also demonstrated that a variety of proteins could be identified for plant where a fully sequenced genome was not available, including enzymes associated with primary and secondary metabolism, photosynthesis, oxidative phosphorylation, and protein synthesis as well as chaperone and structural proteins.

The absence of a fully sequenced genome presents a significant challenge in the proteomic analysis. Several approaches were employed to overcome this problem; for example, (1) sequence homology to *A. thaliana* and other model plant systems was used to identify highly conserved proteins from medicinal plants such as those in *C. roseus*,[24] *P. somniferum*,[26] and *T. cuspidata*;[23] and (2) expressed sequence tags (EST) databases were used for identification of proteins in *P. somniferum*,[25] tomato,[30] and ginseng.[27,28] The ability to perform proteomic analyses of medicinal plants without a fully sequenced and annotated genome will provide a useful platform for exploring and investigating the global metabolism of these medicinal plants.

In this paper, we demonstrate the application of a gel-free shotgun proteomic method and a well-annotated, translated unigene database for identifying proteins in the medicinal plant species, *E. californica*. Specifically, we identified nearly 650 proteins from *E. californica* cell cultures with minimal protein fractionation (i.e., differential centrifugation) followed by peptide separation and analysis using nano-LC-ESI-MS/MS. The number of proteins identified compared favorably to reports in the literature investigating other unsequenced plants, including *C. roseus*[24] and *P. somniferum* cultures.[25] For instance, in Jacobs et al,[24] a 2-D gel proteomic study of *C. roseus* cell cultures resulted in a maximum number of 988 spots; by comparing the protein profiles from different culture days, 88 spots were selected, leading to 58 identified proteins using homology-based search. In Zulak et al.,[25] a 2-D gel proteomic study of *P. somniferum* identified 219 proteins from 340 spots using an EST-database for searching.

Comparing the protein profile of the low and high alkaloid-producing cultures of *E. californica* resulted in the identification of several proteins with significant changes in abundance based on the spectral index method. In particular, we identified several differentially abundant proteins ranging from the glycolytic pathway to the methionine and SAM biosynthetic pathways (i.e., phosphofructokinase, aldose, MS, SAMS, and SAHH) and one enzyme from the BPA biosynthetic pathway (4′OMT). The increased abundance of several enzymes within a specific pathway suggests a coordinated effort by the cell to increase flux through this pathway. The same proteins were identified to be differentially abundant in a duplicate proteomics experiment with our *E. californica* cell line performed using a different subculture, demonstrating the reproducibility of these results. The increased abundance of enzymes associated with the methionine, SAM, and BPA biosynthetic pathways was

also observed in a recent transcript profiling[9] and a proteomic study[25] with fungal-elicited *P. somniferum* suspension cultures, as discussed below.

SAM is a universal methyl donor in biological reactions and is required in 6 steps of the BPA biosynthetic pathway (e.g., 6OMT, CNMT, and 4′OMT shown in Figure 1). Interestingly, using transcript profiling and proteomic studies, Zulak et al.[9,25] observed increased levels of either the transcript (e.g., MS, SAMS, and SAHH) or protein of several SAM biosynthetic enzymes (e.g., SAMS) in fungal-elicited opium poppy cultures, further supporting our findings. SAMS levels were also increased in tobacco cultures treated with methyl jasmonate,[58,59] suggesting that the increased levels of methionine and SAM biosynthesis may be common in several plant species with elicitation and may be important for secondary metabolite production.

In our study, the levels of one enzyme catalyzing early steps in the BPA biosynthetic pathway (4′OMT) were also increased. Zulak et al.[25] observed the increased abundance of other enzymes in the BPA biosynthetic pathway, including 6OMT using 2-D gel proteomics and increased abundance of 6OMT, CNMT, CYP80B3, 4′OMT, and BBE through Western blotting. The differential induction of at least 6 genes coding for enzymes from the BPA pathway (e.g., TYDC, NCS, 6OMT, CNMT, CYP80B1, 4′OMT, and BBE shown in Figure 1) were also observed in fungal-elicited *P. somniferum* cultures.[9,60]

In addition to proteins from the methionine, SAM, and BPA pathways, we observed the following differentially abundant proteins in elicited poppy cultures: stress-related proteins (e.g., glutathione-*S*-transferase), proteasome components, glycolytic enzymes (e.g., fructose-bisphosphate aldolase, phosphofructokinase), and amino acid metabolism (glutamate synthase), which is in agreement with results of Zulak et al.[9,25]

The list of differentially abundant proteins observed in this current study and that of Zulak et al.[25] overlap even though these proteomic studies were conducted using a different proteomic platform, a different but related poppy species (*E. californica* versus *P. sominiferum*), and a different elicitor (i.e., fungal versus yeast elicitor). Hence, the validity of our proteomic findings is supported and suggests that the differentially abundant proteins identified are consistent with the metabolic changes associated with either elicitation or high-alkaloid producing conditions.

