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ORIGINAL PAPER

Proteomic analysis of cell suspension cultures of *Boesenbergia rotunda* induced by phenylalanine: identification of proteins involved in flavonoid and phenylpropanoid biosynthesis pathways

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Abstract *Boesenbergia rotunda* belongs to the Zingiberaceae family. It is widely found throughout Southeast Asia and is commonly used as a food ingredient and in folk medicine. Extracts from this plant contain a number of important bioactive compounds such as boesenbergin, cardamonin, pinostrobin, pinocembrin, panduratin A and 4-hydroxypanduratin A. These compounds have been shown to exhibit anti-HIV protease, anti-dengue NS2B/NS3 protease, antibacterial, antifungal, anti-inflammatory, anticancer, and antioxidant activity. Here we report the use of proteomic approaches to identify proteins that may be involved in the biosynthesis of these compounds. Protein expressions of *B. rotunda* suspension cultures for phenylalanine-treated and normal callus were compared by two-dimensional gel electrophoresis. Following image analysis, protein spots whose expressions were found to be regulated were identified using Matrix Assisted Laser Desorption-Ionization tandem mass spectrometry. In all, thirty four proteins were identified. These proteins were categorized into nine functional categories—defence mechanism, protein biosynthesis, metabolism, terpenoid biosynthesis, cell division, cell organization, energy-related, signaling processes and proteins of unknown function. Eleven of the proteins involved in the phenylpropanoid biosynthetic pathway are related to the biosynthesis of cyclohexenyl chalcone derivatives.

Keywords *Boesenbergia rotunda* · Flavonoids · Panduratin A · Proteomics · 4-Hydroxypanduratin A

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Introduction

Boesenbergia rotunda is a small perennial plant belonging to the Zingiberaceae family. It is widely found in South East Asian countries with local names such as Chinese keys, finger root, and temu kunci. It is commonly used as a food ingredient and folk medicine to treat diseases such as aphthous ulcer, stomach discomfort, leucorrhea, dysentery, rheumatism and muscular pain. It has been shown that the primary bioactive compounds of this ginger are boesenbergin, cardamonin, pinostrobin, pinocembrin, panduratin

A and 4-hydroxypanduratin (Jaipetch et al. 1982, 1983; Trakoontivakorn et al. 2001). These compounds exhibit HIV protease inhibition (Tewtrakul et al. 2003a, b; Cheenpracha et al. 2006) and possess antioxidant activity (Sohn et al. 2005; Shindo et al. 2006), antibacterial (Bhamarapravati et al. 2006; Mahady et al. 2006), antifungal (Phongpaichit et al. 2005; Pattaratanawadee et al. 2006; Pompimon et al. 2009), anti-inflammatory (Tuchinda et al. 2002; Tewtrakul et al. 2009), antitumor (Kirana et al. 2003, 2007; Yun et al. 2006) and anti-parasitic activity (Sawangjaroen et al. 2005). Panduratin A and 4-hydroxypanduratin A, have also been shown to inhibit dengue NS2B/NS3 protease (Kiat et al. 2006).

Most pharmaceutically important plant bioactive compounds are flavonoids. Flavonoids are synthesized from phenylalanine (precursor), into cinnamic acid by phenylalanine ammonia lyase (PAL). PAL catalyzes the non-oxidative deamination of phenylalanine into cinnamic acid. Chalcone will then be produced. It is predicted that cyclohexenyl chalcone derivatives (CCD) are produced in the flavonoids biosynthetic pathway. However, the enzymes that are involved in the CCD biosynthetic pathway derived from this main pathway (flavonoids biosynthetic pathway) are not known. Thus, it is the aim of this project to identify differences in protein expression between normal and phenylalanine-treated cell suspensions as these changes may represent proteins that are involved in the CCD biosynthetic pathway.

Materials and methods

Callus induction

Explants of *B. rotunda* were obtained from a field in Temerloh, Pahang, Malaysia. Sprouts were surface sterilized by soaking in 20 % (v/v) Clorox for 20 min and a further one min in 70 % (v/v) ethanol. This was followed by several rinses in sterile distilled water. Meristems were excised and placed on MS (Murashige and Skoog) media supplemented with 1 mg l^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid), 1 mg l^{-1} NAA (Naphthaleneacetic acid), 1 mg l^{-1} IAA (Indoleacetic acid) and 2 % (w/v) Phytagel (Sigma, US) . The pH of the medium was adjusted to 5.8 with hydrochloric acid (HCl) and/or sodium hydroxide (NaOH) prior to autoclaving at 121 °C for 20 min. All cultures were prepared under aseptic conditions and grown at 26 °C under 16 h light: 8 h dark photoperiod conditions with a light intensity of 31.4 µmol m^{-2} s^{-1} provided by a cool fluorescent lamp. Callus induced from meristem that emerged after 3 weeks of culturing were further propagated in MS agar media supplemented with 3 mg l^{-1} 2,4-D. Friable calli were transferred into liquid media supplemented with 2 mg l^{-1} 2,4-D, 1 mg l^{-1} NAA, 1 mg l^{-1} BAP (6-Benzylaminopurine), 5 g l^{-1} maltose, 100 mg l^{-1} malt extract and 1 mg l^{-1} D-biotin for suspension cultures establishment after 1 month of propagation. The suspension cultures were then subcultured every fortnight and sieved through a 425 µm nylon filter to remove big callus clumps.

