Label-free quantitative proteomic analysis of Panax ginseng leaves upon exposure to heat stress

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1. Introduction

Ginseng (Panax ginseng Meyer), a semi-shade perennial plant, has long been used as a medicinal herb in Asia. It has many bioactive compounds, such as saponins, alkaloids, polysacetylene, polysaccharides, free amino acids, and (poly) phenolics and thus has been used to prepare tonics in Korea for thousands of years. This medicinal plant is mainly used to maintain the homeostasis of the body and exhibits diverse pharmacological effects, including improved brain function, antitumor activity, and antiaging effects, and controls blood pressure [1]. It is a representative Korean medicinal plant and is one of the most important trade goods among agricultural products.

Ginseng is a half-shadow and cryophilic plant requiring optimum growth temperature and light intensity of 21–25°C and 10,000–15,000 lux, respectively [2]. Its exposure to 30°C for 1 week results in leaf burning, which inhibits the dark reaction of photosynthesis. In the recent years, the stable production of this particular plant has been affected because of the abiotic stresses, such as extreme temperatures, drought, and high humidity, caused by global climate changes. Especially, high temperature is a major factor that results in reduced productivity and poor crop quality [3].

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The high-temperature stress affects almost every aspect of plants, including development, growth, reproduction, and yield. In addition, the studies on high-temperature stress in plants are important because plants cannot escape heat stress and therefore are forced to invest valuable resources in modifying their metabolism to prevent heat-induced damage [4]. A decrease in the content of ginsenosides, the major bioactive compound in ginseng, has been reported in ginseng subjected to heat stress because of the reduced photosynthesis [5–7].

Proteomic techniques are routinely used to analyze the changes in the abundance pattern of proteins in response to different environmental stresses [8,9]. Recent advancements in the mass spectrometers and data analysis have provided a platform for the large-scale analysis of proteins in ginseng leaves after heat stress treatment. By comparing the proteins, it is possible to identify specific proteins that appear after heat stress treatment at a specific time points and could be used as biomarkers. Currently, the information on the response of ginseng to heat stress, especially at the protein level, is limited. Majority of the studies related to abiotic stress proteomics have focused on salinity, light, salicylic acid (SA), and cold acclimations [10]. Comparative proteome analyses have been carried out on many crops to examine differentially expressed proteins under adverse conditions [11,12]. It has been used to identify the most relevant proteins in a biological process through expression changes under different conditions. Proteomics studies have been carried out frequently to investigate the effect of heat stress in many plants, including spinach [13], rice [14,15], soybean [16], Arabidopsis [17], alfalfa [18], and tomato [19], among others [20]. These studies have led to the identification of several proteins involved in the heat stress response in plants. Some of the key proteins identified include nucleoside diphosphate kinase, guanosine triphosphate (GTP)–binding protein, annexin, and brassinosteroid-insensitive I-kinase domain–interacting protein 114 [20]. Moreover, several other proteins involved in the signaling, transcription regulation, protein synthesis, primary and secondary metabolism, and reactive oxygen species (ROS) detoxification were also found to play pivotal roles in heat stress tolerance in plants [21]. However, there is no report of proteome analysis of ginseng in response to heat stress to date which is required to understand the biology of this semi-shade medicinal plant.

Therefore, here we used a comparative proteomics approach to investigate the effect of heat stress on ginseng. Ginseng plants were exposed to high-temperature (35 °C) stress for 1 and 3 days and were used to test the photosynthetic efficiency at first, followed by proteome analysis. The MaxQuant-based label-free quantitative proteome analysis was carried out to identify heat-modulated proteins in the leaves of ginseng, followed by validation of eight differential proteins by quantitative real-time polymerase chain reaction (qRT-PCR). The results of the present study improve our understanding of heat stress responses in ginseng leaves at the protein level.

2. Materials and methods

2.1. Plant growth and heat stress treatment

Seedlings of the Korean ginseng (P. ginseng Meyer) cultivar ‘Gumpoong’ were purchased from Eumseong, Korea (36° N, 127° E), and grown in the greenhouse (humidity 50 ± 10%) at the National Institute of Horticultural and Herbal Science, Rural Development Administration (RDA). The temperature of the greenhouse was maintained at 20 °C. Four-week-old ginseng seedlings were subjected to high-temperature stress at 35 ± 1 °C with 75% humidity and 5,000-lux light intensity and used for the chlorophyll fluorescence measurements and isolation of total proteins. Ginseng seedlings grown at 25 °C were used as a control.