Conclusions

Our proteomic study identified approximately 650 proteins and several enzymes from primary and secondary metabolism that were differentially abundant in high-producing cultures of the medicinal plant, *E. californica*. To the best of our knowledge, our analysis of *E. californica*, which relied on an EST database, led to the deepest proteomic coverage of this organism. This paper demonstrates the potential of proteomics to explore and investigate changes in global metabolism associated with enhanced secondary metabolite production.

Abbreviations: BPA, benzophenanthridine alkaloid; DW, dry weight; EST, expressed sequence tags; PYE, purified yeast extract; SAM, (*S*)-adenosyl methionine.

Acknowledgment. This work was supported by Northeastern University (Research and Scholarship Development Fund). We thank Drs. Hwa-Young Cho and Sung-Yong Yoon (Pohang University of Science and Technology) for the *E. californica* suspension cultures and their assistance with the cell culture and alkaloid analysis methods.

Supporting Information Available: Summary of biological and technical replicates analyzed in this proteomic study; complete list of proteins and peptides identified in either control or elicited sample; manual validation of selected MS/MS spectra for proteins from Table 1 (see Nesvizhskii et al.[61] for description of column names and Supplementary Table 1 for description of sample names). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Adhami, V. M.; Aziz, M. H.; Reagan-Shaw, S. R.; Nihal, M.; Mukhtar, H.; Ahmad, N. Sanguinarine causes cell cycle blockade and apoptosis of human prostate carcinoma cells via modulation of cyclin kinase inhibitor-cyclin-cyclin-dependent kinase machinery. *Mol. Cancer Ther.* **2004**, *3* (8), 933–940.
(2) Eley, B. M. Antibacterial agents in the control of supragingival plaque—a review. *Br. Dent. J.* **1999**, *186* (6), 286–296.
(3) Lee, B.; Lee, S.-J.; Park, S.-S.; Kim, S.-K.; Kim, S.-R.; Jung, J.-H.; Kim, W.-J.; Moon, S.-K. Sanguinarine-induced G1-phase arrest of the cell cycle results from increased p27KIP1 expression mediated via activation of the Ras/ERK signaling pathway in vascular smooth muscle cells. *Arch. Biochem. Biophys.* **2008**, *471* (2), 224–231.
(4) Malikova, J.; Zdarilova, A.; Hlobilkova, A.; Ulrichova, J. The effect of chelerythrine on cell growth, apoptosis, and cell cycle in human normal and cancer cells in comparison with sanguinarine. *Cell Biol. Toxicol.* **2006**, *22* (6), 439–453.
(5) Weerasinghe, P.; Hallock, S.; Tang, S. C.; Liepins, A. Role of Bcl-2 family proteins and caspase-3 in sanguinarine-induced bimodal cell death. *Cell Biol. Toxicol.* **2001**, *17* (6), 371–381.
(6) Zhang, Y.-H.; Bhunia, A.; Wan, K. F.; Lee, M. C.; Chan, S.-L.; Yu, V. C. K.; Mok, Y.-K. Chelerythrine and sanguinarine dock at distinct sites on BclXL that are not the classic BH3 binding cleft. *J. Mol. Biol.* **2006**, *364* (3), 536–549.
(7) Ahmad, N.; Gupta, S.; Husain, M. M.; Heiskanen, K. M.; Mukhtar, H. Differential antiproliferative and apoptotic response of sanguinarine for cancer cells versus normal cells. *Clin. Cancer Res.* **2000**, *6* (4), 1524–1528.
(8) Facchini, P. J.; Johnson, A. G.; Poupart, J.; De Luca, V. Uncoupled defense gene expression and antimicrobial alkaloid accumulation in elicited opium poppy cell cultures. *Plant Physiol.* **1996**, *111* (3), 687–697.
(9) Zulak, K. G.; Cornish, A.; Daskalchuk, T. E.; Deyholos, M. K.; Goodenowe, D. B.; Gordon, P. M. K.; Klassen, D.; Pelcher, L. E.; Sensen, C. W.; Facchini, P. J. Gene transcript and metabolite profiling of elicitor-induced opium poppy cell cultures reveals the coordinate regulation of primary and secondary metabolism. *Planta* **2007**, *225* (5), 1085–1106.
(10) Blechert, S.; Brodschelm, W.; Hoelder, S.; Kammerer, L.; Kutchan, T. M.; Mueller, M. J.; Xia, Z.-Q.; Zenk, M. H. The octadecanoic pathway: signal molecules for the regulation of secondary pathways. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (10), 4099–4105.
(11) Gundlach, H.; Mueller, M. J.; Kutchan, T. M.; Zenk, M. H. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89* (6), 2389–2393.
(12) Cho, H.-Y.; Rhee, H. S.; Yoon, S.-Y. H.; Park, J. M. Differential induction of protein expression and benzophenanthridine alkaloid accumulation in *Eschscholtzia californica* suspension cultures by methyl jasmonate and yeast extract. *J. Microbiol. Biotechnol.* **2008**, *18* (2), 255–262.
(13) Cho, H.-Y.; Son, S. Y.; Rhee, H. S.; Yoon, S.-Y. H.; Lee-Parsons, C. W. T.; Park, J. M. Synergistic effects of sequential treatment with methyl jasmonate, salicylic acid and yeast extract on benzophenanthridine alkaloid accumulation and protein expression in *Eschscholtzia californica* suspension cultures. *J. Biotechnol.* **2008**, *135* (1), 117–122.
(14) Odjakova, M.; Hadjiivanova, C. The complexity of pathogen defense in plants. *Bulg. J. Plant Physiol.* **2001**, *27* (1−2), 101–109.
(15) Zhao, J.; Davis, L. C.; Verpoorte, R. Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv.* **2005**, *23* (4), 283–333.
(16) Darvill, A. G.; Albersheim, P. Oligosaccharines. *Sci. Am.* **1985**, *253*, 58–64.
(17) Hahn, M. G.; Albersheim, P. Host-pathogen interactions. XIV. Isolation and partial characterization of an elicitor from yeast extract. *Plant Physiol.* **1978**, *62* (1), 107–111.

