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Proteomic variation in Korean ginseng (*Panax ginseng* C.A. Meyer) isolates from different geographic regions

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Korean ginseng is a traditional medicine that is widely used in Korea. In this study, a proteomic approach was used to investigate variations in Korean ginseng isolates that are associated with ecologic and geographic differences. Ginseng samples were collected from four geographically isolated locations in Korea: North gyeonggi, Gochang, Geumsan and Kanghwa. Two-dimensional gel electrophoresis (2-DE) and peptide fingerprinting of tryptic digests by mass spectrometry (MALDI-TOF) revealed primary ginseng root region-specific variations in protein profiles in these distinct areas. Thirty seven (37) major proteins that are common to the main root of ginseng at all four geographic sites and six proteins that are specific to the main root of a local ginseng (Kanghwa) were identified. Most of the major common proteins identified could be classified into the following functional categories: (i) stress response; (ii) transcription and translation; (iii) nucleotide metabolism; (iv) plant hormone response; (v) signal transduction; (vi) protein degradation; (vii) protein destination and storage; and (viii) unassigned. The results show that Korean ginseng species can be distinguished on the basis of classical proteomics.

Key words: *Panax ginseng* C.A. Meyer, 2-DE, peptide fingerprinting, classical proteomics.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a traditional medicine for more than a thousand years in Korea to increase stamina and the capacity to cope with fatigue and physical stress. Ginseng has many reported health benefits, including regulation of blood sugar level and anti-stress, anti-cancer, anti-oxidant and anti-aging activities (Helms, 2004; Yoo et al., 2006; Koo et al., 2007). Korean ginseng is found to have such main properties as ginsenoside, polyacetylene, acid polysaccharide, anti-oxidative aromatic compound, and insulin-like acid peptides. The number of ginsenoside types contained in Korean ginseng (38 ginsenosides) is substantially more than that of ginsenoside types contained in American ginseng (19 ginsenosides). Furthermore, Korean ginseng has been identified to contain more main non-saponin compounds, phenol compounds, acid polysaccharides and polyethylene compounds than American ginseng and

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Figure 1. Locations of ginseng plantations from which the ginseng was collected in this study.

Sanchi ginseng (Choi, 2008). Vast quantities of ginseng are consumed in Korea, where the ginseng trade is an important business. Traditionally, the authentication of Korean ginseng sites of origin has relied on morphologic inspection (Hong et al., 2012). In many cases, this approach is unreliable because the roots of different Korean ginseng isolates are often morphologically similar. Thus, a more quantitative analysis of Korean ginseng sites of origin may be a useful reference tool for promoting fair trade of ginseng in the Korean herbal industry. Recently, high performance liquid chromatography (HPLC) separation of different ginsenosides (Fuzzati et al., 1999; Lee and Marderosian 1981; Li et al., 2000) and amplification of polymorphic DNA (Tochika-Komatsu et al., 2001; Um et al., 2001; Mihalov et al., 2000) have been used to screen various types of ginseng. However, as a tool for distinguishing ginseng isolates with different origins, these approaches have several limitations, including reproducibility. A potentially efficient and reliable alternative for characterizing regional ginseng isolates would be a proteomic approach.

The proteome is the entire complement of proteins expressed by a genome in a cell, tissue or organism. More specifically, it is the set of proteins expressed at a given time under defined conditions. Recent technical improvements in two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) have made it possible to compare hundreds of proteins and identify patterns of differential protein expression, thus providing a framework for understanding target tissue function. Here, a proteomic approach was used to investigate natural variations in total protein profiles among Korea ginseng isolates from four different geographic regions. A proteomic approach for comparing within-species variations among Korean ginsengs could be a useful separation system for resolving questions of sites of origin and distinguishing different ginseng subspecies.

