Communication

Proteomic Analysis of Ginseng (Panax ginseng C. A. Meyer) Fluid Proteins under Salt Stress

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Abstract: Ginseng (Panax ginseng C. A. Meyer), due to its relatively longer cultivation time, is often exposed to environmental stresses such as heat, salt, and drought. Particularly, salt-stress-derived oxidative damages greatly affect photosynthetic efficiency and consequently cause reduction of growth, development, and yield of ginseng. Thus, efforts have been made to understand the salt-stress-induced changes at proteome levels; however, the overall understanding of possible salt-responsive proteins in ginseng is still limited because of their low-abundance. A growing body of evidence suggests that plants secrete various low-abundant proteins localized in the intra- and extracellular spaces during stress conditions, and those proteins may have a key role for salt tolerance. Therefore, here, we report the ginseng fluids proteome to identify the potential salt-responsive proteins. This approach led to the identification of 261 secreted fluid proteins, and functional categorization revealed that identified proteins were majorly associated with photosynthesis, protein synthesis, cell binding, and various metabolisms. Further validation using qRT-PCR analysis showed similar expression profiles of heat-shock protein 70, glycosyl hydrolase 17, and fructose-bisphosphate aldolase class-I with proteome results. Overall, ginseng fluid proteomic analysis successfully identified the potential salt-responsive proteins, which might be helpful for understanding of salt-tolerance mechanisms in ginseng.

Keywords: fluids proteome; panax ginseng; salt stress; shotgun proteomics

1. Introduction

Ginseng (Panax ginseng C. A. Meyer) has been a backbone of the Korean and Chinese traditional medical systems and has long been used to promote health and for the treatment of various diseases [1]. Therefore, extensive research has been conducted worldwide to understand the medicinal properties of ginseng and to increase the concentration of its active compounds [2]. Recently, a few studies have reported that the associated medicinal benefits of ginseng are attributed to saponins, protopanaxadiols, and protopanaxatriols, collectively known as ginsenosides [3]. These active ingredients have been demonstrated to have various therapeutic potentials as anti-cancer and anti-inflammatory agents and in the treatment of neurological disorders and cardiovascular disease, among others [4]. Owing to its pharmacological and medicinal properties, the annual demand for ginseng is increasing. However, ginseng requires, over the long period that is cultivated for growth, placement under artificial shade for 4 to 6 years for sufficient accumulation of ginsenosides, during which it is exposed to multiple stressors including salt, which can significantly limits its productivity [5,6].
The optimum soil salinity for ginseng growth is relatively low (0.5 dS/m), and, thus, ginseng is considered a salt-sensitive crop [7]. Therefore, the stable production of ginseng is difficult because of salt-induced damage caused by rapid changes in climate and the use of high amounts of chemical fertilizers. When soil salinity is high, crops are severely damaged because of the increased osmotic potential and increased concentrations of ions and toxins [8]. High salinity also leads to the inhibition of various enzyme activities involved in metabolic pathways, the reduction in carbon-use efficacy, and the breakdown of protein/membrane structures [9–11]. Recently, several comparative proteomic analyses have been performed to identify and understand the potential salt-responsive proteins in response to salt stress in ginseng [12,13]. Those studies have shown that various proteins involved in photosynthesis, detoxification, and defense, among others, were differentially modulated during salt-stress conditions in ginseng [12,13]. Nonetheless, those studies ended up with the repeated identification of the high-abundant proteins, as well as the understanding of the molecular mechanism and role of salt-stress-responsive proteins in ginseng is currently elusive.

Recently, a few studies suggested that plant fluids containing sap and apoplastic fluid proteins localized in intra- and extracellular spaces were secreted by external stress such as biotic and abiotic stress conditions, and those secreted proteins have an important role for various physiological processes including water and nutrient trafficking, defense, and development, among others [14–16]. Therefore, plant-fluid proteomics has recently become one of the major fields that is defining plant biology during development and various environmental stress conditions [15,17]. The vascular system, through sap and apoplastic fluids, is a transporter of water, nutrients, and developmental and stress signals [14,16]. The major advantage of plant-fluid proteome analysis is that it is free from high-abundance intracellular proteins that hinder the identification of stress-specific markers [15,17]. Therefore, attempts have previously been made to analyze the fluid proteome in Solanum lycopersicum [18], Brassica oleracea [19,20], Glycine max [21], and grapevine [22], among others. In addition, Fernadez-Garcia et al. 2011 reported that salt-stress-responsive proteins were identified through the analysis of the sap proteome of Brassica oleracea, and, in particular, proteins involved in xylem differentiation and ligation under salt-stress conditions were identified as markers of salt stress [23].

