Chapter 8

Comparative Proteomic Analysis of *Panax ginseng* C. A. Meyer × *Panax quinquefolius* L. Leaves and Parental Lines

Xiujuan Lei, Zhiqing Wang, Juan Song, Shao Liang,

Lina Yao, Zhifang Hou and Yingping Wang

Additional information is available at the end of the chapter

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Abstract

Heterosis refers to the superior performance of heterozygous F1 hybrid plants with respect to those of their genetically distinct parents. Despite its wide use in crops, heterosis is seldom applied in the Panax genus, and its molecular basis remains unclear. Thus, this study is aimed to obtain hybrid F1s and identify the proteins associated with heterosis. Hybrid F1 plants and parental inbred lines were obtained using the embryo rescue technique, and the proteomes of their leaves were analyzed using two-dimensional gel electrophoresis. A total of 236 differentially expressed proteins were found, among which 84 nonadditive proteins indicated a heterosis pattern in the hybrid. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes analysis revealed that photosynthesis, carbohydrate metabolism, and protein and amino acid synthesis were the most abundant classes of nonadditive proteins. Of the proteins in these categories, 10, 6, and 4 proteins, respectively, showed above high parent expression in the hybrid leaves. These results imply that the increment in photosynthetic capacity, carbohydrate decomposition, and nitrogen fixation might be related to the heterosis of the hybrid biomass and ginsenoside production in the hybrid leaves. This study could provide a basis for hybrid breeding of the Panax genus.

Keywords: hybridization, heterosis, proteomic analysis, morphological traits, ginsenoside, Panax genus

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1. Introduction

Hybridization is a commonly used breeding method for plants because it allows gene transfer and optimization of the best plant features [1]. Wheat, maize, and soybean hybrids have been produced from conventional hybrid breeding, and these hybrids tend to exhibit better traits than those of their parents [2–4]. This phenomenon is called heterosis. *Panax ginseng* C. A. Meyer (Asian ginseng) and *Panax quinquefolius* L. (American ginseng) are two medicinal plants of the Araliaceae ginseng species widely used in Asia because of their purported therapeutic effects on cancer, diabetes, and Parkinson's disease [5–7]. The ability to enhance the hybridization of Panax plants may improve yield and quality, which is an essential goal of the ginseng industry. However, research into the hybrid breeding of each species, especially the molecular basis of heterosis, remains to be clarified, although several hypotheses and models have been proposed, such as locus-specific overdominant effects and genome dominance complementation [8, 9]. Recently, several studies on maize, rice, and Arabidopsis analyzed heterosis at the genome, transcriptome, and proteome levels by applying a variety of molecular tools [10–13].

Protein profiling plays an important role in comparative proteomic analysis, thereby enabling the comparison of proteins across different genotypes, organs, or treatments. Two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS) is commonly used to appoint correlations among the polymorphism of individual protein amounts, indications, and hybrid vigor for agronomic traits [14–17]. However, to date, no report is available on the differences in the proteomic profiling of the leaves of the Panax hybrid and its parents. In this study, we report research into the differences in leaf proteome profiles between the hybrid F1 and its parental inbred lines at the seedling stage. The proteomic analysis in this work reveals that the proteins involved in photosynthesis, carbohydrate metabolism, and protein and amino acid synthesis may be responsible for heterosis in Panax. The molecular insights provided by this study might help in improving understanding of the possible molecular networks involved in Panax heterosis.

2. Materials and methods

2.1. Plant materials and hybridization

All plant materials were collected from Fusong, Jilin province, China, in 2012. Among them, "FX01" (*Panax ginseng*, female parental inbred line) is a landrace with high yield and ginsenoside content, and "ZNYS01" (*Panax quinquefolius*, male parental inbred line) was introduced from America in 1975. Samples were cultivated in an experimental greenhouse of Special Wild Economic Animals and Plants Institute of Chinese Academy of Agricultural Sciences, Changchun, China. To ensure matching flowering periods, seeding stage was regulated for four years for "ZNYS01" and "FX01" on 5 April and 1 May, respectively. When flowering, pollen was collected from "ZNYS01," dried with silica gel, and kept at 4°C until use. Interspecific hybridization was accomplished by selecting the unopened but fully developed flowers of "FX01" emasculating and pollinating them with dried pollen of "ZNYS01," and bagging the

pollinated flowers immediately to prevent contamination from other pollen sources. Abortive seeds were removed 21 days after pollination and stored at 4°C.