MATERIALS AND METHODS

Fresh 6-year-old Korean ginsengs were collected from four well-known Korean ginseng cultivation regions (North gyeonggi, Kanghwa, Geumsan, and Gochang) in Korea: North gyeonggi (between 37°53′11″N 127°11′25″E to 37°54′26″N 127°12′39″E), Kanghwa (between 37°44′46″N 126°30′03″E to 37°54′26″N 126°30′43″E), Geumsan (between 36°00′45″N 127°30′38″E to 36°06′19″N 127°31′48″E), and Gochang (between 35°48′07″N 127°25′50″E to 35°49′52″N 127°26′45″E) (Figure 1). The Northern regions (that is, North gyeonggi, Kanghwa) were colder than the southern regions (that is, Gochang, Geumsan). The Geumsan region showed the highest percentage of the days (25.02%), which was about a quarter of a year. North gyeonggi and Kanghwa showed a percentage of approximately 22 to 24%. The Gochang region showed that the lowest days with a peak air temperature above 30°C was counted.

Sample preparation

Ginseng samples were stored at 4°C until protein extraction. The

main roots (body) of the ginseng plants were cut and weighed before being ground in extraction buffer. Samples (10 g) were ground in a mortar with liquid nitrogen and incubated with sample buffer (0.3% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl pH8.0, 200 mM DTT) at 100°C for 10 min. The solution was transferred to ice and incubated with sample buffer II (DNase I, RNase A, 50 mM Tris-HCl pH 8.0, 50 mM $MgCl_2$) for 10 min. After centrifugation at 15,000 × g for 30 min, supernatants were collected and precipitated with 10% trichloroacetic acid (TCA) solution overnight at -20°C. Protein pellets were washed with ice-cold acetone at least three times to remove contaminants and solubilized in a solution containing 8 M urea, 2 M thiourea, 100 mM DTT and 4% (w/v) CHAPS. Protein concentrations in samples were determined using the 2-D Quant protein assay kit (Amersham Biosciences).

Two-dimensional electrophoresis and image analysis

For 2-DE gels, samples were diluted into isoelectric focusing (IEF) buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.8% carrier ampholytes (pH 4.0 to 7.0 and pH 3-10 NL) and a trace of bromophenol blue to yield the desired protein amount in a volume that could be adsorbed by the immobilized pH gradient (IPG) strip. These diluted samples were used to rehydrate 11 cm IPGs for 12 h at 50 V. All IEFs were performed using the Protean IEF Cell (Bio-Rad) with an 11 cm IEF tray. After rehydration, the following voltage program was applied to the IPG strips: a linear ramp to 250 V over 15 min, followed by a linear ramp to 8,000 V over 2.5 h and then a constant 8,000 V for 4.3 h, for a total of 44,000 Vh. For cup-loading gels, IPG strips were passively rehydrated overnight in IEF buffer and the rehydrated strips were placed gel-side up in a cup-loading tray. A set of cups was placed 1 cm away from the anode end. A 65 µg sample was loaded into the cups, and movable electrodes were placed on both ends of the strips. The strips were focused according to the following protocol: 500 V for 5 min, 4,000 V for 1.5 h, a linear ramp to 8,000 V for 3 h, and 8,000 V for 20,000 Vh. Focused IPG strips were stored at -80°C before equilibration and separation in the second dimension by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels. After IEF, IPG strips were equilibrated by immersion in 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 30 mM DTT for 10 min, followed by immersion in 6 M urea, 50 mM Tris-HCl, pH 8.0, 30% glycerol, 2% SDS, and 5% iodoacetamide for 10 min. The IPG strips were then placed on top of 10% polyacrylamide gels and embedded in hot 0.5% agarose (about 70°C) containing bromophenol blue. Separation was performed at a 80 mA constant current with external cooling until the tracking dye migrated to within 1 cm of the bottom of the gel. Upon completion of 2-DE SDS-PAGE, gels were stained with SilverQuest (Invitrogen, Carlsbad, CA, USA) stain as directed by the manufacturer. All experiments were performed in triplicate, and the representative single gel images represented in the figure. Spot detection and analysis was performed using the PDQuest version 8.0.1 software (Bio-Rad).