The culture was then induced with phenylalanine. The determination of optimum inoculum age and phenylalanine concentration was performed as previously described (Tan 2005). Briefly, cultures were harvested on day 10, 14 and 21, filtered and air dried. The mass of five flavonoid compounds were then determined and compared using HPLC. Based on these results, induction with different concentrations of phenylalanine (0, 20 and 40 mg/l) was then performed by mixing with liquid media. The media were renewed in 1:4 old media to new media ratio. Again, the mass of five flavonoid compounds were then determined and compared using HPLC. The growth of the suspension cell culture was then measured through Settle Cell Volume (SCV).

Total protein extraction and precipitation

Protein extraction was performed as previously described (Park et al. 2006; Pan et al. 2010; Wang et al. 2010; Chong et al. 2011; Pavoković et al. 2012). Briefly, extraction buffer was prepared by adding 160 µl of Tris–HCl (pH 9.5, 40 mM), 100 mg Polyvinyl polypyrrolidone (PVPP), 40 µl protease inhibitor mix and 60–63 µl (approximately 1,200 units) of nuclease mix to distilled water. This solution was made up to a final volume of 4 ml and is sufficient for 2 g of suspension culture (Chong et al. 2011). Total protein was extracted from fresh and frozen *B. rotunda* suspension cultures. Approximately 2 g of frozen cells were ground to fine powder in a pre-chilled mortar with a small amount of liquid nitrogen. Extraction buffer was then added and the mixture was ground thoroughly until it was homogenised. 500 µl of homogenate was then aliquoted into a 2 ml microcentrifuge tube, vortexed vigorously for 1 min and sonicated. This was repeated three times. The homogenate was then centrifuged twice at 5,000*g* for 5 min and about 350–450 µl of the supernatant was recovered. Protein extracts were concentrated by Trichloroacetic acid (TCA) precipitation. Briefly, 37.5 % TCA (w/v) with 1 % β-mercaptoethanol (v/v) was added to 400 µl of sample to achieve a final volume of 2 ml. The mixture was then incubated at −20 °C for at least 1 h. This was followed by centrifugation at 13,000*g* (4 °C for 15 min). The resulting protein pellet was washed three times in 80 % acetone (v/v) containing 0.05 % β-mercaptoethanol (v/v) and then air-dried in a laminar flow hood at room temperature. The pellet was then solubilized in isoelectric focusing

buffer (8 M urea, 2 % CHAPS, 0.5 % IPG buffer (GE Healthcare), 1 M Thiourea and trace amounts of Orange G) and incubated at room temperature for at least 30 min. The solubilized protein was then centrifuged (10,000*g* at 4 °C for 20 min) and either used immediately or stored at −80 °C.