2.2. Embryo rescue and plant regeneration

Embryo rescues were performed according to Suputtitada et al. [18] with modifications. Three weeks after pollination, abortive fruits were collected and surfaces were washed with running water for 2 h, and then, seeds were removed and sterilized with 75% ethanol for 1 min, followed by 0.1% $HgCl_2$ treatment for 10 min, and five washes in sterilized water. Ovules were excised from seeds and cultured in Erlenmeyer flasks containing solid embryo induction medium (Murashige and Skoog basal medium supplement with 1.0 mg/L 6-benzyladenine, 2.0 mg/L gibberellin, 0.5% lactalbumin hydrolysate, 3.0% sucrose, and 6.0% agar), with 3–4 ovules/flask [19]. Cultures were kept in the dark at 25 ± 2°C. After 3–4 weeks of culture, developed embryos were excised from ovules and then transferred to White's basal medium [20], containing 1.0 mg/L 6-benzyladenine, 0.2 mg/L α-naphthalene acetic acid, 3.0% sucrose, and 6.0% agar for plant regeneration, and cultured under a 15-/9-h photoperiod with a light intensity of 40 μE/s/min provided by cool white fluorescent light (25 ± 2°C). pH of media was adjusted to 5.8 before autoclaving. Subculture was performed every two months. Samples were collected from the middle leaf of cultured plantlets (palmate compound leaves) after 30 days from the third subculture. For each experiment, 30 plantlets were propagated from one embryo to ensure genetic background consistency.

2.3. Molecular identification

Leaves of the F1 hybrid and parental inbred lines were frozen in liquid nitrogen and ground into fine powders. Genomic DNA was isolated with the cetyltriethylammonium bromide method. DNA was separated on agarose gel and quantified with a DNA/Protein analyzer. PCR amplification of ribosomal external transcribed spacer regions was performed on plant DNA samples. Oligonucleotide primers (**Table 1**) were designed according to Wang et al. [21] and the 20 μL PCR reaction mixture consisted of 10 ng of template DNA, 0.5 μM of each primer, 1 UE × Taq, 2 μL 10 × PCR buffer, and dNTP 0.2 mM. PCR amplification was performed using 1 predenaturation cycle of 4 min at 94°C, 39 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were analyzed on a 1.0% agarose gel stained with ethidium bromide.

2.4. Morphological traits measurement

Plants were carefully harvested and dried in an oven at 60°C for 96 h, for dry weight determination. Six plant traits were characterized including plant height (PH), total leaf number (TLN), leaf area (LA), leaf thickness (LT), leaf fresh weight (LFW), and leaf dry weight (LDW). Statistical analysis of the five traits was performed using F-test.

2.5. Ginsenoside analysis

For ginsenoside analysis, 250 mg of leaf samples (dry weight) was soaked in 50 mL methanol for 1 day and filtered. Filtrate was removed to a 100-mL volumetric flask, and residue was

re-extracted twice. Extracts were concentrated with methanol and diluted to 100 mL. Then, 2 μL samples were analyzed with a Waters XEVE-TQ ultra-high performance liquid chromatography-tandem mass spectrometry system. Separation was achieved using a BEH C18 column (1.7 μm, 50 mm × 2.1 mm). MS/MS analyses were carried out under positive and negative ion modes. Gradient elution and ion source parameters were set as Wang et al. reported [22]. Ginsenosides were detected under negative multiple reaction monitoring mode.

2.6. Protein extraction and separation

Total protein extraction and separation were performed according to Lei et al. [23]. Leaves of hybrid F1 and two parents were removed, and each ~0.5 g was ground to powder under liquid nitrogen and homogenized in 3.0 mL of ice-cold acetone containing 10% (w/v) trichloroacetic acid and 0.07% β-mercaptoethanol. After incubation at -20°C overnight, the mixture was centrifuged at 15,000×*g* at 4°C for 15 min. The upper fraction was removed, and precipitate was collected and washed three times with 3 mL of ice-cold acetone containing 0.07% β-mercaptoethanol. Each washing was followed by centrifugation as described above. All obtained precipitates were air-dried at 4°C and dissolved in sample rehydration buffer containing 7 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid, 1% phenylmethanesulfonyl fluoride, and 1% protein inhibitors. After dissolution, sample protein solutions were vortexed at 38°C for 30 min and centrifuged at 15,000×*g* at 15°C for 10 min. Protein concentration was measured with a Bradford assay [24].