2.2. Chlorophyll fluorescence measurements

Chlorophyll fluorescence transients were measured using a portable FluorPen FP100 fluorimeter (Photon Systems Instruments, Brno, Czech Republic) [22,23]. All measurements were performed on the adaxial surface of a leaf disc (5 mm diameter) after a dark adaptation period of 15 min during which time leaf disks were prepared using the leaf clips supplied by the manufacturer. OJIPs measurements were repeated at time 0 (25 °C) and then after 3, 6, 9, 12, 24, and 48 h of heat treatment (35 °C) at approximately 5,000-lux light intensity. Recorded data were analyzed using a FluorPen 1.0.4.1 software (Photon Systems Instruments, Brno, Czech Republic). The maximum quantum efficiency of photosystem II (PS II) photochemistry, Fv/Fm (Fv; maximal variable fluorescence, Fm; maximal fluorescence intensity), was calculated from the OJIP analysis.

2.3. qRT-PCR analysis

Total RNA was isolated from 200 mg of ground ginseng leaves using the Easy Spin kit (iNtRON Biotechnology, Korea) according to the manufacturer’s instructions. For the first strand complementary DNA (cDNA) synthesis, reverse transcription was performed using iScriptTM cDNA synthesis kit (Bio-Rad, Korea) according to the manufacturer’s instructions. qRT-PCR was performed with the LightCycler system (Bio-Rad) using SYBR Green Sensimix Plus Master Mix (Bio-Rad) under the following conditions: 15 min at 95 °C; 40 cycles of 30 s at 95 °C, annealing for 30 s for the different primers at 60 °C, 30 s at 72 °C, and then 7 min at 72 °C. Data were subjected to an analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) statistics v. 18 (SPSS Inc., IBM, Armonk, NY, USA). The means were compared using Tukey’s honest significant difference test at p < 0.05 to identify significant differences in levels of gene expression.

2.4. Total protein extraction

Total leaf proteins were isolated as described previously [24,25]. Ginseng leaf powder (1 g) was homogenized in 5 mL of ice-cold Tris-Mg/NaPO4 extraction buffer [0.5 M Tris-HCl (pH 8.3), 2% (v/v) NP-40, 20 mM MgCl2, 2% (v/v) β–mercaptoethanol, and 2% (w/v) polyvinylpolypyrrolidone]. After homogenization, samples were centrifuged at 12,000 × g for 15 min at 4 °C followed by addition of four volumes of 12.5% (w/v) trichloroacetic acid (TCA)/acetone containing 0.07% (v/v) β–mercaptoethanol to the supernatant. After incubation at −20 °C for 1 hr, tubes were centrifuged at 12,000 × g for 10 min at 4 °C, and the pellet thus obtained was washed twice with cold 80% (v/v) acetone in distilled water containing 0.07% (v/v) β–mercaptoethanol. Finally, the pellet was solubilized in 80% acetone and stored at −20 °C until analysis.

2.5. Label-free quantitative proteome analysis

Label-free quantitative proteomic analysis of samples was carried out as described previously [26,27]. In brief, 200 μg of proteins were used for the in-solution trypsin digestion by filter-aided sample preparation (FASP) method [28]. Obtained peptides after FASP were dissolved in Solvent A (water/ACN, 98:2 v/v; 0.1% formic acid) and separated by reversed-phase chromatography using an ultra-high-performance liquid chromatography (UHPLC) Dionex UltiMate 3000 (Thermo Fisher Scientific, USA) instrument [29]. For trapping the sample, the UHPLC instrument was equipped with an Acclaim PepMap 100 trap column (100 μm × 2 cm, nanoViper C18, 5 μm, 100 Å) and subsequently washed with 98% Solvent A for 6 min at a flow rate of 6 μL/min. The sample was continuously
separated on an Acclaim PepMap 100 capillary column (75 μm × 15 cm, nanoViper C18, 3 μm, 100 A) at a flow rate of 400 nl/min. The LC analytical gradient was run with 2% to 35% Solvent B for more than 90 min, then 35% to 95% for more than 10 min, followed by 90% Solvent B for 5 min, and finally 5% Solvent B for 15 min. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was coupled with an electrospray ionization source to the quadrupole-based mass spectrometer QExactive Orbitrap High-Resolution Mass Spectrometer (Thermo Fisher Scientific, USA). Resulting peptides were electrosprayed through a coated silica-emitted tip (PicoTip emitter, New Objective, USA) at an ion spray voltage of 2000 eV. The MS spectra were acquired at a resolution of 70,000 (200 m/z) in a mass range of 350–1,800 m/z. A maximum injection time was set to 100 ms for ion accumulation. Eluted samples were used for MS/MS events (resolution of 17,500), measured in a data-dependent mode for the 10 most abundant peaks (Top10 method), in the high–mass accuracy Orbitrap after ion activation/dissociation with higher energy c-trap dissociation at 27 collision energy in a 100–1,650 m/z mass range [29]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the proteomics identifications [30], partner repository with the data set identifier PXD009124.