2-D gel analysis

Protein concentration was determined using 2-D Quant kit (GE Healthcare) using BSA as standards. First-dimension IEF was performed on an Ettan IPGPhor 3 IEF System (GE Healthcare Bio-Sciences, Uppsala, Sweden) according to the manufacturer's recommendations on 13 cm immobiline drystrips (pH 3–10). Briefly, drystrips were first rehydrated for 16 h with 450 µl of rehydration buffer containing 8 M urea, 2 % CHAPS, 0.5 % IPG Buffer (pH 3–10) and trace amounts of bromophenol blue, and 20 µg of protein sample from plant. The drystrips were focused for a total of 35.5 kVh. Focused IPG strips were equilibrated for 15 min in equilibration buffer (6 M urea, 75 mM Tris–HCl pH 8.8, 29 % glycerol, 2 % SDS and trace amounts of bromophenol blue) containing 1 % DTT. It was then alkylated in the same buffer containing 2.5 % iodoacetamide for a further 15 min. Electrophoresis of reduced and alkylated samples were then performed on 13 cm, 10.0 % SDS-PAGE gels on an IPGphor III electrophoresis platform (GE Healthcare). The gels were run at 50 mA per gel until the bromophenol blue tracking dye reached the bottom of the gel. Following SDS-PAGE, Protein spots were visualized using protocols described in the PlusOne^{TM} Silver staining kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). The complete protocol was followed for analytical gels. For preparative gels, the protocol was modified so that glutaraldehyde was omitted from the sensitization step and formaldehyde omitted from the silver reaction step. Silver-stained gels were scanned (ImageScanner III, GE Healthcare Bio-Sciences, Uppsala, Sweden) and protein profiles compared (Image Master Platinum software 7.0, GE Healthcare Bio-Sciences, Uppsala, Sweden). Gel images were analyzed using the Image Master Platinum software 7.0 (GE Healthcare Bio-Sciences, Uppsala, Sweden). Protein expression between the normal and phenylalanine-treated callus was compared. A total of 4 gels were run for each group (quadruplicates) for gels pH 3–10, and, 6 gels for each group for gels pH 4–7. This was performed to eliminate experimental and biological variations. To further select against variations between individual samples, the selection criteria for spots were very stringent. Firstly, gels were compared within their respective groups and a representative (virtual) gel was generated. For the generation of the virtual gel, only spots present across all gels within the groups were considered. This was performed to eliminate differences due to inter-individual gel variations. The virtual gels were then compared between the different samples to identify differentially expressed proteins. Only spots that were found to be differentially expressed by more than two folds were accepted for further consideration. Statistical analysis of all spots that were found to be differentially expressed was then performed using the student's *t* test. A P value <0.05 was considered as statistically significant.

Protein identification

Protein spots were excised and in-gel digested with trypsin (Promega) for mass spectrometric analysis according to published protocols (Shevchenko et al. 1996; Wilm et al. 1996). Briefly, excised spots were first destained in destaining solution (15 mM potassium ferricyanide/50 mM sodium thiosulphate, 1:1 [v/v]). The spots were then reduced in a solution containing 10 mM DTT/100 mM ammonium bicarbonate for 30 min at 60 °C and alkylated in 55 mM iodoacetamide/100 mM ammonium bicarbonate for 20 min in the dark. The gel pieces were then washed (3× 20 min) in 50 % acetonitrile/ 100 mM ammonium bicarbonate. This was followed by dehydration of the gel pieces with 100 % acetonitrile and drying in a vacuum centrifuge (SpeedVac, Thermo Scientific, Savant DNA 120). Subsequently the dried gel pieces were rehydrated with 25 µl of 7 ng/µl trypsin (Promega trypsin gold) in 50 mM ammonium bicarbonate buffer and digested at 37 °C for 18–20 h. Tryptic peptides were then extracted using 50 % acetonitrile for 15 min, followed by 100 % acetonitrile for 15 min. The extracted solutions were then pooled into a single tube and dried in a SpeedVac concentrator and solubilized with 10 µl of 10 % acetonitrile/ 40 mM ammonium bicarbonate (Shevchenko et al. 1996; Wilm et al. 1996).

Protein identification was essentially performed as previously described (Dahlan et al. 2011). Briefly, extracted peptides were first desalted using ZipTip C18 (Millipore, USA) according to protocols described by the manufacturer. The final elution volume following ZipTip cleanup was 1.5 µl. The peptide samples were then mixed (1:1) with a matrix consisting of a saturated solution of CHCA (α-cyano-4-hydroxycinnamic acid, Sigma) prepared in 50 % 6 ACN/0.1 % TFA. Aliquots of samples (0.7 µl) were spotted onto stainless-steel sample target plates. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass spectrometer (ABI 4800 plus, Applied Biosystems) in the positive ion reflector mode. For precursor ion selection, all fractions were measured in single MS before MS/MS was performed. For MS/MS spectra, the peaks were calibrated by default. The 20 most abundant precursor ions *per* sample were selected for subsequent fragmentation by high energy

CID. The collision energy was set to 1 keV and air was used as the collision gas. The criterion for precursor selection was a minimum S/N of 5. The mass accuracy was within 50 ppm for the mass measurement and within 0.1 Da for CID experiments. The other parameters for searching were of trypsin, 1 missed cleavage, variable modification of carbamidomethyl and oxidation of methionine, peptide charge of 1+, and monoisotopic. For database searches, known contamination peaks such as keratin and autoproteolysis peaks for trypsin were removed before searching. Spectra were processed and analyzed by the Global Protein Server Explorer 3.6 software (Applied Biosystems). This uses an internal MASCOT (Matrix Science, UK) program for matching MS and MS/MS data against database information. The data obtained were screened against plant databases downloaded from the Swiss-Prot/TrEMBL homepage (http://www.expasy.ch/sprot).