Before the first dimension [isoelectric focusing (IEF)], 1.5 mg of sample protein (350 μL) was loaded on an immobilized pH gradient dry strip (17 cm, pH 4–7, linear; Bio-Rad) and rehydrated for 15 h. IEF was then performed under the following conditions: 200 V for 1.5 h, 500 V for 1.5 h, 1000 V for 3 h, and 10,000 V for 7 h. IEF was terminated after reaching 70,000 Vh. Gel strips were subsequently equilibrated for 15 min in 5 mL equilibration buffer [75 mM Tris-HCl (pH 8.8), 6 M urea, 2 M thiourea, 30% glycerol, 2% sodium dodecyl sulfate, 0.002% bromophenol blue, and 1% (w/v) dithiothreitol (DTT)] and then soaked again for an additional 15 min with the same buffer but replacing DTT with 2.5% (w/v) iodoacetamide. For the second dimension, the equilibrated strips were then separated with 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sealed with 0.5% (w/v) low temperature agarose. Gels were stained with Coomassie brilliant blue R-250 and destained with a solution containing 10% ethanol and 10% acetic acid. Three biological replicates were carried out for all samples to obtain statistically reliable results. Then, the 2-DE gels were scanned using Image Scanner 6.0 (Amersham Biosciences, location) with a resolution of 300 dpi and analyzed on the PDQuestTM 2-DE analysis Software Version 8.01 (Bio-Rad). Protein expression was estimated by the spot percent volume (vol.%), a value normalized as a percent of the total volume of all gel spots present. Percent volumes can be used to correct the variability caused by sample loading, gel staining, and destaining. Fold-changes in protein expression were calculated according to spot percent volumes, and only spots with more than twofold quantitative variation (increase/decrease) in three replicates and statistically significant when calculated by ANOVA ($p < 0.05$) were considered significantly differentially expressed proteins.

2.7. Protein MS analysis and classification

Gel digestion was performed according to Deng et al. [25] with modifications. Protein spots with significant differences in abundance were manually excised from gels. Peptide MS and MS/MS analyses were carried out on an ABI–5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, location). General MS parameters were as follows: laser, 200 Hz (UV, 355 nm); acceleration voltage, 2 kV; scans per laser spot, 2500–3000 times; mass range from 800 to 4000 kDa; eight most intense peaks were selected on each mass spectrum for further MS/MS analysis.

Data were acquired in a positive MS reflector by using a CalMix5 standard to calibrate the instrument (ABI–5800 Calibration Mixture). The following parameters were used as follows: one allowed missed cleavage site; fixed modifications of carbamidomethyl; variable modifications of oxidization; 100 ppm for precursor ion tolerance and 0.3 Da for fragment tolerance. Both MS and MS/MS data were integrated and processed by using GPS Explorer V3.6 software (Applied Biosystems) with default parameters.

Protein identification was performed by searching Viridiplantae sequences in the nonredundant National Center for Biotechnology Information (NCBI) database and ginseng Expressed Sequence Tags database using the GPS-Mascot V 2.4 search engine (www.matrixscience.com Matrix Science Ltd., London, UK). Only proteins with Mascot scores >75 based on 95% or greater confidence intervals were considered identified. Protein functions were classified using Gene Ontology (GO) annotation according to their biological processes and molecular functions (http://geneontology.org/). When no GO annotation was available, protein classification was based on literature retrieval and closely related homologous sequences.

3. Results

3.1. Plant regeneration and molecular identification

One major difficulty in interspecific hybridization is the embryogenic abortion of hybrids [26]. To overcome this problem, the embryo rescue technique was used as depicted in the "Methods," and the embryo germination rate was influenced by basal medium, hormones, and the embryonic developmental stages. **Figure 1a–d** shows "FX01 × ZNYS01" and the parent plants regenerated from immature embryos; both growth and reproduction of the hybrid plants showed the highest speed and stability. Although "FX01 × ZNYS01" displayed significant differences in trait with their parents, identification at the molecular level was considered essential. Thus, single-nucleotide polymorphism molecular markers in the ribosomal external transcribed spacer region were adopted to identify such differences. Two primers P1 and P2 were specific to *P. quinquefolius* and *P. ginseng*, respectively. Another primer P3 was used as the corresponding reverse primer of P1 and P2 (**Table 1**). The combination of three primers generated different fragment patterns for the hybrid F1s and their parents (**Figure 2**). "FX01" produced specific 388-bp bands, whereas "ZNYS01" yielded specific 501-bp bands. Two bands were detected when the DNA of "FX01 × ZNYS01" was used.