2.6. Data processing using MaxQuant software and quantification

The obtained spectra from control and heat-stressed samples were cross-referenced against the in-house—developed RNA sequencing (RNA-seq, PacBio) database (SRA: SUB2796783; containing 135,317 sequences) [31]. Label-free quantification (LFQ) data processing was performed using default precursor mass tolerances set by Andromeda, which were set to 20 ppm for the data processing was performed using default precursor mass tolerances (FDR) that was set to 1% for peptide identifications. A reverse nonsense version of the original database (SRA: SUB2796783; containing 135,317 sequences) [31]. Label-free quantification (LFQ) was conducted within MaxQuant using MaxLFQ [32,33], followed by data analysis using Perseus software (ver. 1.5.8.5, Max Planck Institute of Biochemistry, Martinsried, Germany) [34]. Missing values indicating protein intensities were performed from a normal distribution (width: 0.3, down shift: 1.8) using Perseus software [34]. Hierarchical clustering analysis (HCL), a statistical analysis, was carried out using Perseus software. Multi-sample test (one-way ANOVA), controlled by Benjamini–Hochberg method—based FDR threshold of 0.01, was used to identify the significant differences in the protein abundance during heat stress in ginseng leaves. Functional annotation of the identified proteins was carried out using MapMan and PageMan tools.

3. Results and discussion

3.1. Effects of high-temperature stress on photosynthesis in the seedlings of ginseng

Photosynthesis is a complex process that converts light energy into chemical energy, which is required for the growth and development of plants. Photosynthesis integrates many components of the photosynthetic apparatus, including PS II, which is the most thermal-sensitive component. To measure the activities of the PS II under high-temperature conditions, chlorophyll fluorescence analysis was carried out using an OJIP curve of transient fluorescence [35]. The OJIP measures the polyphasic rise in chlorophyll fluorescence and is a useful method to detect photosynthetic efficiency because of its short measurement time and easy handling under both field and laboratory conditions. The four important levels of chlorophyll fluorescence are distinguished into O, J, I, and P [22]. In the present study, phenotypic changes in leaves were not observed until 48 h at 35°C (Fig. 1A) and the OJIP measurements did not show significant changes until 48 h of heat stress treatment (Fig. 1B). The optimal temperature range for the growth of ginseng is 20–25°C, and high-temperature injury is generally observed at temperatures of 30°C or above for ≥ 5 days [3]. In the preliminary test, we detected severe wilting of ginseng seedlings exposed to 35°C for 5 days. Furthermore, the OJIP patterns of the heat-treated plants were lower than that of the control, and the lowest OJIP transient was identified 48 h after high-temperature treatment.

The chlorophyll fluorescence parameters can be derived from the OJIP analysis, and Fv/Fm value represents the maximal photosynthetic efficiency of PS II [36]. The minimal fluorescence (F0) was measured at 50 μs when all the PS II reaction centers are open, and it is defined as the O step, followed by the J step (at 2 ms) and I step (at 60 ms). The Fv was measured when all the PS II reaction centers are closed—the P step. Several studies have reported that the measurement of OJIP transients is a sensitive and reliable way to detect and quantify heat stresses in PS I and PS II [37,38]. In the present study, the OJIP curves were significantly decreased by heat stress for 48 h. The decrease in the OJIP curves indicates the inhibition of photosynthesis, probably because of the nonfunctioning of PS II [39]. Furthermore, the Fv/Fm values are also used as a stress indicator of chlorophyll fluorescence. We observed a reduction in Fv/F0 values in the leaves of heat-treated plants compared with those in the leaves of the controls (Fig. 1C). The Fv/Fm value of the control was 0.768, whereas that of the high temperature–treated plants ranged from 0.501 to 0.718. In a previous study, high temperature–resistant “Chunpoong” exhibited Fv/Fm value of 0.8 and 0.6, respectively [2]. From these results, it is evident that the high temperature affects the Fv/Fm value due to the inhibition of photosynthesis.