Results and discussion

Effect of inoculum age and phenylalanine concentration towards compounds production in *B. rotunda* suspension cultures

The amount of flavonoids was highest in cultures harvested on the 14th day (Table 1). The masses of pinotrobin, alpinetin, 4-hydroxypanduratin A and panduratin A of 14th day cultures were all three digit percent higher than those of the 10th day culture, only pinocembrin recorded a 69 % increase. The largest gain was alpinetin at 694 % increase followed by panduratin A at 586 % increase. Compound masses from 21st day cultures showed a decline of all five compounds when compared to 14th day cultures (Table 1). These results indicated that 14th day cultures gave the highest yield for all the five flavonoids when no fresh medium was used. Hence cultures were harvested on the 14th day for all subsequent investigations to ensure highest possible yield. It was also found that the level of flavonoids increased dose-dependently with increasing phenylalanine concentration. Table 2 is a tabulation of the mean masses obtained. It was apparent that supplementing standard culture media with 40 mg/l of phenylalanine brought about a high yield of most *B. rotunda* flavonoids with an increase of between 188 to over 1,000 % increase is mass yield. Thus, for the proteomics experiments, all cultures were harvested at the 14th day with phenylalanine supplementation at 40 mg/l.

Proteomics analysis

A total of ~1,000 (pH 3–10 2DE gels) and ~2,000 (pH 4–7 gels) individual protein spots were resolved on silver stained 2DE gels. Representative gels are shown in Fig. 1a, b. The gels were found to be highly reproducible with little variation in terms of spot profile and numbers. Following image analysis, 76 proteins were found to be differently expressed. Out of these, 34 were identified by matrix-assisted laser desorption/ ionization tandem time of flight (MALDI TOF/ TOF). Eleven proteins were found to be associated with the phenylpropanoid biosynthesis pathway. Despite the inavailability of *B. rotunda* genome and protein databases, we were able to identify 45 % of the selected protein spots. Thirty-four proteins were identified and they were categorized into nine functional groups which were proteins involved in plant defence mechanism, amino acids and protein synthesis, metabolism, terpenoid biosynthesis, cell division, cytoskeleton (cell organization), energy-related, signaling processes and proteins of unknown function. The identities of these proteins are shown in Supplementary Table 1. From this point forward these proteins will be referred to by their abbreviated names as shown in Supplementary Table 1. Here we discuss the proteins that were found to be differentially expressed under the various functional categories.

Table 1 Effect of inoculum age on five selected flavanoids

Compounds	Compound mass (μg ± SE) and percentage mass change based on 10-day harvest mass as standard				
	Harvest age in days				
	10	14		21	
	Mass	Mass	% Change	Mass	% Change
Pinostrobin	1.374 ± 0.009	6.032 ± 0.029	350.15 ± 4.04	4.149 ± 0.017	209.63 ± 2.27
Pinocembrin	0.789 ± 0.009	1.333 ± 0.021	68.95 ± 2.05	1.317 ± 0.006	66.92 ± 1.24
Alpinetin	0.221 ± 0.005	1.754 ± 0.006	693.67 ± 19.07	1.147 ± 0.010	419.00 ± 13.13
4-hydroxypanduratin A	0.430 ± 0.005	1.800 ± 0.020	318.60 ± 7.24	1.460 ± 0.009	239.53 ± 4.26
Panduratin A	0.042 ± 0.001	0.288 ± 0.023	585.71 ± 22.08	0.255 ± 0.000	507.14 ± 12.67

SE standard error

Table 2 Effect of phenylalanine concentration on five selected flavanoids

Compounds	Mean compound mass (μg/g ± SE) and percentage mass change as based on standard medium C1 (no phenylalanine) under indirect light				
	Concentration of phenylalanine (mg/l)				
	0, C1	20		40	
	Mass	Mass	% Change	Mass	% Change
Pinostrobin	19.902 ± 1.287	72.202 ± 0.037	262.79 ± 4.89	183.773 ± 0.030	827.77 ± 1.67
Pinocembrin	1.892 ± 0.011	10.861 ± 0.024	474.05 ± 3.80	26.038 ± 0.173	1,276.22 ± 15.90
Alpinetin	1.490 ± 0.005	5.260 ± 0.077	253.02 ± 4.55	20.342 ± 0.181	1,265.23 ± 15.50
4-hydroxypanduratin A	0.829 ± 0.004	1.729 ± 0.079	108.56 ± 5.48	2.388 ± 0.034	188.06 ± 3.58
Panduratin A	0.164 ± 0.000	0.309 ± 0.012	88.41 ± 3.43	1.066 ± 0.035	550.00 ± 18.06