MALDI-TOF mass spectrometric analysis and protein identification

For protein identification, spots were excised from the gels and subjected to *in situ* digestion with trypsin as described previously (Savijoki et al., 2005). The digested supernatant fluid was mixed with MALDI matrix (α-cyano-4-hydroxycinamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) and spotted onto an MTB AnchorChip TM 600/384 MALDI plate (Bruker Daltonik), and peptide masses were determined using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker Daltonik). Calibration was carried out based on the internal mass of trypsin. Peptide masses were matched with the theoretical peptides of plant proteins in the National Center for Biotechnology Information (NCBI) database, using the MASCOT software and the MS-Fit software of Protein Prospector (website: http//prospector.ucsf.edu). The peptide mass fingerprint search included a few plants (*Arabidopsis thaliana* and *Oriza sativa*).

RESULTS AND DISCUSSION

Protein maps resolution and analysis

An optimization of 2D-PAGE protocol was used to obtain sharp protein maps from 4 origins of *Panax ginseng*. A side-by-side comparison of 2-DE gels of 65 µg samples of ginsengs from four different geographic regions was prepared by in-gel rehydration (Figure 2 *upper panel*) or cup loading (Figure 2 *lower panel*). Protein precipitation can be clearly seen as a collapsed line of unresolved spots in the in-gel rehydration sample; this was not observed when the sample was cup loaded. More proteins were present in the pH 4 to 7 range in the cup-loaded gel, and proteins were better resolved, with less horizontal streaking and improved spot quantification.

Protein spot identification

Three replicates of 4 origins of *Panax ginseng* were run on 2-DE. Artifacts or protein spots that could not be confidently verified as true matches, were disregarded rather than manually edited. Cut-off values for which 95% of observed data were valid was determined. An analysis of individual ginseng samples collected from different geographic regions in Korea by 2-DE showed clear proteomic variations, revealing both region-specific proteomic similarities and differences among ginseng samples. The total number of main root protein spots in ginsengs grown at North gyeonggi, Gochang, Geumsan and Kwanghwa was 400, 580, 313 and 414, respectively (Figure 3). A comparison of ginseng protein patterns obtained from the four different geographic regions revealed that 165 protein spots were common to all four regions. Small amounts of total proteins with masses ~17 to 40 kDa and pI values ~5.0 to 6.6 were detected in ginseng main roots collected from North gyeonggi and Geumsan, whereas those from Gochang and Kwanghwa contained high amounts of these proteins (Figure 3). This 2-DE analysis also clearly showed that the proteome pattern for ginseng main roots collected in Kwanghwa was distinct from those of ginsengs collected from North gyeonggi, Gochang and Geumsan. These differences in the Kwanghwa ginseng proteome may reflect genetic diversity caused by geographic isolation and time, as well as by variations in local environmental conditions and breeding cycles.

Kwanghwa region has a profitable environmental condition such as soil and regional climate for the