(18) Roos, W.; Viehweger, K.; Dordschbal, B.; Schumann, B.; Evers, S.; Steighardt, J.; Schwartze, W. Intracellular pH signals in the induction of secondary pathways: The case of *Eschscholzia californica*. *J. Plant Physiol.* **2006**, *163* (3), 369–381.
(19) Viehweger, K.; Schwartze, W.; Schumann, B.; Lein, W.; Roos, W. The Ga protein controls a pH-dependent signal path to the induction of phytoalexin biosynthesis in *Eschscholzia californica*. *Plant Cell* **2006**, *18* (6), 1510–1523.
(20) Dittrich, H.; Kutchan, T. M. Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88* (22), 9969–9973.
(21) Marques, I. A.; Brodelius, P. E. Elicitor-induced L-tyrosine decarboxylase from plant cell suspension cultures. II. Partial characterization. *Plant Physiol.* **1988**, *88* (1), 52–55.
(22) Tanahashi, T.; Zenk, M. H. Elicitor induction and characterization of microsomal protopine-6-hydroxylase, the central enzyme in benzophenanthridine alkaloid biosynthesis. *Phytochemistry* **1990**, *29* (4), 1113–1122.
(23) Cheng, J.-S.; Yuan, Y.-J. Proteomic analysis reveals the spatial heterogeneity of immobilized *Taxus cuspidata* cells in support matrices. *Proteomics* **2006**, *6* (7), 2199–2207.
(24) Jacobs, D. I.; Gaspari, M.; van der Greef, J.; van der Heijden, R.; Verpoorte, R. Proteome analysis of the medicinal plant *Catharanthus roseus*. *Planta* **2005**, *221* (5), 690–704.
(25) Zulak, K. G.; Khan, M. F.; Alcantara, J.; Schriemer, D. C.; Facchini, P. J. Plant defense responses in opium poppy cell cultures revealed by liquid chromatography-tandem mass spectrometry proteomics. *Mol. Cell. Proteomics* **2009**, *8* (1), 86–98.
(26) Decker, G.; Wanner, G.; Zenk, M. H.; Lottspeich, F. Characterization of proteins in latex of the opium poppy (*Papaver somniferum*) using two-dimensional gel electrophoresis and microsequencing. *Electrophoresis* **2000**, *21* (16), 3500–3516.
(27) Kim, S. I.; Kim, J. Y.; Kim, E. A.; Kwon, K.-H.; Kim, K.-W.; Cho, K.; Lee, J. H.; Nam, M. H.; Yang, D.-C.; Yoo, J. S.; Park, Y. M. Proteome analysis of hairy root from *Panax ginseng* C. A. Meyer using peptide fingerprinting, internal sequencing and expressed sequence tag data. *Proteomics* **2003**, *3* (12), 2379–2392.
(28) Nam, M. H.; Kim, S. I.; Liu, J. R.; Yang, D. C.; Lim, Y. P.; Kwon, K.-H.; Yoo, J. S.; Park, Y. M. Proteomic analysis of Korean ginseng (*Panax ginseng* C.A. Meyer). *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *815* (1−2), 147–155.
(29) Nawrot, R.; Kalinowski, A.; Gozdzicka-Jozefiak, A. Proteomic analysis of *Chelidonium majus* milky sap using two-dimensional gel electrophoresis and tandem mass spectrometry. *Phytochemistry* **2007**, *68* (12), 1612–1622.
(30) Sheoran, I. S.; Ross, A. R. S.; Olson, D. J. H.; Sawhney, V. K. Proteomic analysis of tomato (*Lycopersicon esculentum*) pollen. *J. Exp. Bot.* **2007**, *58* (13), 3525–3535.
(31) Morozova, O.; Hirst, M.; Marra, M. A. Applications of new sequencing technologies for transcriptome analysis. *Annu. Rev. Genomics Hum. Genet.* **2009**, *10*, 135–151.