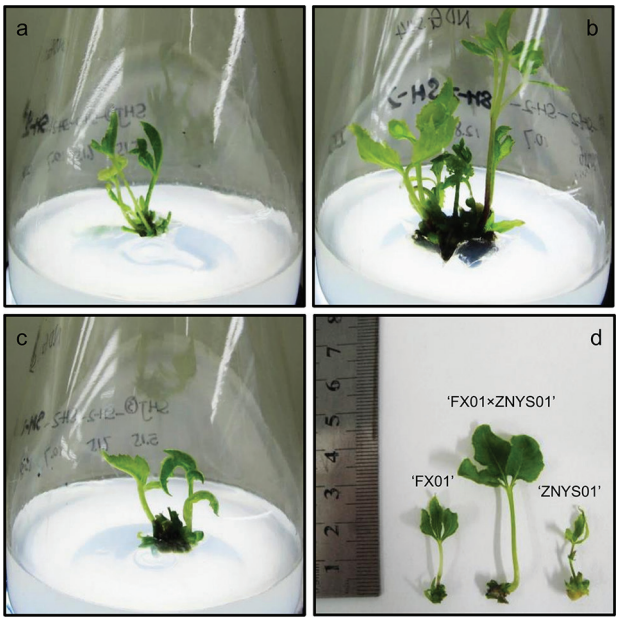

Figure 1. Hybrid F1 plants and parental inbred lines differentiated from embryos and morphology. Cultured (a) "FX01," (b) "FX01 × ZNYS01," and (c) "ZNYS01." (d) The leaf trait of plant height and leaf area in "FX01 × ZNYS01" was significantly different from that in the parents.

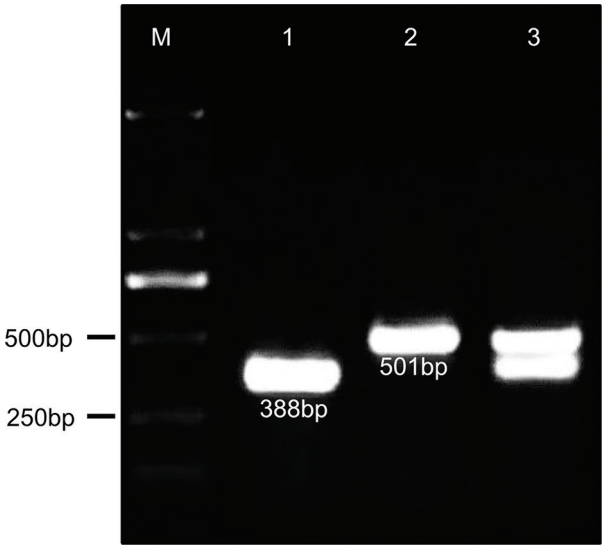

Figure 2. Multiplex allele-specific PCR products using the DNA extracted from the leaves of the hybrid and its parental inbred lines. Lane M: 2000 bp DNA marker; Lane 1: "FX01"; Lane 2: "ZNYS01"; and Lane 3: "FX01 × ZNYS01".

Primer name	Nucleotide sequence (5′–3′)
P1	GTGTTGGCATAGTGTACGTTA
P2	AGAGCAGTAAGCCTTGGAAAAT
P3	AGACAAGCATATGACTACTGGCAGG

Table 1. Oligonucleotide sequences of the primers used.

3.2. Morphological traits and ginsenoside measurement

Hybrid plants often perform better than their parents; this is a phenomenon called heterosis. This better performance manifests as increased growth speed, yield, or vigor of hybrid F1 plants compared with the average levels of traits in their parents. In this study, six plant traits, namely, PH, TLN, LA, LT, LFW, and LDW, were measured. Statistical analysis revealed the significant differences in morphological traits between the hybrid and parental inbred lines (**Table 2**). Ginsenoside is the major effective component of Panax; thus, ginsenoside production was measured as another important heterosis factor. The quantified ginsenosides and related data are listed in **Table 3**. The results on both the total ginsenosides and 20(S)-Rg2 indicate that "FX01 × ZNYS01" F1 exhibited the highest yield and showed a strong transgressive inheritance. The other seven ginsenosides also displayed increased mid-parent advantage.

SV[a]	DF[b]	PH (cm)	TLN	LT (mm)	LA (cm^2)	LFW (mg)	LDW (mg)
Genotype	2	8.04**	102.4**	3.89×10^{-2} NS[c]	1.41**	22.58**	0.66**
Error	15	0.28	4.82	3.67×10^{-2}	0.18	2.3	0.05

[a] SV, source of variance.