3.2. Transcriptomics changes in response to heat stress

To further validate the sample conditions, we checked the expression of four heat shock proteins (hsp), which are considered as the marker genes for heat stress. These included an 18.2-kDa hsp, a 17.6-kDa hsp, and two 17.4-kDa hsps. The expression pattern of these nine genes was analyzed by first synthesizing cDNAs from the samples that were measured for OJIP. Of these, expression of all the genes increased significantly after heat treatment when compared to that of the controls (Fig. 1D), validating the effect of heat stress on ginseng. After confirmation of the samples, these were used for the large-scale proteome analysis (Fig. S1).

3.3. LFQ proteomics for identification of differentially expressed proteins

For the comparative proteome analysis, a LFQ proteomic approach was used using MaxQuant followed by the data analysis using Perseus software. Complete genome sequence of the ginseng has recently been released [40,41] and was not available when this study was carried out. Unavailability of genome sequence for ginseng was a major bottleneck in the comprehensive identification of ginseng. Therefore, to overcome this problem, we used a database from a large cDNA library of flower, leaf, stem, and root tissues of ginseng, generated by the PacBio isoform sequencing ( Iso-Seq) [31]. This in-house developed RNA-seq (PacBio) database
(SRA: SUB2796783; containing 135,317 sequences and 67.5% unigenes) was used to search the proteins in the MaxQuant analysis. To increase the reliability of protein identification, each sample was run in four biological replicates.

The MaxQuant analysis led to the identification and quantification of 905, 938, and 1,000 proteins in the control, 1-day heat-stressed samples, and 3-day heat-stressed samples, respectively. The Venn diagram showed 724 common proteins among the three samples (Fig. 2A). Among the 905 proteins identified in the control, 59 and 28 proteins were common in 1-day and 3-day heat-stressed samples, respectively. Furthermore, 121 proteins overlapped between 1-day and 3-day heat-stressed samples. The principal component analysis (PCA) led to the separation of all the three samples into different principal components (PCs). The control and heat-stressed samples were separated in PC1, which accounted for 64.9% of the total variation (Fig. 2B). Furthermore, 1-day and 3-day heat-stressed samples were included in PC2, accounting for 12.9% of the total variation (Fig. 2B). These results suggest that heat stress alters more than 60% of the leaf proteome of ginseng, with >10% differences between the 1-day and 3-day heat-stressed samples.

For the identification of heat stress–modulated proteins, multiple ANOVA test, controlled by a Benjamini–Hochberg FDR threshold of 0.01, was performed. The results showed that 847 proteins were significantly modulated in response to heat treatment as compared to control (Table S1 and Fig. 2C). The multiscatterplots were generated using the Perseus software to confirm the reproducibility of the label-free protein quantification between different replicates of the samples (Fig. S2). The Pearson correlation coefficient value of all the replicates of a sample was > 0.9, suggesting a high degree of correlation among the different replicates of a sample.

3.4. Multivariate analysis and functional classification of heat stress–responsive proteins

The HCL of the differentially regulated proteins was carried out to identify the proteins groups with a similar expression pattern.

Fig. 1. (A) Morphology of the leaves after heat stress treatment. (B) OJIP fluorescence transient plotted on a logarithmic time scale. (C) Maximum photochemical efficiency, Fv/Fm, analysis of the same samples. (D) qRT-PCR analysis of heat shock protein genes.
For the HCL, log₂ intensities after Z-score normalization of the data and Euclidean distances between averages were used. The HCL grouped all the differential proteins into five major clusters (Fig. 2D). Cluster 1 included 7 proteins with maximal expression at 1-day high-temperature treatment, with a subsequent decrease in abundance after 3 days. These proteins might be involved in the early perception of heat stress and early heat stress–induced signaling. Clusters 2 and 3 included proteins with increased abundance during heat stress with 74 proteins of Cluster 2 increased specifically after 3 days and 178 proteins of Cluster 3 increased gradually from Days 1 to 3 of heat stress. These proteins might be positive regulators of the heat stress response. Clusters 4 and 5 contained 308 and 270 proteins, respectively, with decreased abundance in heat-stressed samples. The proteins of Cluster 4 decreased in abundance gradually, whereas those of Cluster 5 maintained abundance until 1 day and decreased in abundance after 3 days (Fig. 2E).