(32) Carlson, J. E.; Leebens-Mack, J. H.; Wall, P. K.; Zahn, L. M.; Mueller, L. A.; Landherr, L. L.; Hu, Y.; Ilut, D. C.; Arrington, J. M.; Choirean, S.; Becker, A.; Field, D.; Tanksley, S. D.; Ma, H.; de Pamphilis, C. W. EST database for early flower development in California poppy (*Eschscholzia californica* Cham., Papaveraceae) tags over 6000 genes from a basal eudicot. *Plant Mol. Biol.* **2006**, *62* (3), 351–369.
(33) Zenk, M. H. The formation of benzophenanthridine alkaloids. *Pure Appl. Chem.* **1994**, *66* (10/11), 2023–2028.
(34) Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (17), 9390–9395.
(35) Wolff, S.; Otto, A.; Albrecht, D.; Zeng, J. S.; Buettner, K.; Glueckmann, M.; Hecker, M.; Becher, D. Gel-free and gel-based proteomics in *Bacillus subtilis*. A comparative study. *Mol. Cell. Proteomics* **2006**, *5* (7), 1183–1192.
(36) Wienkoop, S.; Glinski, M.; Tanaka, N.; Tolstikov, V.; Fiehn, O.; Weckwerth, W. Linking protein fractionation with multidimensional monolithic reversed-phase peptide chromatography/mass spectrometry enhances protein identification from complex mixtures even in the presence of abundant proteins. *Rapid Commun. Mass Spectrom.* **2004**, *18* (6), 643–650.
(37) Natera, S. H. A.; Ford, K. L.; Cassin, A. M.; Patterson, J. H.; Newbigin, E. J.; Bacic, A. Analysis of the *Oryza sativa* plasma membrane proteome using combined protein and peptide fractionation approaches in conjunction with mass spectrometry. *J. Proteome Res.* **2008**, *7* (3), 1159–1187.
(38) Larrainzar, E.; Wienkoop, S.; Weckwerth, W.; Ladrera, R.; Arrese-Igor, C.; Gonzalez, E. M. *Medicago truncatula* root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. *Plant Physiol.* **2007**, *144* (3), 1495–1507.
(39) Park, J.-J.; Yoon, S.-Y.; Cho, H.; Son, S.; Rhee, H.; Park, J. Patterns of protein expression upon adding sugar and elicitor to the cell culture of *Eschscholtzia californica*. *Plant Cell, Tissue Organ Cult.* **2006**, *86* (2), 257–269.
(40) Cho, H.-Y.; Lee-Parsons, C. W. T.; Yoon, S.-Y. H.; Rhee, H. S.; Park, J. M. Enhanced benzophenanthridine alkaloid production and protein expression with combined elicitor in *Eschscholtzia californica* suspension cultures. *Biotechnol. Lett.* **2007**, *29* (12), 2001–2005.
(41) Linsmaier, E. M.; Skoog, F. Organic growth factor requirements of tobacco tissue cultures. *Physiol. Plant.* **1965**, *18* (1), 100–127.
(42) Phillips, J. P.; Bates, D.; Feuer, H.; Thyagarajan, B. S. *Organic Electronic Spectral Data*; Wiley & Sons: New York, 1984, Vol. 20.; p 1040.
(43) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72* (1−2), 248–254.
(44) Iseli, C.; Jongeneel, C. V.; Bucher, P. ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* **1999**, 138–148.
(45) Rhee, S. Y.; Beavis, W.; Berardini, T. Z.; Chen, G.; Dixon, D.; Doyle, A.; Garcia-Hernandez, M.; Huala, E.; Lander, G.; Montoya, M.; Miller, N.; Mueller, L. A.; Mundodi, S.; Reiser, L.; Tacklind, J.; Weems, D. C.; Wu, Y.; Xu, I.; Yoo, D.; Yoon, J.; Zhang, P. The Arabidopsis Information Resource (TAIR): a model organism database providing a centralized, curated gateway to Arabidopsis biology, research materials and community. *Nucleic Acids Res.* **2003**, *31* (1), 224–228.