[b] DF, degree of freedom.

[c] NS, nonsignificant at the 0.05 probability level.
** Significant at the 0.01 probability level.

Table 2. Mean squares of variance analysis for the different characteristics of the hybrid and its parental inbred lines.

3.3. 2-DE separation of the leaf proteins of the hybrid and its parental inbred lines

The protein expression profiles of the leaves from the hybrid F1 and parental inbred lines were obtained by 2-DE. Means of 679 ± 21, 869 ± 32, and 987 ± 16 (mean ± standard deviation; $n = 3$) spots per gels were detected in "FX01," "FX01 × ZNYS01," and "ZNYS01," respectively (**Figure 3**). The spot intensities on each replicate gel were normalized with PDQuest software to compensate for the non-expression-related variations in spot intensity. The 2-DE map of the leaf proteins of the hybrid F1 was used as a reference map for comparison with the proteins of the parental inbred lines. After normalization, the average protein spot intensities of the three replicate gels per genotype were compared between "FX01 × ZNYS01" and the parental inbred lines. A total of 236 protein spots were present in significantly different quantities across the three genotypes.

Ginsenoside	"FX01" (mg/g)	"FX01 × ZNYS01" (mg/g)	"ZNYS01" (mg/g)
Rg1[&]	22.62[a] ± 1.65	2.98[b] ± 0.21	0.61[c] ± 0.07
Re[&]	56.69[a] ± 1.13	51.41[b] ± 2.36	26.78[c] ± 2.28
20(S)-Rg2[&]	1.16[c] ± 0.09	7.38[a] ± 0.63	2.98[b] ± 0.13
Rb1[&]	5.43[a] ± 0.32	5.49[a] ± 0.45	1.12[b] ± 0.02
Rb2[&]	3.47[c] ± 0.49	6.88[b] ± 0.71	8.97[a] ± 0.57
Rb3[&]	0.41[c] ± 0.07	16.52[b] ± 0.39	30.39[a] ± 1.29
Rc[&]	3.52[b] ± 0.41	4.66[a] ± 0.22	4.01[a] ± 0.19
Rd[&]	10.21[c] ± 0.83	39.22[b] ± 1.28	40.58[a] ± 3.02
Total yield[#] (mg/g DW)	103.51[c] ± 2.61	134.54[a] ± 1.52	115.44[b] ± 0.98

[&]Means with the different letters in a single line are significantly different according to Tukey's honestly significant difference multiple comparisons with (family error 0.05).
[#]Total yield = (Rg1 + Re + 20(S)-Rg2 + Rb1 + Rb2 + Rb3 + Rc + Rd).

Table 3. Ginsenoside analysis of the hybrid and its parental inbred lines.

3.4. Identification of nonadditively accumulated proteins in hybrid

The target of this study was to identify proteins in the leaf proteome of hybrid "FX01 × ZNYS01" that accumulated significantly different traits from those of the mid-parent level of the parental inbred lines. Only these so-called nonadditive proteins might be associated with heterosis in the leaf of Panax genus. Among the 236 differentially expressed protein spots, 84 (36%) displayed significant heterosis patterns and were identified. The nonadditive proteins are shown in **Figure 3**. These nonadditively accumulated proteins were then categorized on the basis of the system suggested by Stupar and Springer [27] (**Figure 4**). The proteins with significantly higher expression in the hybrid than in the better performing parental line were classified as "above high parent" (++). The proteins with significantly lower expression in the hybrid than in the less performing parent line were named "below low parent" (--). The hybrid proteins that exhibited a significantly higher expression than that of the less performing parent but with no significant difference with that of the better performing parent were classified as "high parent" (+). The hybrid proteins with significantly lower expression than in the better performing parent but with no significant difference in expression with the less performing parent were designated as "low parent" (-). Moreover, the hybrid proteins with significantly higher expression than that in the less performing parent and significantly lower expression than that in the better performing parent were classified as "partial dominance" (±). Among the different heterotic classes, 25 proteins (30% of the nonadditive expressed protein) displayed "above high parent" expression, and 2 proteins (2%) showed "below low parent" expression. Moreover, 14 proteins (17%) revealed "high parent" expression, 12 proteins (14%) demonstrated "low parent" expression, and 31 (37%) of the nonadditive proteins exhibited "partial dominance." Results showed that the class with "partial dominance" expression exhibited the highest degree of expression among the different expression patterns, followed by the "above high parent" class.