In this study, we carried out fluid proteomic analyses through the collection of secreted fluid proteins from ginseng shoots, which are in direct contact with the salts, to decipher the proteome alteration and identify the potential salt-responsive proteins. The cultured fluids were collected after salt-stress treatment at 24, 72, and 120 h and used for the shotgun proteomic analysis. This is the first report of ginseng fluid proteome analysis, which will aid further understandings of salt-stress responses and the identification of potential salt-responsive proteins in ginseng.

2. Materials and Methods

2.1. Plant Materials and Protein Extraction Using Ginseng Fluids and Leaves

The salt-resistant ginseng cultivar “Cheonryang” was obtained from the National Institute of Horticultural and Herbal Science, Rural Development Administration (RDA), Eumseong-gun, South Korea (36° N, 127° E). Five-year-old ginseng plants were grown in a greenhouse at an average temperature of 22.5 ± 2.5 °C and average humidity of 50 ± 10%. Salt stress was induced as described by [13]. For the collection of fluid proteome samples, the cut ends of ginseng shoots were kept in hydroponic solution (5 dS/m of salt concentration, 21 mM KNO₃, 6.1 mM KH₂PO₄, and 6.2 mM MgSO₄·7H₂O) and the culture solution chosen as the culture fluid and harvested at 0, 24, 72, and 120 h, respectively. Sequentially, ginseng leaves were harvested for validation of the abundance profile of the antioxidant enzyme using Western blot analysis.

For the isolation of the total protein from ginseng leaves, we carried out TCA/Acetone precipitation combined with a methanol/chloroform washing method as followed in previous studies [15]. Moreover, for the extraction of fluid proteins from ginseng cultured fluids,
15 mL of cultured fluids were mixed with an equal volume of tris-saturated phenol (15 mL, pH 7.6) and centrifuged at 12,000 × g for 10 min at 4 °C. The phenol phase was transferred to a new centrifuge tube. Then, four volumes of methanol containing 0.1 M ammonium acetate each were added to the phenol phase, mixed thoroughly, and precipitated at −20 °C for 1 h. After centrifugation at 12,000 × g for 5 min at 4 °C, the pellet was washed thrice with methanol containing 0.1 M ammonium acetate and twice with ice-cold 80% (v/v) acetone containing 0.07% (v/v) β-mercaptoethanol. Finally, the pellet was suspended in 80% acetone and stored at −20 °C until further analysis.

2.2. Validation of Physiological Changes Using Ginseng Leaves and Culture Fluids and Western Blot

To determine the photosynthetic efficiency of ginseng leaves during exposure to salt-stress conditions, chlorophyll fluorescence imaging was performed using the saturation pulse method using an image fluorometer, as described previously (Handy FluorCam, Photo System Instruments, Drasov, Czech Republic) [24]. The relative changes of H$_2$O$_2$ in the culture fluids were quantified using luminol (Sigma-Aldrich, St. Louis, MO, USA) and horseradish peroxidase (HRP; Thermo Fisher Scientific, Waltham, MA, USA), as previously described [25]. Stock solutions of luminol (3.4 mg/mL) and HRP (2 mg/mL) were prepared. Culture fluids (35 µL) were mixed with luminal (100 µL) and HRP (15 µL) stock solutions and loaded onto a 96-well plate. The luminescence-detection assay was performed over 30 min with a signal integration time of 1 s using the Glomax® multi-detection system (Promega, Madison, WI, USA). For comparison of H$_2$O$_2$ accumulation in the fluid samples, the chemiluminescence intensities detected at 30 min were used.