(46) Tuskan, G. A.; DiFazio, S.; Jansson, S.; Bohlmann, J.; Grigoriev, I.; Hellsten, U.; Putnam, N.; Ralph, S.; Rombauts, S.; Salamov, A.; Schein, J.; Sterck, L.; Aerts, A.; Bhalerao, R. R.; Bhalerao, R. P.; Blaudez, D.; Boerjan, W.; Brun, A.; Brunner, A.; Busov, V.; Campbell, M.; Carlson, J.; Chalot, M.; Chapman, J.; Chen, G. L.; Cooper, D.; Coutinho, P. M.; Couturier, J.; Covert, S.; Cronk, Q.; Cunningham, R.; Davis, J.; Degroeve, S.; Dejardin, A.; dePamphilis, C.; Detter, J.; Dirks, B.; Dubchak, I.; Duplessis, S.; Ehlting, J.; Ellis, B.; Gendler, K.; Goodstein, D.; Gribskov, M.; Grimwood, J.; Groover, A.; Gunter, L.; Hamberger, B.; Heinze, B.; Helariutta, Y.; Henrissat, B.; Holligan, D.; Holt, R.; Huang, W.; Islam-Faridi, N.; Jones, S.; Jones-Rhoades, M.; Jorgensen, R.; Joshi, C.; Kangasjarvi, J.; Karlsson, J.; Kelleher, C.; Kirkpatrick, R.; Kirst, M.; Kohler, A.; Kalluri, U.; Larimer, F.; Leebens-Mack, J.; Leple, J. C.; Locascio, P.; Lou, Y.; Lucas, S.; Martin, F.; Montanini, B.; Napoli, C.; Nelson, D. R.; Nelson, C.; Nieminen, K.; Nilsson, O.; Pereda, V.; Peter, G.; Philippe, R.; Pilate, G.; Poliakov, A.; Razumovskaya, J.; Richardson, P.; Rinaldi, C.; Ritland, K.; Rouze, P.; Ryaboy, D.; Schmutz, J.; Schrader, J.; Segerman, B.; Shin, H.; Siddiqui, A.; Sterky, F.; Terry, A.; Tsai, C. J.; Uberbacher, E.; Unneberg, P.; Vahala, J.; Wall, K.; Wessler, S.; Yang, G.; Yin, T.; Douglas, C.; Marra, M.; Sandberg, G.; Van de Peer, Y.; Rokhsar, D. The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* **2006**, *313* (5793), 1596–1604.
(47) Ouyang, S.; Zhu, W.; Hamilton, J.; Lin, H.; Campbell, M.; Childs, K.; Thibaud-Nissen, F.; Malek, R. L.; Lee, Y.; Zheng, L.; Orvis, J.; Haas, B.; Wortman, J.; Buell, C. R. The TIGR Rice Genome Annotation Resource: improvements and new features. *Nucleic Acids Res.* **2007**, *35* (suppl_1), D883–887.
(48) Velasco, R.; Zharkikh, A.; Troggio, M.; Cartwright, D. A.; Cestaro, A.; Pruss, D.; Pindo, M.; FitzGerald, L. M.; Vezzulli, S.; Reid, J.; Malacarne, G.; Iliev, D.; Coppola, G.; Wardell, B.; Micheletti, D.; Macalma, T.; Facci, M.; Mitchell, J. T.; Perazzolli, M.; Eldredge, G.; Gatto, P.; Oyzerski, R.; Moretto, M.; Gutin, N.; Stefanini, M.; Chen, Y.; Segala, C.; Davenport, C.; DemattlAdA , L.; Mraz, A.; Battilana, J.; Stormo, K.; Costa, F.; Tao, Q.; Si-Ammour, A.; Harkins, T.; Lackey, A.; Perbost, C.; Taillon, B.; Stella, A.; Solovyev, V.; Fawcett, J. A.; Sterck, L.; Vandepoele, K.; Grando, S. M.; Toppo, S.; Moser, C.; Lanchbury, J.; Bogden, R.; Skolnick, M.; Sgaramella, V.; Bhatnagar, S. K.; Fontana, P.; Gutin, A.; Van de Peer, Y.; Salamini, F.; Viola, R. A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. *PLoS One* **2007**, *2* (12), e1326.
(49) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215* (3), 403–410.
(50) Li, L.; Stoeckert, C. J., Jr.; Roos, D. S. OrthoMCL: identification of ortholog groups for eukaryotic genomes. *Genome Res.* **2003**, *13* (9), 2178–2189.