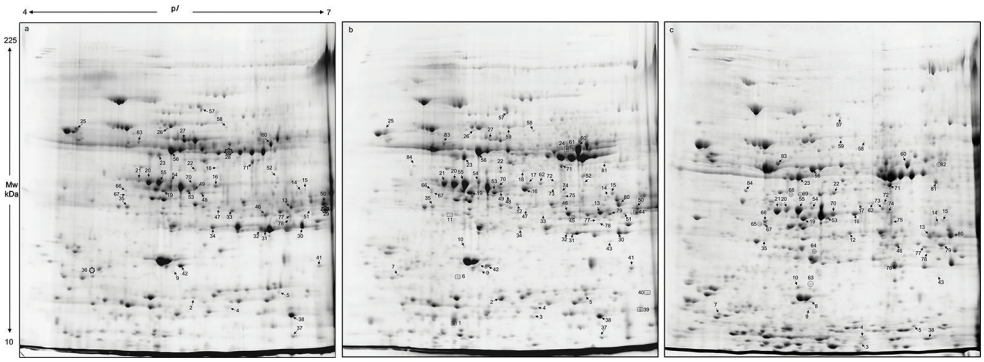

Figure 3. 2-DE profile of proteins extracted from the leaves of the hybrid and its parental inbred lines. (a) "FX01," (b) "FX01 × ZNYS01," and (c) "ZNYS01." The nonadditive accumulation proteins were marked with symbols in gel images. The spots only found in "FX01," "FX01 × ZNYS01," and "ZNYS01" are marked with stars, squares, and circles, respectively.

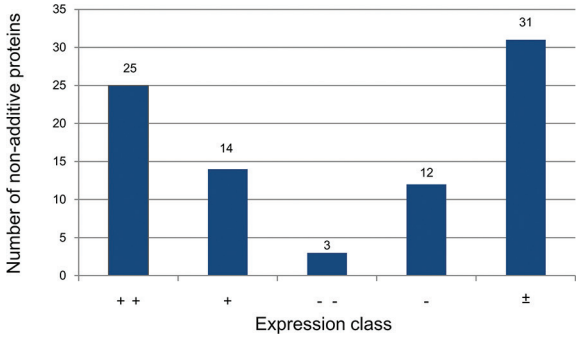

Figure 4. Classification of the nonadditive proteins expressed in the hybrid leaves and its parental inbred lines. "Above high parent expression" (++); "high parent expression" (+); "below low parent expression" (--); "low parent expression" (-); and "partial dominance" (±).

3.5. Biological functional classification

Molecular annotation and GO of these proteins were obtained using blast2GO (http://www.blast2go.com/b2ghome) and NCBI annotation (**Figure 5**). Photosynthesis, carbohydrate metabolism, and protein metabolism were the top three functional categories of the nonadditive expression proteins.

4. Discussion

P. ginseng and *P. quinquefolius* have been traditionally used as precious herbal medicines in Asia for many years. Despite its commercial importance, interspecific hybrid breeding, especially the molecular basis of heterosis, is poorly understood. In this study, comparative

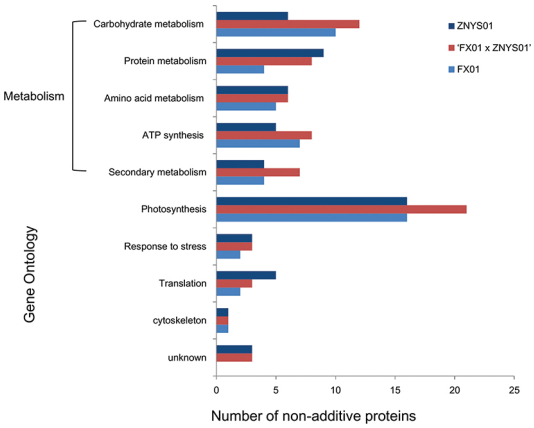

Figure 5. Functional classification of the nonadditive proteins.

proteomic analysis was employed to uncover the proteins related to heterosis in the hybrid plants. Among the 236 differentially expressed proteins, 84 exhibited a nonadditive pattern (**Figure 3**). According to GO annotation, most of these proteins were involved in the metabolism (60%) and photosynthesis (25%) categories. Mohayeji et al. [28] studied the nonadditive protein accumulation in sunflower leaves of hybrid F1 and reported that the main categories of nonadditive proteins belonged to energy metabolism and photosynthesis. Hoecker et al. [29] and Marcon et al. [4] also reported that the first group of nonadditive proteins in maize roots was classified under metabolism. These findings suggest the importance of metabolism and photosynthesis in illuminating the molecular basis of heterosis. In this light, the most important proteins that showed nonadditive patterns are discussed on the basis of their functional categories.