In addition, for validation of salt-stress-derived changes of antioxidant enzymes in ginseng leaves, a Western blot analysis was carried out using the superoxide dismutase (SOD) antibody. For production of the SOD antibody, a protein sequence was obtained, and the genes for the respective proteins were cloned in a pQE30 expression vector system, as described in a previous study [26]. Subsequently, for antibody generation, the recombinant protein was purified according to the manufacturer’s instruction (Qiagen, Valencia, CA, USA), and polyclonal antibodies were generated by immunizing the rabbits. Finally, the antibody was then purified using the antigen–antibody interaction method as followed by a previous report [26]. For Western blot analysis, ginseng leaves proteins were resolved on SDS-PAGE and transferred to a PVDF membrane. Membrane was incubated with the SOD antibody (1:667) and anti-rabbit IgG conjugated with horseradish peroxidase (1:6667) as used for primary and secondary antibodies, respectively. Finally, the signal was detected using EzWestLumi plus kit (ATTO Corporation, Tokyo, Japan).

2.3. Shotgun Proteomic Analysis and Data Processing

A shotgun proteomic analysis using fluid protein samples was performed as described previously [27,28]. SDS-PAGE analyses of fluid proteins collected from the shoots of five-year-old ginseng in the ddH$_2$O and the hydroponic solution, respectively, were carried out. Fluid proteins extracted from the five-year-old ginseng cultured with the hydroponic solution loaded on SDS-PAGE gels were cut out into five pieces and subjected to in-gel trypsin digestion (trypsin gold, MS grade, Promega, Madison, WI, USA) for LC-MS/MS analysis [29]. LC-MS/MS analysis was performed using a high-performance liquid chromatograph interfaced with a linear trap quadrupole-orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nano-electrospray ionization (nESI) source and fitted with a fused silica emitter tip (New Objective, MA, USA), as described previously [30]. The acquired MS raw data were analyzed using MaxQuant (ver. 1.5.3.30) [31]. All the raw data were cross-referenced against the RNA-sequencing (RNA-seq) database using 14 tissues, 4 differently aged ginseng roots (SRP 066368, 215,879 sequences), and an RNA-seq (PAC-BIO) database developed in-house (SRA: SUB2796783, containing 135,317 sequences) [32,33]. Furthermore, raw data processing was carried out using the default precursor mass tolerance set by the Andromeda search engine, which is set at 20 ppm for
the first search and 4.5 ppm for the main search. In addition, a maximum of two missed tryptic cleavages was allowed, and the carbamidomethylation of cysteine residues was specified as a fixed modification. For variable modifications, the acetylation of lysine residues and the oxidation of methionine residues were specified. A reverse-nonsense version of the original database was generated and used to determine the FDR, which was set to 1% for peptide identifications [31]. Raw data obtained from MS were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010322 [34]. Functional annotations of the identified proteins were performed using AgriGO v2 web-based software for gene-ontology (GO) enrichment analysis [35] and MapMan for metabolic pathway analysis [36].

2.4. Validation with Quantitative Real-Time PCR (qRT-PCR)

RNAs were extracted from an adventitious root exposed to 150 mM NaCl condition at different time points (0, 24, and 48 h) using an RNA isolation kit (Intron Biotechnology Inc., Seongnam, Korea) according to the manufacturer’s instructions. The total RNA was subjected to reverse transcription using the HelixCrix First-Strand cDNA Synthesis Kit (NanoHelix, Korea). qRT-PCR was performed using the RealHelix™ qPCR kit (NanoHelix, Korea) and SYBR Green on a Bio-Rad CFX96™ thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). The ratios of the transcript levels in the salt-stress-treated adventitious root samples at 0 h to 48 h were compared to the β-actin transcript levels in the corresponding samples. The primer sequences used for the qRT-PCR are listed in Table S1.