The MapMan and PageMan analyses were carried out for the functional annotation of the identified proteins. The PageMan analysis showed that both 1-day and 3-day heat-stressed samples exhibited an elevation in the level of proteins involved in stress, protein synthesis, and protein degradation, whereas the down-regulated proteins were related to the major CHO metabolism, cell wall, lipid metabolism, secondary metabolism, tetrapyrrole and 5 contained 308 and 270 proteins, respectively, with decreased abundance in heat-stressed samples. The proteins of Cluster 4 decreased in abundance gradually, whereas those of Cluster 5 maintained abundance until 1 day and decreased in abundance after 3 days (Fig. 2E).

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synthesis, and nucleotide metabolism (Fig. 3). Furthermore, the primary metabolism overview of the MapMan analysis showed an overall downregulation of metabolism, especially light reactions, photosynthesis, starch metabolism, and sucrose metabolism (Fig. 4A). In the defense response overview, the proteins involved in abiotic stress, heat shock, signaling, and proteolysis were increased in abundance, especially in the 3-day heat-stressed sample (Fig. 4B). Most of the proteins related to abiotic stress were hsps and chaperones. However, some other proteins, such as calnexin1 (CNX1), calreticulin 1b, HSP40 heat shock N-terminal domain-containing protein (ATERDJ3A), aldolase-type TIM barrel family protein (HSA32), shepherd (SHD), and chaperone protein htpG family protein (CR88) were also identified. In addition, several transcription factors such as argonaute-4 (AGO4), ankyrin repeat-containing 2B (AKR2B), and proliferating cell nuclear antigen 2 were also identified which were differentially modulated in response to heat stress (Table S2).

Expression pattern of some of the identified differentially modulated proteins was further validated by qRT-PCR (Fig. S3). We selected 11 differentially modulated proteins, of which five including Rab GTPase homolog, GTP-binding, general regulatory factor (GRF), COP9-signalosome, and inositol-3-phosphate synthase showed similar increased expression as observed by the proteome analysis. Inositol-3-phosphate synthase is an enzyme involved in inositol synthesis which functions as a general ROS scavenger [42]. Therefore, the expression of this gene is expected to increase to relieve the oxidative stress caused by heat stress. Six proteins were found to have reduced expression patterns, and they include Rab GTPase homolog, RAS superfamily, G-box regulating factor, cysteine-rich receptor-like protein kinase (RLK), serine carboxypeptidase and cytochrome P450 3A21. The levels of carboxypeptidase, which is a protease enzyme that hydrolyzes peptide bonds, and cytochrome P450 were decreased. Furthermore, carboxypeptidase is associated with resistance to biotic and oxidative stress, and cytochrome P450, a monooxygenase belonging to the heme-containing protein group, catalyzes various oxidative reactions [43,44]. The decrease in the expression of these two genes is presumed to be due to the fact that ginseng does not exhibit a proper resistance reaction because of its sensitivity to heat stress.

3.5. Inhibition of photosynthesis by heat stress

The reduction in photosynthesis by high temperatures is a well-known phenomenon in the tropical and subtropical plants [45]. In particular, many studies have reported that PS II is the most heat-

Fig. 4. Functional annotation of the identified proteins using MapMan program in (A) primary metabolism and (B) defense response overview. Color codes represent abundance patterns of the proteins, where red and green colors, respectively, indicate increased and decreased abundance of the proteins.