4.1. Photosynthesis-related proteins

Photosynthesis is a highly important biological process in plant growth. A total of 21 nonadditive proteins belonged to photosynthesis, and 10 of these proteins showed a "above high parent" pattern. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; spots 24 and 60) is a major Calvin cycle enzyme composed of eight large and eight small subunits. The high expression of these proteins indicates that the hybrid F1 could fix more CO_2 in the Calvin cycle [30, 31]. Ribulose bisphosphate carboxylase/oxygenase activase (RCA; spots 20, 54, and 55) regulates the activity of Rubisco in a light-dependent manner [32]. In most species, RCA is found in two forms (RCAI and RCAII), both of which can activate Rubisco. Studies showed that RCA physically interacts with Rubisco, thereby catalyzing ATP and facilitating the release of ribulose-1,5-bisphosphate and other tight-binding sugar phosphates from the active site of Rubisco [33, 34]. RCA expression increased, thereby suggesting increased Rubisco activation in the hybrid F1. The light-harvesting chlorophyll a/b binding protein (spot 6) was upregulated, suggesting a greater degree of light harvesting in the hybrid leaves than in the parent leaves. Carbonic anhydrase (CA; spots 2 and 39), which catalyzes the

reversible interconversion of HCO_3^- and CO_2, participates in many biochemical pathways [35]. In photosynthesis, CA supports the CO_2 production for the Rubisco reaction [35]. CA was upregulated in the hybrids, thereby suggesting increased CO_2 delivery in photosynthesis. These results suggest that the enhancement of the proteins involved in photosynthesis may increase the photosynthetic capacity and efficiency of the hybrid F1 and consequently increase biomass production.

4.2. Carbohydrate metabolism-related proteins

Among the several processes involved in metabolism, carbohydrate metabolism is a critical plant process [36]. Glycolysis, the citric acid cycle, and the pentose phosphate pathway (PPP) are chief metabolic pathways for carbohydrate breakdown, which can provide not only energy but also intermediates for various activities [12]. Our results showed that most of the nonadditive proteins involved in carbohydrate metabolic pathways were expressed with "high parent" and "partial dominance" patterns. However, fructose-bisphosphate aldolase 1 (FBA1; spot 13), fructose-bisphosphate aldolase 3 (FBAIII; spot 46), and malate dehydrogenase (MDH; spots 44, 45, and 51) were expressed with an "above high parent" pattern in the "FX01 × ZNYS01" F1. In glycolysis, FBA reversibly catalyzes fructose 1,6-bisphosphate (F1,6BP) into triose phosphates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (GA3-P) [37]. The increased abundance of various isoforms of FBA indicates the enhancement of the glycolytic pathway in the hybrids. MDH reversibly catalyzes the oxidation of malate to oxaloacetate as part of multiple metabolic pathways, including secondary metabolism [38]. However, decreased or no expression of enzymes, such as enolase (spot 28), 6-phosphogluconate dehydrogenase (6-PGDH; spot 81), and some MDHs (spots 29 and 32), occurred in the hybrids. Enolase is an essential phosphopyruvate hydratase in glycolytic catabolism [39], and 6-PGDH is needed for the PPP pathway. The enzyme 6-PGDH catalyzes the reversible oxidative decarboxylation of 6-phosphogluconate to ribulose-5-phosphate and CO_2 [40]. The lower expression of proteins in the hybrid F1 is likely due to the inherence of the parent line traits. In particular, the parents naturally express such traits in lower amounts.

4.3. Protein and amino acid synthesis-related proteins

Protein and amino acid metabolism is crucial for plant growth. High accumulation of amino acid synthesis-related enzymes (spots 1 and 53) in the hybrid is evidence of the higher amino acid production. Ribonuclease-like storage protein (spot 4) and elongation factor Tu (spot 62) also showed an "above high parent" expression pattern. Elongation factor Tu is a protein that promotes the GTP-dependent binding of aminoacyl–tRNA to the A site of ribosomes during biosynthesis in mitochondria [41]. These results reveal that the amino acid synthesis capacity and protein biosynthesis are higher in the hybrid than in its parental inbred lines.