3. Results and Discussion

3.1. Salt-Stress-Triggered Accumulation of Reactive Oxygen Species in Ginseng

The osmotic stress caused by high soil salinity leads to the reduction in external water potential and water uptake by plants [37]. Previous studies have shown that osmotic stress, triggered by high salt concentrations, induces the generation of reactive oxygen species (ROS), such as superoxide (O$_{2}^-$), hydroxyl radical (OH), H$_2$O$_2$, and singlet oxygen ($^1$O$_2$), which are known as major inhibitors of plant growth [38]. Moreover, the accumulation of ROS during the stress conditions induced the oxidative damage and resulted in the inhibition of various enzymes related to the photosynthetic activities in ginseng [37]. Therefore, the effects of salt stress on photosynthesis and ROS homeostasis were investigated. First, chlorophyll fluorescence imaging was conducted to determine the photosynthetic activity in the ginseng leaves after exposure to salt stress at 0, 24, 72, and 120 h, followed by the saturation pulse method [39]. The wilting and drying of leaf margins in ginseng plant were among the earliest visible symptoms of the salt stress [12,13]. In this result, we successfully confirmed the decrease of the photosynthetic activity by the measurement of chlorophyll fluorescence at the leaf margin at 120 h as compared with the 0, 24, and 72 h samples (chlorophyll fluorescence changed to red color) (Figure 1A). For measurement of the oxidative burst in the ginseng fluids, H$_2$O$_2$ was detected using a luminescence assay. We successfully observed 114-, 281-, and 633-fold increases of H$_2$O$_2$ in the cultured fluid after treatment with salt stress at 24, 72, and 120 h, respectively, with that at 0 h (Figure 1B). Moreover, the Western blot analysis revealed salt-stress-induced abundance of superoxide dismutase (SOD), a salt-stress marker protein in plants [40] (Figure 1C). The effect of salt stress was clearly visible at the time points selected for analysis, and, thus, these samples were used further for the high-throughput proteome analysis of the ginseng fluid proteins.
woody plant species and can extract large amounts of sap proteins for further downstream analysis. In this method, the cut plants were first immersed in EDTA for 1 h to prevent the sealing of phloem followed by transferring the plants into water for the enrichment of fluid proteins, including sap and apoplastic proteins during salt-stress condition, in which the cut ends of the ginseng shoots were immersed in a salt solution over 0, 24, 72, and 120 h, and the secreted fluid proteins were collected. A SDS-PAGE analysis was carried out for validating the extraction efficiency of the used method, which revealed a clear separation of fluid proteins after treatment with salt stress at 72 and 120 h. However, fluid proteins were not observed in the 0 and 24 h samples, probably because of their low amounts. The higher amounts of fluid proteins observed at 72 h and 120 h could be because of the salt-stress-induced accumulation of ROS that led to the membrane peroxidation and allowed the higher secretion of proteins from the cut ends (Figure S1). In contrast, the proteins from the ginseng shoot cultured with ddH$_2$O did not show the secretion of enough amount of fluid proteins for SDS-PAGE during 0 to 120 h, indicating that some of salt-responsive proteins were specifically secreted in ginseng when harvested under high salt conditions (Figure 2).

**Figure 1.** Analysis of salt-stress-induced changes in ginseng. (A) Chlorophyll fluorescence measurement at different timepoints following salt-stress treatment in ginseng leaves. (B) H$_2$O$_2$ measurement in the harvested culture fluid at different timepoints of salt-stress treatment. (C) SDS-PAGE showing changes in ginseng proteins in response to salt stress and the Western blot of salt-marker superoxide dismutase. Different letters of each sample indicated statistically significant (p-value < 0.0001).

### 3.2. Extraction of Fluid Protein from Ginseng and SDS-PAGE Analysis

To analyze the changes in the fluid proteome in response to salt stress, the five-year-old salt-tolerant ginseng “Cheonryang” was used. The major bottle-necks in the extraction of fluid proteins include the difficulty in determining the harvesting time and extraction steps [41,42]. Previous studies showed that xylem sap proteins are constantly produced over a 28 h period in soybeans regardless of stress conditions; moreover, various stress resulted in variations of the xylem composition such as nitrate concentration and pH in *Zea mays* [43]. In addition, a few studies demonstrated that the successful extraction of sap from a few plants by the cut end of the petiole or stem; however, the major disadvantage of those methods is that they work only on several selected cultivars [44]. On the other hand, Tetyuk et al. 2013 reported a sap-protein-extraction method using EDTA and distilled water that allowed the exudation of sap proteins from the cut ends of the plants [44]. Interestingly, this simple method for the isolation of sap proteins was applicable to many herbaceous and woody plant species and can extract large amounts of sap proteins for further downstream analysis. In this method, the cut plants were first immersed in EDTA for 1 h to prevent the sealing of phloem followed by transferring the plants into water for the enrichment of sap proteins [44]. Following this method, in this study, we used distilled water for the enrichment of fluid proteins, including sap and apoplastic proteins, during salt-stress condition, in which the cut ends of the ginseng shoots were immersed in a salt solution over 0, 24, 72, and 120 h, and the secreted fluid proteins were collected. A SDS-PAGE analysis was carried out for validating the extraction efficiency of the used method, which revealed a clear separation of fluid proteins after treatment with salt stress at 72 and 120 h. However, fluid proteins were not observed in the 0 and 24 h samples, probably because of their low amounts. The higher amounts of fluid proteins observed at 72 h and 120 h could be because of the salt-stress-induced accumulation of ROS that led to the membrane peroxidation and allowed the higher secretion of proteins from the cut ends (Figure S1).
3.3. Application of Shotgun Proteomics for Identification of Salt-Responsive Proteins