(51) Rauch, A.; Bellew, M.; Eng, J.; Fitzgibbon, M.; Holzman, T.; Hussey, P.; Igra, M.; Maclean, B.; Lin, C. W.; Detter, A.; Fang, R.; Faca, V.; Gafken, P.; Zhang, H.; Whitaker, J.; States, D.; Hanash, S.; Paulovich, A.; McIntosh, M. W. Computational Proteomics Analysis System (CPAS): An extensible, open-source analytic system for evaluating and publishing proteomic data and high throughput biological experiments. *J. Proteome Res.* **2006**, *5* (1), 112–121.

(52) Elias, J. E.; Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. *Nat. Methods* **2007**, *4* (3), 207–214.

(53) Liu, H.; Sadygov, R. G.; Yates, J. R., III. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **2004**, *76* (14), 4193–4201.

(54) Sadygov, R. G.; Cociorva, D.; Yates, J. R., III. Large-scale database searching using tandem mass spectra: looking up the answer in the back of the book. *Nat. Methods* **2004**, *1* (3), 195–202.

(55) Fu, X.; Gharib, S. A.; Green, P. S.; Aitken, M. L.; Frazer, D. A.; Park, D. R.; Vaisar, T.; Heinecke, J. W. Spectral index for assessment of differential protein expression in shotgun proteomics. *J. Proteome Res.* **2008**, *7* (3), 845–854.

(56) Lemenager, D.; Ouelhazi, L.; Mahroug, S.; Veau, B.; St-Pierre, B.; Rideau, M.; Aguirreolea, J.; Burlat, V.; Clastre, M. Purification, molecular cloning, and cell-specific gene expression of the alkaloid-accumulation associated protein CrPS in *Catharanthus roseus*. *J. Exp. Bot.* **2005**, *56* (414), 1221–1228.

(57) Ounaroon, A.; Decker, G.; Schmidt, J.; Lottspeich, F.; Kutchan, T. M. (R, S)-reticuline 7-O-methyltransferase and (R, S)-norcoclaurine 6-O-methyltransferase of *Papaver somniferum*—cDNA cloning and characterization of methyl transfer enzymes of alkaloid biosynthesis in opium poppy. *Plant J.* **2003**, *36* (6), 808–819.

(58) Goossens, A.; Hakkinen Suvi, T.; Laakso, I.; Seppanen-Laakso, T.; Biondi, S.; De Sutter, V.; Lammertyn, F.; Nuutila Anna, M.; Soderlund, H.; Zabeau, M.; Inze, D.; Oksman-Caldentey, K.-M. A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (14), 8595–8600.

(59) Imanishi, S.; Hashizume, K.; Nakakita, M.; Kojima, H.; Matsubayashi, Y.; Hashimoto, T.; Sakagami, Y.; Yamada, Y.; Nakamura, K. Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures. *Plant Mol. Biol.* **1998**, *38* (6), 1101–1111.

(60) Facchini, P. J.; Park, S.-U. Developmental and inducible accumulation of gene transcripts involved in alkaloid biosynthesis in opium poppy. *Phytochemistry* **2003**, *64* (1), 177–186.

(61) Nesvizhskii, A. I.; Keller, A.; Kolker, E.; Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* **2003**, *75* (17), 4646–4658.

PR1000412