SE standard error

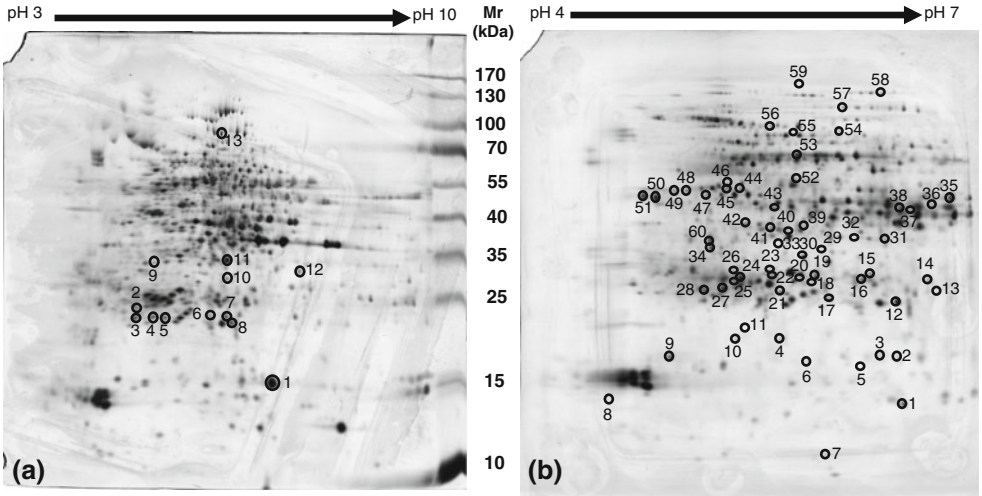

Fig. 1 Two dimensional gel electrophoresis proteome maps of *B. rotunda* explant cultures. Protein spots were selected and classified as shown in Supplementary Table 1. First dimension focusing used 13 cm IPG drystrips with a linear pH. **a** Shows the gel with pH 3–10 while **b** shows the gel with pH 4–7. Each gel was loaded with 20 μg of total protein sample into the drystrips during IEF and focused for 35.5 kVhr. In the second dimension, 10 % of SDS-PAGE gels were used to determine the whole protein profiles. The gels were visualized by silver staining and analyzed by using Image Master Platinum 7 (GE Healthcare)

Defense mechanism

Five proteins involved in plant defense mechanism were found to be differentially expressed (identities shown in Supplementary Table 1). NSH is a plant protein that contains heme groups and thus has high binding, reversible affinity towards oxygen (O_2) and nitric oxide (NO). This protein can be elevated when the plant is exposed to stress conditions. NSH is found ubiquitously in the stems, leaves, roots, seeds and flowers of plants. It is also found in growing, differentiating seedlings (Perazzolli et al. 2004). AP is an antioxidative agent. Genetically, it is highly conserved among proteins of the heme peroxidase family of enzymes which can be found in most life forms. It is involved in oxidation-reduction processes. Under normal or stress (biotic or abiotic) environments, plants produce a quantity of radical oxygen species (ROS) from metabolic processes. This can cause damage at the cellular level. Therefore, antioxidative agents, such as ascorbate peroxidase are needed to control the damage from oxidants. AP has been reported to exhibit apoptotic suppression effect in cells (de Pinto et al. 2006). Two forms of this protein were found to be differentially expressed. HPosJ is categorised in the low genetically conserved cytochrome P450 superfamily. It is involved in oxidative degradation processes and are classified based on electron transfer from NAD(P)H to the catalytic site of the protein (Kim et al. 2004; Sasaki et al. 2010). COM plays a vital role in the production of feruloyl-CoA from caffeoyl-CoA by the methylation of 3-hydroxyl group of caffeoyl-CoA, resulting in an end product, sinapoyl-CoA which a main component in lignin production. The suppression of this

enzyme will decrease the production of lignin in plants especially woody plants (Zhong et al. 2000). This enzyme is also involved in the plant defense mechanism where it triggers the production of defensive compounds, such as phytoalexin (Yang et al. 2004). The fifth identified protein—GP—is an ROS-scavenging enzyme that catalyses the oxidative homeostasis in plants and animals, during biotic or abiotic stress (Faltin et al. 2010).

Amino acid and protein biosynthesis

Four proteins—PSB3, PSB6 (two different spots), MET and PGD—were found to be regulated. Proteins under this category play important roles in plant growth and development. PSB3 and PSB6 (2 different forms) are the subunits of proteosome β-type. Proteosomes are ATP-dependent proteases that degrade intracellular missfolded and short-lived proteins. It is involved in cell cycle regulation of plants and animals. Some proteosomes are involved in the defense mechanism of plants and the immune system in human (Santner and Estelle 2010). MET is an enzyme involved in the biosynthesis of methionine in animals and plants. Methionine is used in protein synthesis, mRNA initiation process and as a regulatory molecule (Hesse et al. 2004; Dancs et al. 2008). PGD is a multiconformation enzyme related to serine biosynthesis, a process before glycolysis in plants and animals. It catalyses the conversion of phosphoglycerate into phosphohydroxypyruvate using NAD/ NADH as a cofactor (Boland and Schubert 1983).