The 120 h salt lane of the SDS-PAGE gel was directly subjected to in-gel trypsin digestion and LC-MS/MS analysis for the identification of salt-responsive proteins in ginseng. A total of 216 proteins were identified, of which 7 proteins were commonly available in both databases. Moreover, 158 and 96 proteins were specifically identified in the RNA-seq and PAC-BIO database, respectively (Tables S2 and S3). Further functional characterization of identified proteins, carried out using the GO enrichment analysis, revealed that the proteins with catalytic activity (46%), structural molecule activity (16%), and ATPase-coupled ion transmembrane transporter activity (3%) were dominant in the molecular function category (Figure 3A and Table S4). Moreover, response to stimulus (42%) and nitrogen-compound metabolic process (26%) were the most prominent terms in the biological process category (Figure 3B and Table S4). Responses to stimulus were divided into response to stress (33%), response to abiotic stimulus (27%), response to heat (9%), response to oxidative stress (7%), and response to cold (11%), Table S4). In addition, 20 proteins were involved in the catabolic process (11%), 12 proteins involved in protein folding (7%), and 11 proteins involved in ribonucleoprotein complex biogenesis (6%) (Figure 3B).

Further MapMan functional characterization revealed that proteins related to glycolysis (11 proteins), lipid metabolism (7 proteins), and amino-acid metabolism (6 proteins) were mainly annotated in the metabolism category (Figure 4). Moreover, in the cell function category, 23, 19, 13, and 7 proteins were related to protein synthesis, biotic and abiotic stress, cell, and redox, respectively (Figure 4).
Photosynthesis is an important physiological process that is highly affected by changes in environmental conditions [45]. A salt-stress-induced reduction in photosynthetic efficiency is a well-known phenomenon [46,47]; thus, in this study, multiple proteins associated with photosynthesis were identified. For instance, ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) and RuBisCO activase (RCA) were observed in the culture fluid in response to salt stress (Figure 5, Tables S2 and S3). RuBisCO is a key enzyme of the Calvin cycle involved in the fixation of carbon dioxide (CO₂), while RCA is a member of the ATPases associated with the diverse cellular activity protein family, which has various chaperone-like functions. The major function of RCA is to remove inhibitory sugars from the active site of RuBisCO, and the decreased abundance of these two proteins highlights the decreased rate of photosynthesis under salt-stress conditions in ginseng [48]. A previous study showed that salinity stress leads to an oxidative burst, which subsequently leads to the degradation of the small subunits of RuBisCO and RCA [49]. Fructose bisphosphate aldolase (FBPA) is an enzyme associated with glycolysis, gluconeogenesis, and the Calvin cycle; its decreased abundance in response to salt stress has already been reported in various plants including wheat [50]. The secretion of these three photosynthesis-related proteins in the culture fluid of ginseng was predicted to be an outcome of the inhibition of carbon fixation due to salt stress. Furthermore, we identified various other photosynthesis-related proteins in the culture fluid, including oxygen-evolving enhancer (OEE), PS II oxygen-evolving complex 1 (PsbO), PS II subunit P (PsbP), PS I subunit D (PsaD), chlorophyll a-b binding protein (CAB), and manganese-stabilizing protein (MSP) (Tables S2 and S3). MSP is an element comprising the photosystem in vascular plants and playing a role in maintaining the structural stability of photosystem II (PSII) [51]. Similar to MSP, the specific role of PsbO in maintaining PSII stability and plant growth was reported previously [52]. In addition, the PSII oxygen-evolving complex (OEC), which consists of OEE1 and 2 proteins, and PsaD, which is a small extrinsic polypeptide of the PSI reaction center complex, were reported previously [53]. Several reports have shown an increased abundance of OEE proteins during salt-stress conditions in plants; moreover, these proteins play a role in repairing the damage caused by dissociation and maintaining a putative function of OEC in plants [54]. In parallel with the results obtained by chlorophyll fluorescence imaging analysis, these results clearly showed an inhibition of photosynthesis in ginseng plants in response to salt stress.