ABA, abscisic acid; bZIP, basic region leucine zipper; DOF, DNA-binding with one finger; ERF, ethylene responsive factor; hsp, heat shock protein; JA, jasmonic acid; MAPK, mitogen activated protein kinase; MYB, myeloblast; OPP, oxidative pentose phosphate; PR, pathogenesis-related; SA, salicylic acid; TCA, trichloroacetic acid.
labile component of the photosynthetic apparatus and that the electron transport capacity is unaffected at temperatures, where CO₂ fixation is inhibited [45]. As expected, the proteins involved in the light reaction and Calvin cycle decreased in abundance in the heat-stressed ginseng (Fig. 5). There was a decrease in PS I subunits, such as photosystem I reaction center subunit N (PSAN) and photosystem I reaction center subunit F (PSAF); PS II subunits, such as photosystem II subunit R (PSBR) and photosystem II subunit Q (PSBQ); PS II reaction center proteins, such as photosystem II CP43 protein (PSBC) and photosystem II reaction center protein G (PSBG); low PS II accumulation 3 (LPA3), which is required to assemble the subunit of PS II; and photosynthetic electron transfer proteins, such as apocytochrome f (PETA) and cytochrome b6/f-complex iron-sulfur subunit (PETC). Contrarily, there was an increase in the level of early light-induced protein 1. It belongs to the light-harvesting complexes and was temporarily accumulated under bright-light condition. Furthermore, it has been reported to perform protective function [46].

3.6. Downregulation of primary metabolism by heat stress

Heat stress treatment led to the overall downregulation of metabolism-related proteins, particularly those involved in the carbohydrate, lipid, amino acid and secondary metabolism. In addition, proteins related to the cell wall modification and degradation were also reduced in response to heat stress (Fig. 3). Previous studies have also shown similar results of downregulation of primary and secondary metabolism in response to heat stress, probably because of the deactivation of metabolic enzymes at higher temperatures [20]. Here, some of the important enzymes such as starch synthase, alpha-amylase, starch-branching enzyme 2.1 and 2.2, adenosine diphosphate (ADP) glucose pyrophosphorylase family protein, uridine-5’-triphosphate (UTP):galactose uridylyltransferase, ADP glucose pyrophosphorylase-1, and ADP glucose pyrophosphorylase large subunit-1 were strongly inhibited upon heat stress. However, some other proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) C subunit-1 and GAPDH C2 were strongly induced by the heat stress. Increased abundance of GAPDH was also observed in rice leaves and seedlings upon exposure to heat stress [14,47]; however, it was previously speculated that the GAPDH subunits are generally increased in the heat-tolerant cultivars and decreased in the heat-sensitive cultivars [20]. These results contradict the case of ginseng which is a heat-sensitive plant, meanwhile increased abundance of GAPDH here also suggests that this plant tries to cope with increasing temperatures, at least partially.

3.7. Accumulation of hsp in response to heat stress

The Hsps and chaperones are well-known stress response proteins, which are responsible for protein folding, assembly, translocation, and degradation. These proteins play a crucial role in protecting the plants by protein refolding under stress conditions [48]. Currently, five major families of Hsps/chaperones have been identified in plants, including Hsp70 (DnaK) family, chaperonins (GroEL and Hsp60), Hsp90 family, and small Hsp (sHsp) family. Among these, the level of several Hsps, such as Hsp70, Hsp60, Hsp90, casein lytic protease B3 (CLPB3), AtHsp101, sHsp, and AtHsp22, increased in the leaves of ginseng under heat stress. The level of other proteins related to Hsps and chaperones, such as CNX1, calreticulin 1b, ATERDJ3A, HSA32, SHD, and CR88, were also increased in response to heat stress. Furthermore, CNX and calreticulin are calcium-binding molecular chaperones, especially calreticulin, which is known to increases in response to stress and functions as a positive regulator of stress response in plants [49,50]. ATERDJ3A is a J-domain protein that functions as a cochaperone of the Hsp70 machinery. It is a key regulator of the function of Hsp70s and regulates it by stimulating

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**Fig. 5.** Mapping of identified proteins in respective metabolic pathways to visualize the overall changes in the metabolism in response to heat stress on ginseng leaves. Abundance pattern of the proteins is shown by the green-yellow-red color scheme. DHA, dehydroascorbate; MDHA, monodehydroascorbate; NADP, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate.
the activity of Hsp70s ATPase [51]. HAS32 has been reported to be induced in heat stress–treated Arabidopsis and was found to be related to the maintenance of acquired thermotolerance [52]. The SHD gene encodes the hsp90-like protein of ER, and CR88 encodes the chloroplast-targeted 90-kDa Hsp90 [53,54]. Modulation of these chaperones in the leaves of ginseng suggests that the protein folding machinery in the leaves of ginseng functions appropriately in response to heat stress, similar to that of other plants. Therefore, the susceptibility of the ginseng plant to heat stress might be because of other yet unknown factors.