Plant metabolism

This group of proteins is involved in plant metabolism—lipid metabolism, nitrogen metabolism and assimilation, and nucleic acid metabolism. Five proteins were found to be regulated.

Lipid metabolism

GDSL was found to be regulated. GDSL is a multifunctional enzyme within the SGNH hydrolase/ esterase superfamily. It plays a vital role in seed germination, plant development and morphogenesis. GDSL hydrolyses phenolic esters, fatty acyl-ester, lipids or fatty acids and polysaccharides groups (Akoh et al. 2004; Clauss et al. 2008).

Nitrogen metabolism and assimilation

FNR and GS were found to be regulated. GS is a key enzyme in nitrogen metabolism where it equalizes the intracellular nitrogen level (Miflin and Habash 2002). This enzyme also induces photosynthetic activity and growth rate at low nitrogen levels (Fuentes et al. 2001). Increased level of nitrogen in cells will also be equalized by FNR.

Nucleic metabolism-related proteins

HIS and NDK were found to be regulated. HIS is an enzyme that regulates gene expression, specifically chromatin remodeling in nuclear and plant development. It is involved in cell proliferation and differentiation, meristem function, and organogenesis (Servet et al. 2010). NDK is a highly conserved multiple signaling enzyme that catalyzes the phosphorylation of nucleoside diphosphates (NDPs) to equilibrate the amount of nucleoside triphosphate (NTPs) for RNA, DNA and protein biosynthesis. It is also involved in the autophosphorylation of GTP and GTPase stimulating protein in plants. Recent studies have shown that this enzyme is also involved in signaling processes (Hasunuma et al. 2003; Shen et al. 2008).

Terpenoid biosynthesis

Two proteins involved in terpenoid biosynthesis—HMDPR and DXPR—were found to be up-regulated, as shown in Supplementary Table 1. These proteins are involved in the non-mevalonate or 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway in plant plastids, which produce isoprenoids and terpenoids, such as, taxol. DXPR is involved in the biosynthesis of 2-C-methyl-erythritol 4-phosphate and 1-deoxy-D-xylulose 5-phosphate, while HMDPR is involved in the biosynthesis of 1-hydroxy-2-methyl-butenyl 4-diphosphate, isopentenyl diphosphate and dimethylallyl diphosphate (Takahashi et al. 1998; Hasunuma et al. 2008). Furthermore, HMDPR is currently being studied as the target in the production of the cancer drug, taxol, from the plants of the taxus family (Sun et al. 2009).

Cell division

Three proteins involved in cell division were found to be regulated—STK, PP and CYC. STK is an enzyme that is crucial in animal and plant cell division. This enzyme is categorized under the serine/ threonine-protein kinase superfamily which is a biological switch in microtubule phosphorylation, metabolism, gene expression and cell growth and division. It also functions in the defense system by initiating the immune response in animals. Although the proteins are highly homologous between animals and plants, its function, particularly in plants is still poorly understood (Hardie 1999; Reddy and Rajasekharan 2007). CYC is a member of a large family of cyclins that is the regulator for kinases. These proteins mediate the plant cell