Figure 4. Functional classification of the fluid proteins using MapMan software. Identified fluid proteins were annotated as metabolism and cell-function categories.

3.4. Inhibition of Photosynthesis by Salt Stress in Ginseng
3.5. Accumulation of Energy Metabolism Related Proteins during Salt Stress

In this study, we identified nine proteins associated with energy metabolism (glycolysis) in ginseng, including phosphoglucomutase, FBPA, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase, enolase, ATP synthase subunit, alcohol dehydrogenase, and malate dehydrogenase in the culture fluid of 5Y-120H ginseng shoot (Figure 5). In plants, the glycolytic enzymes are involved in the production energy and intermediates of various sugars required for the biosynthesis of other metabolites, such as amino acids, many of which function as compatible solutes that participate in salt-stress tolerance [55,56]. A previous study reported an increased abundance of glycolytic enzymes in rice and Aeluropus lagopoides, while a decrease in the abundance of enzymes was observed in soybeans during salt stress [57–59]. In addition, glyoxalase, which functions in the detoxification of methylglyoxal was identified in the culture fluids from ginseng shoot. The accumulation of methylglyoxal, a byproduct of triosephosphate in glycolysis, triggers glyoxalase activation in plants as a tolerance response to stress conditions [60]. Thus, the accumulation of energy-metabolism-related proteins, especially glycolysis, plays important roles in energy production and osmotic balance and may have a positive correlation with salt-stress tolerance in ginseng.

3.6. Activation of ROS Scavenging Enzymes by Oxidative Stress

Stomatal closure triggered by salt stress inhibits the CO₂ fixation and induces the oxidative stress by generating ROS [61]. In this study, we successfully identified various ROS-scavenging enzymes, such as Cu/Zn SOD, Mg SOD, Glutathione peroxidase (GPX), peroxidase, and catalase (CAT) 1, 2, and 3 in the culture fluids from the ginseng shoot (Figure 5, Tables S2 and S3). Antioxidant enzymes such as SODs, CATs, and peroxidases are important components of ROS scavenging in plant systems [62]. The SODs involved in the glutathione-ascorbate cycle protect the plant cells by converting O₂⁻ into H₂O₂ and/or O₂ [63]. During this cycle, the produced H₂O₂ is scavenged by CATs through the decomposition of H₂O₂ into water and molecular oxygen, while the peroxidases oxidize the phenolic compounds and antioxidants to decompose H₂O₂ in plants [64]. The GPX pathway is a major enzymatic defense mechanism against oxidative membrane damage that uses reduced glutathione or other reducing equivalents to convert H₂O₂ to the corresponding

Figure 5. Salt-responsive proteins identified from fluid proteomic analysis. Identified proteins were classified according to the categories of energy metabolism, protein metabolism, cell reconstruction, ROS scavenging, and defense.
·OH compounds [65]. Moreover, the up-regulation of various antioxidant enzymes such as peroxidases, Mg SOD, and Cu/Zn SOD enzymes in salt-tolerant crops was reported in a previous report [66], and the accumulation of these enzymes in ginseng suggests activation of the tolerance response against salt-stress-induced oxidative stress.

### 3.7. Identification of Proteins Related to Various Metabolism

In this study, various chaperones were also identified (Figure 5). The function of heat-shock proteins (Hsps), such as Hsp17.6 II, Hsp70, Hsp81-2, and Hsp90.1, and the TCP-1/cpn60 chaperonin is required for protein folding, assembly, translocation, and degradation, and their role in the stress responses are well established in plants [67]. Hsp70 functions in cooperation with Hsp100 and caseinolytic proteases ClpB or ClpB3 for the removal or repair of misfolded proteins, which has been extensively studied in Arabidopsis, previously [68,69]. In particular, previous studies have shown that Hsp70 is up-regulated to activate defense mechanisms against various stress conditions and the overexpression of this protein elevated tolerance in plants during salt stress [70]. Therefore, the secretion of these chaperones in ginseng fluid suggests a key role for these proteins in the refolding of salt-stress-damaged proteins in ginseng.