3.8. Role of G-protein and calcium signaling during heat stress

The guanine nucleotide–binding proteins (G-proteins) and 14-3-3 proteins are involved in signal transduction in plants. The levels of several G-proteins, such as RAS-related protein (RABB1C) and guanosine nucleotide diphosphate dissociation inhibitor 1 (AtGDI1), which affect various physiological processes in cells, were found to be increased after the high-temperature treatment. Furthermore, RABB1C is a member of a small GTPases superfamily and plays an important role in membrane trafficking [55]. In addition, the expression of RAS superfamily of proteins, such as AtRABH1c, AtRAB7B, and AtSAR2, was also found to be increased in response to heat stress in ginseng (Fig. 6A). The small GTPases, which constitute the RAS superfamily, control the GTP/GDP exchange reaction to regulate the commitment of transport vesicles in specific receptor membranes. AtGDI1 regulates most of the Rab proteins by inhibiting the dissociation of GDP from them and the subsequent binding of GTP [56]. In the high temperature treated leaves of ginseng, the expression of GRF2 and GRF6 was increased. Furthermore, GRF6 encodes lambda isoform of the 14-3-3 family. The GRF is associated with the G box DNA/protein complex and encodes the 14-3-3 gene family. GRF6 interacts with ascorbate coxidase (APX3) and ankyrin repeat–containing protein (AKR2), suggesting its role in mediating oxidative metabolism in response to stress [57]. Furthermore, APX1 and AKR2B also increased, which might have correlations with the oxidative metabolism response to heat stress with GRF. These results indicate a central role of G-protein signaling in the heat stress response in ginseng. However, the information on the role of G-protein signaling during heat stress is limited, which needs to be confirmed by further studies.

The recent studies have demonstrated that calcium protects plants from oxidative damage induced by heat stress. When the temperature increases, the calcium channels open, and calcium is transported into the cell as a response to heat stress [58]. However, levels of most of the proteins associated with calcium signaling, such as calcium-dependent lipid-binding protein, calcium ion–binding receptor, and calcium-sensing receptor decreased in high temperature–treated ginseng leaves. Only the plasma membrane–associated Ca2+-binding proteins (PCaPs), which might act as molecular switches in Ca2+ signaling mediated by phosphatidylinositol phosphates (PtdInsPs), were upregulated [59]. In addition, H2O2 is broadly accepted as the major ROS in plant cells and can trigger increases in cytosolic Ca2+ by activating the Ca2+-permeable channels [60]. This suggests that the flux of calcium ions is required to prevent or repair oxidative damage caused by heat stress. Many studies have demonstrated that the increase in the production of ROS by various environmental stress is related to phosphatidic acid (PA) [61,62]. PA is involved in various cellular processes, including mitogenic signaling, vesicular trafficking, and oxidative burst and targets protein kinase (APDPK1), protein phosphatase (AB1), phosphoenolpyruvate carboxylase (PEPC), and calcium-dependent protein kinase in plants [63]. In ginseng, there was an increase in the level of PEPC, phospholipase D alpha 2, and cysteine-rich receptor-like protein kinase 2 (CRK2), whereas the level of PECK, phospholipase C (PLC), and phospholipase D alpha 1 and delta decreased (Fig. 6B). CRKs involved in disease resistance and cell death are involved in ROS signaling, redox regulation, and response to oxidative stress at the transcriptional level [64]. PLC, which is involved in the PA pathway, performs important functions as a competitor of the classic second messengers Ca2+ and cAMP in response to various abiotic stresses. All these proteins are expected to be associated with PA, and PA appears to be involved in the multifunction stress signal. It might be interpreted that the resistance mechanism is not expressed properly because ginseng is susceptible to high temperatures.
3.9. Downregulation of phytohormones signaling by heat stress

Several proteins involved in phytohormone signaling have been shown to be differentially modulated in ginseng leaves upon heat stress. The level of zeaxanthin epoxidase, which is involved in abscisic acid (ABA) biosynthesis, decreased after heat treatment, suggesting the downregulation of ABA signaling. However, previous studies have reported that ABA induces thermotolerance in maize [65] and bromegrass [66], which is contrary to the findings of the present study.