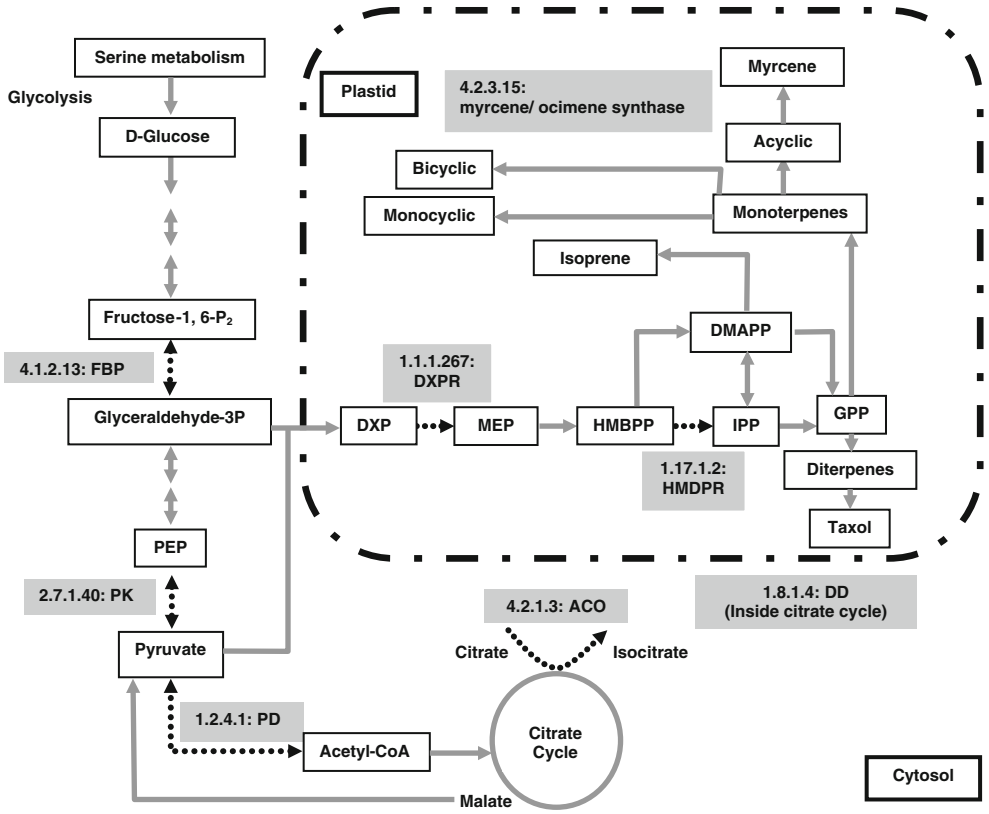

Fig. 2 Glucose metabolism and terpenoid biosynthesis pathway highlighting the observed proteome changes. Proteins that changed in expression are highlighted in *grey*. When phenylalanine was added, the glycolysis pathway was regulated in the cytosol and the products entered the plastid, going through the MEP/ DOXP pathway, for terpenoid biosynthesis to produce monoterpenes, diterpenes and taxol. *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methyl-erythritol 4-phosphate, *HMBPP* 1-hydroxy-2-methyl-butenyl 4-diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate

cycle, cell growth and differentiation, hormonal signals, and meiosis (Cockcroft et al. 2000; d'Erfurth et al. 2010). A predicted protein, PP was shown to be a possible targeting protein from the Xklp2 (TPX2) family. These central spindle regulator family members are kenesin-like proteins that are localized on centrosomes and spindle pole microtubules, before nuclear envelop breakdown and during nuclear assembly (Vos et al. 2008; Evrard et al. 2009).

Cytoskeleton

Two different spots that were regulated were identified as actins. Actins are involved in cytoskeleton building (cell wall), cell division, expansion and intracellular trafficking, all of which are important for plant morphogenesis (Thomas et al. 2009).

Energy-related processes

Seven proteins involved in energy production were found to be regulated as shown in Supplementary Table 1. DD was found to be up-regulated. This enzyme functions in the glycolysis pathway where it is located in the pyruvate-acetyl-CoA reaction. Its' role is in the regulation and maintenance of the production of acetyl-CoA. Glycolysis also involves a few enzymes which are tightly-regulated in order to produce glyceraldehyde-3P from fructose-1, 6-diphosphate by FBP. Glyceraldehyde-3P will be further catalyzed to produce phosphoenolpyruvate (PEP) and then altered to pyruvate by PK. Pyruvate will then be catalyzed by PD to form acetyl-CoA and enter the citrate cycle. ACO in the citric cycle involves catabolism process which produces energy by catalyzing the dehydration of isocitric and citric acid to aconitic acid, in microbs, animals and plants. This process happens in mitocondria. Cytosolic aconitase has the ability to regulate the iron homoestasis in microbs and animals but not in plants (Arnaud et al. 2007). The remaining two up-regulated energy-related proteins were AK and ATPS. AK is one of the enzymes that catalyze conversion of ATP to ADP by releasing a phosphate group as an energy source for plants. After ADP was produced from cellular processes, ATPS will catalyze the