Moreover, several proteins related to protein metabolism, defense and stress response, and cell composition, including glycosyl hydrolase 17 (GH17, β-1,3-Glucanases), peptidyl-prolyl cis-trans isomerase (PPIase), actin, and tubulin, were also identified in this study (Figure 5). Among these, the physiological roles of the GH family proteins have been studied in plants using various molecular genetics approaches [71]. These proteins are involved in the biosynthesis and remodeling of glycans, mobilization of storage reserves, and defense response, among others [71,72]. Particularly, GH17 belongs to pathogenesis-related protein-2 (PR-2) and catalyzes the endo-type hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucans. Regulation of this protein, both at the expression and activity levels, has been reported in response to viral and bacterial pathogen infections [71]. In addition, some salt-responsive GH family proteins, especially GH1, have been reported in Arabidopsis [73]. Here, we successfully identified GH17 proteins in ginseng fluids; however, the distinct function of GH17 proteins during salt stress in ginseng is largely elusive. PPIase catalyzes the cis–trans isomerization of proline imidic peptide bonds in oligopeptides to accelerate protein folding [74]. Moreover, the diverse functions of PPIase have been implicated in photosynthesis [75], growth and development [76], redox homeostasis [77], and stress responses [78,79]. Similar to GH family proteins, the overexpression of the PPlase gene in Arabidopsis resulted in the tolerance of various abiotic stresses such as salt, drought, and heat, while this protein promoted plant growth under normal conditions [80].

In addition, the proteins related to cytoskeletal components, such as actin and tubulin, were also found to accumulate in the culture fluid (Figure 5). The dynamic function of cytoskeleton networks is important for cell division, cell movement, cell morphogenesis, and cell signal transduction in plant [81]. Moreover, the depolymerization and reorganization of the cytoskeleton, such as active filaments and microtubules, were reported to play a pivotal role in salt-stress tolerance in Arabidopsis [81,82], and the re-organization of the cells’ structural components may play an important role in salt-stress tolerance in ginseng.

### 3.8. Validation of Identified Proteins Using qRT-PCR Analysis

To validate the expression profile of the identified proteins during salt stress, we performed a qRT-PCR analysis using RNA isolated from salt-treated ginseng root. A total of three proteins including Hsp70, GH17, and FBPA were selected from the fluid proteome analysis to determine their expression at the transcript level (Figure 6). In parallel with their corresponding proteins, 1.3-, 33.89-, and 1.6-fold increases were observed in Hsp70, GH17, and FBPA, respectively, against salt stress in ginseng roots (Figure 6). However, further investigation is required to determine their role in salt-stress tolerance in ginseng.
Salt stress is a major environmental stress that affects plant growth and causes severe damage to ginseng plants. Recently, a few studies have investigated the effects of salt stress to identify the key proteins responsible for salt-stress tolerance in plants; however, few studies have been conducted to define the salt-responsive mechanism in ginseng. Therefore, to expand our understanding of the salt-stress responses and investigate the salt-responsive proteins in ginseng, we employed a shotgun proteomic approach using fluid proteins collected from the cut ends of five-year-old ginseng shoots. This approach resulted in the identification of various photosynthesis-related enzymes with decreased abundance, while the accumulation of various other proteins associated with ROS scavenging, energy metabolism, and protein metabolism was also observed in the culture fluids of ginseng. Moreover, the expression levels of three proteins Hsp70, GH17, and FBPA were also confirmed to show parallel expression profiles with corresponding proteins in ginseng roots. Overall, this study not only provides a simple method for extraction of fluid proteins, but also identified potential salt-responsive proteins in ginseng. Moreover, this study will be helpful for the further understanding of the molecular mechanism of salt-stress tolerance and improving the salt tolerance of salt-sensitive ginseng cultivars by next-generation breeding programs.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/10.3390/agronomy12092048/s1](https://www.mdpi.com/10.3390/agronomy12092048/s1), Figure S1: SDS-PAGE showing profile of secreted fluid protein in each sample. Five-year-old (5Y)-120 h sample showing clear separation of fluid protein, and this line further applied shotgun proteomic analysis for identification of proteins. Table S1: List of primers used in this study, Table S2: List of identified proteins by RNA-seq database, Table S3: List of identified proteins by PAC-BIO database. Table S4: Gene ontology enrichment analysis of identified proteins

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