Furthermore, COP9-signalosome complex subunit 5b (CSN5B) is a multiprotein complex involved in photomorphogenesis and is associated with protein degradation and auxin and jasmonic acid (JA) response [67,68]. In the present study, there was a decrease in the level of CSN, indicating the inhibition of JA and auxin response. Moreover, the level of lipoxygenase, allene oxide synthase, and allene oxide cyclase 3, associated with the biosynthesis of JA, also decreased, and the level of probable aldo-keto reductase 4 and cullin-associated NEDD8-dissociated protein 1 (CAND1) associated with the auxin pathways also changed. These results indicate that the key proteins of ABA, JA, and auxin signaling are downregulated. As these phytohormones are positive regulators of heat stress tolerance in plants, downregulation of these proteins indicates that the susceptibility of ginseng to heat stress might be due to the inactivation of hormone signaling. The modulation of these hormone signaling pathways in ginseng can lead to enhanced heat stress tolerance in future.

3.10. Heat-responsive proteins involved in ginsenoside biosynthesis

Ginsenoside is a major bioactive compound of ginseng and is one of the most important factors affecting the quality of ginseng as a food. As mentioned earlier, ginsenoside content of ginseng is known to decrease under high-temperature stress. The biosynthesis of ginsenosides involves three steps: 2, 3-Oxidosqualene is cyclized, hydroxylated, and glucosylated [69]. The results of protein identification showed that the content of isopentenyl diphasphate isomerase 1 (IPP1), lupeol synthase 2 (LUP2), and glycosyltransferases involved in ginsenoside synthesis was altered in response to heat stress, indicating that the heat stress can cause changes in ginsenoside production (Fig. 6C). Furthermore, IPP1, a precursor involved in the synthesis of squalene (precursor of ginsenoside), decreased in 1 day after heat treatment and recovered in 3 days, whereas LUP2, which converts 2, 3-oxidosqualene to lupeol during the synthesis of ginsenoside from squalene, increased 1 day after heat treatment and decreased in 3 days. Many glycosyltransferases have been identified, which synthesize various ginsenosides by attaching one or several monosaccharides to triterpene aglycones. Among these, the level of uridine diphosphate (UDP)-glycosyltransferase superfamily of protein and UDP-glycosyltransferase superfamily protein (HYR1) was increased and/or decreased, whereas the level of dolichyl-diphosphooligosaccharide-protein glycosyltransferase (a 48-kDa subunit family protein) increased after the heat stress treatment. These results indicate that heat stress directly affects the abundance of proteins involved in the biosynthesis of ginsenoside, thus altering the production of ginsenosides.

3.11. Activation of RNA transport and ribosome biogenesis upon heat stress

The transport of RNA and the expression of ribosome synthesis–related, heat shock, and ubiquitin-mediated proteolysis–related proteins were increased (Fig. 6D). This indicates that some of the proteins are degraded because of heat stress, whereas some are newly synthesized. Therefore, synthesis and degradation of proteins must be considered during the adaptation to environmental stress. Heat is a major stress factor that induces protein degradation, leading to aging and death [70]. Among the newly generated proteins are the Hsps, which are the molecular chaperones involved in protein folding, assembly, translocation, and degradation during normal growth and stress conditions. Many molecular chaperones are originally identified as Hsps, and these protect plants against stress by reestablishing the normal protein conformation [48].

4. Conclusion

We applied a label-free quantitative proteomics approach to investigate the proteomic changes in ginseng leaves upon heat stress, which led to the identification of 847 proteins. The physiological and proteomic data together showed that the heat stress results in the decrease of the photosynthetic efficiency of the leaves of ginseng, especially after 3 days. Moreover, proteins related to photosynthesis and phytohormone signaling were reduced, whereas proteins associated with RNA transport and ribosome biogenesis were activated with an accumulation of Hsps. Decreased modulation of photosynthesis-related proteins and accumulation of Hsps are well-known high-temperature stress responses in plants, and the same was observed for ginseng. Activation of calcium and phytohormone signaling are considered as the positive responses of stress tolerance in plants; however, here we observed a down-regulation of these proteins which could be one of the reasons of ginseng susceptibility to high-temperature stress. The aforementioned protein changes forced to invest valuable resources in modifying their metabolism to avoid damage by heat stress. Taken together, this study provided a global view of heat stress response in ginseng leaves and their possible effects of ginsenoside production.

Conflicts of interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2018.09.005.

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