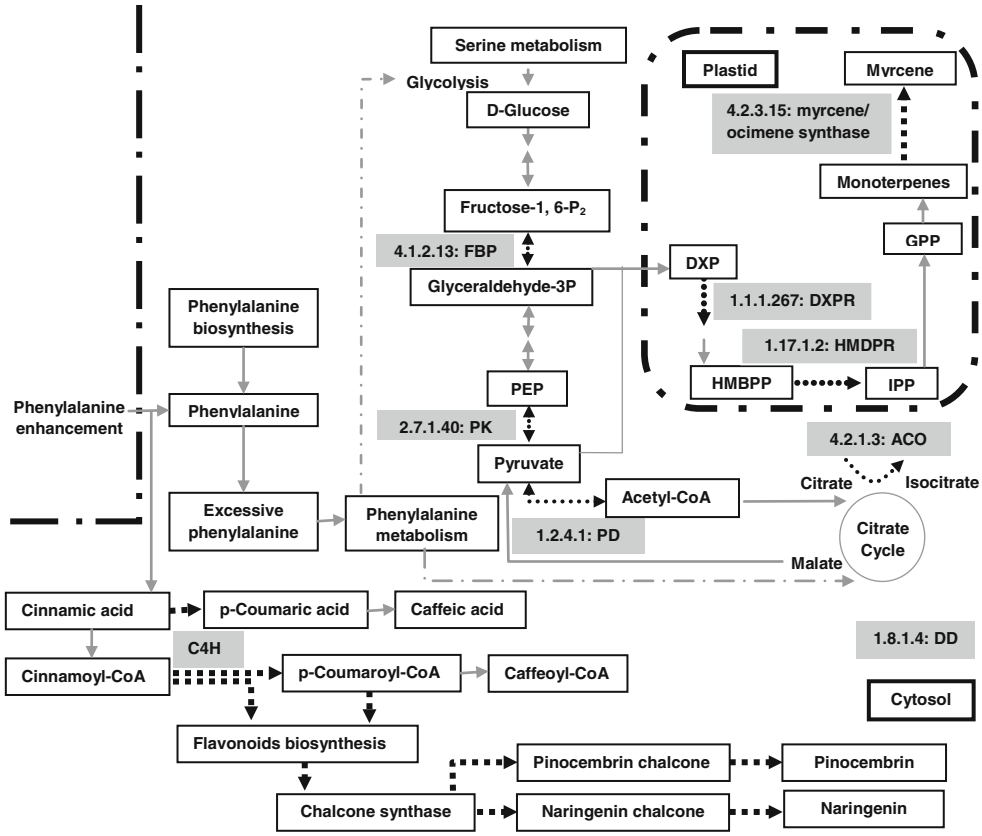

Fig. 3 Proposed general CCD biosynthesis pathway showing proteins that undergo change following induction with phenylalanine. Proteins that changed in expression are highlighted in *grey*. In the glycolysis pathway, glucose from the phenylalanine metabolism was broken down into products that go through the MEP/DOXP pathway, in the plastid, for terpenoid biosynthesis to produce monoterpenes, diterpenes and taxol. Hypothetically, flavonoids biosynthesis end product, pinocembrin chalcone, will interact with the products within the terpenoid biosynthesis pathway, ocimene, to produce CCD. *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2-C-methyl-erythritol 4-phosphate, *HMBPP* 1-hydroxy-2-methyl-butenyl 4-diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *C4H* cinnamate-4-hydroxylase

synthesis of ATP from ADP and this was shown in the identified protein of ATPS catalytic subunit A which was up-regulated after the phenylalanine treatment on the cell suspension (Beke-Somfai et al. 2010).

Signaling and hormone processes

Two signaling processes-related proteins were found to be regulated. These proteins were GTPR and ScD. GTPR is a Ras-related nuclear small GTP protein that is highly conserved in animals, microbes and plants. It is a multifunctional protein with roles in protein and RNA transportation, cell cycle regulation, plant hormone regulation and suppresses mutation (Kim et al. 2001; Lee et al. 2007). ScD is a member of a superfamily of oligomeric enzymes in plants, humans and microbes. ScD has been shown to be mostly an NAD(P)(H)-dependent dehydrogenase/ reductases. It is also a multifunctional protein involved in the mediation of programmed cell death, plant growth hormone biosynthesis or hormones conversion, and nutrient signaling processes. These signals and hormones are molecular switches for plant growth and development (Cheng et al. 2002).

Proteins of unknown function

Four proteins of unknown function were also found to be regulated (as shown in Supplementary Table 1). These proteins were either hypothetical proteins or predicted proteins without any known function.

Regulated proteins involved in the flavonoid and phenylpropanoid biosynthesis pathways

In this study, *B. rotunda* tissue cultures were exposed to an excess of phenylalanine. The cells utilize phenylalanine to produce higher amounts of chalcones. This "overproduction" is expected to affect the expression dynamics of various proteins including those involved in and/or related to the flavonoid and phenylpropanoid biosynthesis

pathways. Among the proteins that were found to be regulated, 11 were known to be related to the flavonoid and phenylpropanoid biosynthesis pathways. These proteins were the hypothetical protein OsJ_02583 (HPosJ), caffeoyl-CoA-O-methyltransferase (COM), fructose biphosphate aldolase (FBP), pyruvate kinase (PK), pyruvate dehydrogenase (PD), dihydrolipoyl dehydrogenase (DD), aconitate hydratase/ aconitase (ACO), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXPR), 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase (HMDPR), ferredoxin-nitrite reductase (FNR) and glutamine synthetase (GS). The locations of these proteins on the flavonoid and phenylpropanoid biosynthesis pathways are shown in Figs. 2 and 3. However, exactly how they affect the production of these compounds remained unclear.

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