4.4. Two ginsenoside synthesis-related proteins identified in "FX01 × ZNYS01"

Two ginsenoside synthesis-related proteins, namely, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (IspH; spot 61) and uridine diphosphate–glucosyltransferase (UGT;

spot 14), were identified, although 2-DE is limited for the study of low-abundance proteins. IspH is involved in the final step of the methylerythritol phosphate pathway, generating two pathway products isopentyl pyrophosphate and dimethylallyl pyrophosphate, which are needed for ginsenoside synthesis [42]. UGT is assumed to play an important role in producing different ginsenosides by adding monosaccharides to triterpene aglycones [43, 44]. The difference in UGT expression between the F1 hybrid and parents might be an important reason for the difference in ginsenoside monomers produced by the plants.

5. Conclusion

In this study, we demonstrated that "FX01 × ZNYS01" F1 exhibited heterosis in morphology and ginsenoside yield. At the proteome level, 236 differentially expressed proteins were found, among which 36% (84/236) accumulated in a nonadditive pattern. Eighty-four nonadditive proteins were identified, among which 60% (50/84) and 25% (21/84) were involved in the metabolism and photosynthesis categories, respectively. These results indicate that the greater biomass production in the hybrids than in the parental inbred lines is related to the increased carbon fixation, protein synthesis, and carbohydrate metabolism in the former. Enhanced protein and carbohydrate metabolism is important for producing additional organic compounds. Moreover, the increased release of energy for plant growth and photosynthesis

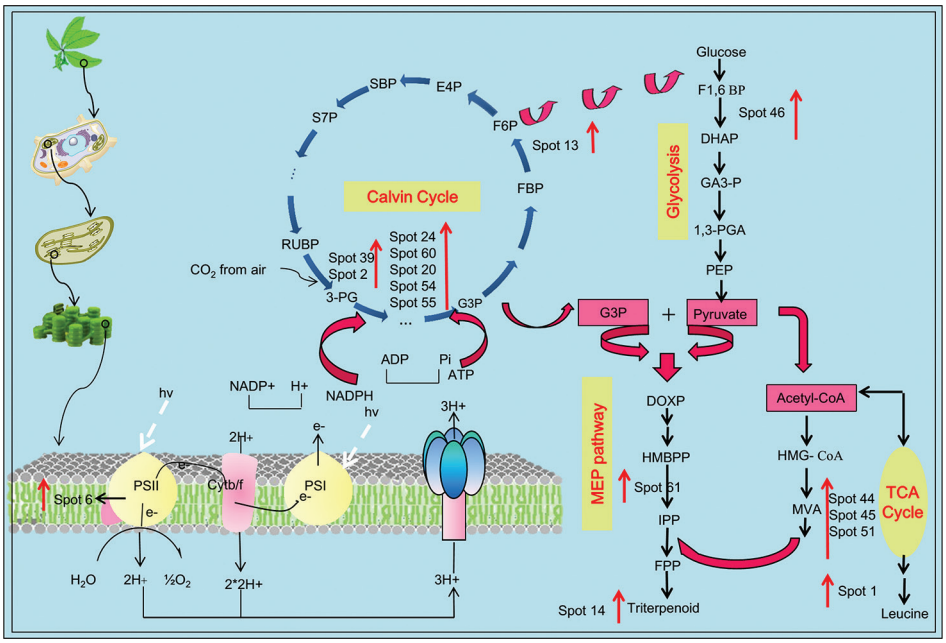

Figure 6. Schematic of heterosis in the hybrid ("FX01 × ZNYS01") from the Kyoto Encyclopedia of Genes and Genomes and UniProt database. The proteins and metabolites are indicated by spot number and red arrows (above high parent).

as the only source of assimilation plays an important role in biomass production. Leaves are the sites of ginsenoside synthesis, and a higher leaf yield implies greater ginsenoside production in the hybrid F1. Furthermore, the enhanced photosynthesis can offer a higher amount of intermediate products for plant secondary metabolism, which then improves ginsenoside yield (**Figure 6**). These data offer a foundation to better understand heterosis in the Panax genus and provide a basis for hybrid breeding.

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Author details

Xiujuan Lei, Zhiqing Wang, Juan Song, Shao Liang, Lina Yao, Zhifang Hou and Yingping Wang*

*Address all correspondence to: yingpingw@126.com

Institute of Special Wild Economic Animal and Plant Science of Chinese Academy of Agricultural Sciences, Changchun, P. R. China

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