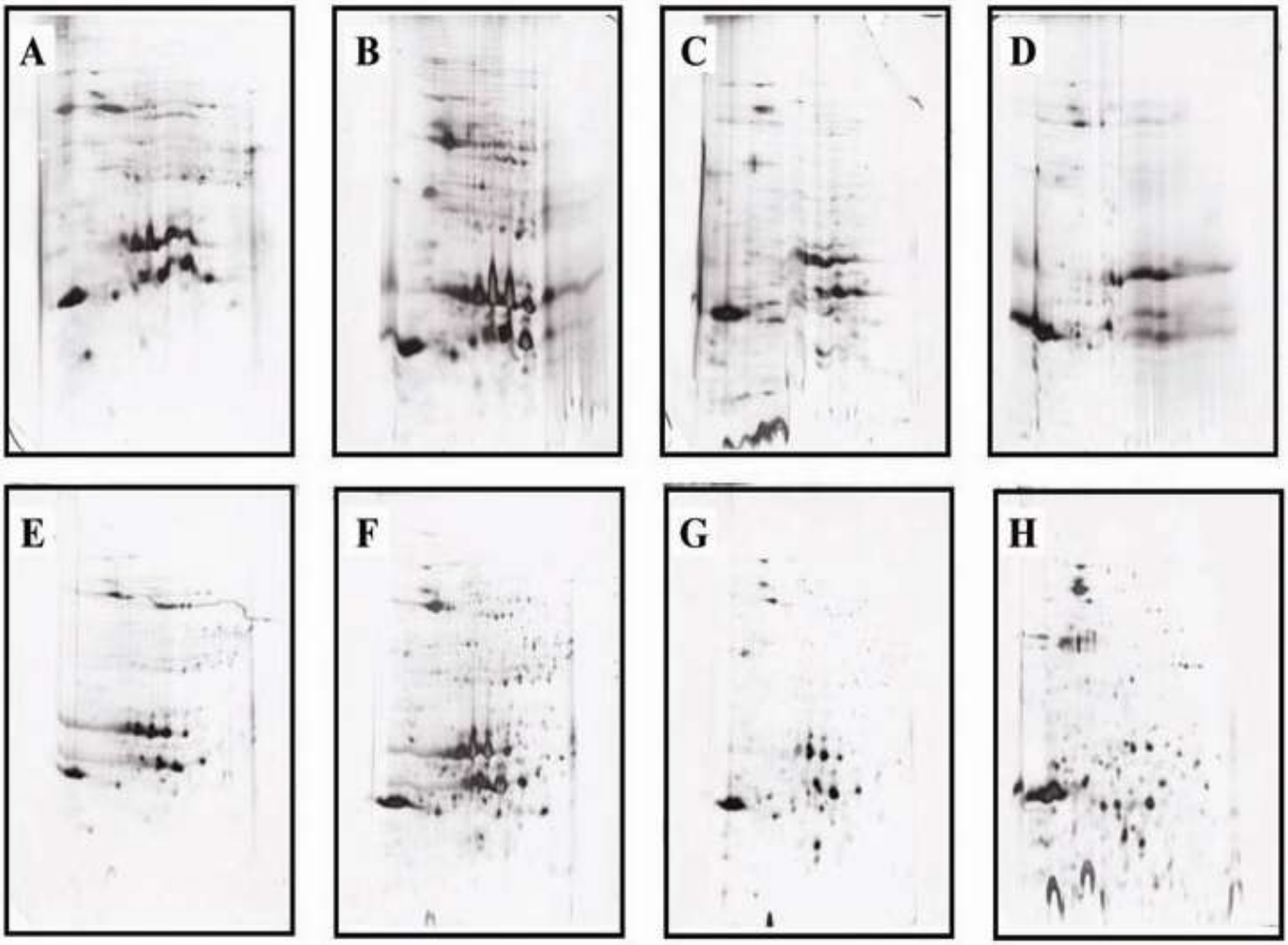

Figure 2. In-gel rehydration loading compared to the cup-loading technique. Representative 2-DE gels of different ginsengs (65 µg/sample) from four different geographic regions. *Upper panel:* pH 4 to 7 IPGs loaded using in-gel rehydration; *lower panel:* cup-loaded pH 4 to 7 IPGs. (A, E) North gyeonggi; (B, F) Gochang; (C,G) Geumsan; (D,H) Kwanghwa.

ginseng species. The climate of Kwanghwa is characterized by the relatively low daily temperature and large diurnal variation with plenty of solar radiation, long sunshine duration and less cloudiness. Because of the sea surrounding Kwanghwa island with low salinity and moderate wind, the salt contained in sea breeze is relatively low compared to other regions. It is also found that moderately coarse texture or fine loamy soils known as good for water drainage and for the growth and cultivation of the 'Kwanghwa-ginseng' are distributed throughout the areas around mountainous districts in Kwanghwa. A lower summer air temperature contributes to xylem compaction and hardness of ginseng, and in consequence, enriching crude saponin content and helping growth of ginseng. The implication of these differences is that there has been selective pressure on the Kwanghwa ginseng isolate to produce a particular proteome.

Thirty-seven protein spots (indicated in Figure 3) from among the 165 proteins common to all four regions were excised from 2-DE gels and digested with trypsin. Following extraction, the tryptic peptides were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The results are summarized in Table 1. A literature search revealed that the identified proteins could be categorized into the following functional categories: stress-responses (two proteins), transcription and translation (four proteins), nucleotide metabolism (one protein), plant hormone response (one protein), signal transduction (three proteins), protein degradation (one protein), and protein destination and storage (one protein). There were 13 proteins of unknown function and 11 unidentified proteins.

Among the differentially expressed proteins, the most highly represented categories were stress-response and transcription and translation. Two spots (spots 6 and 30)

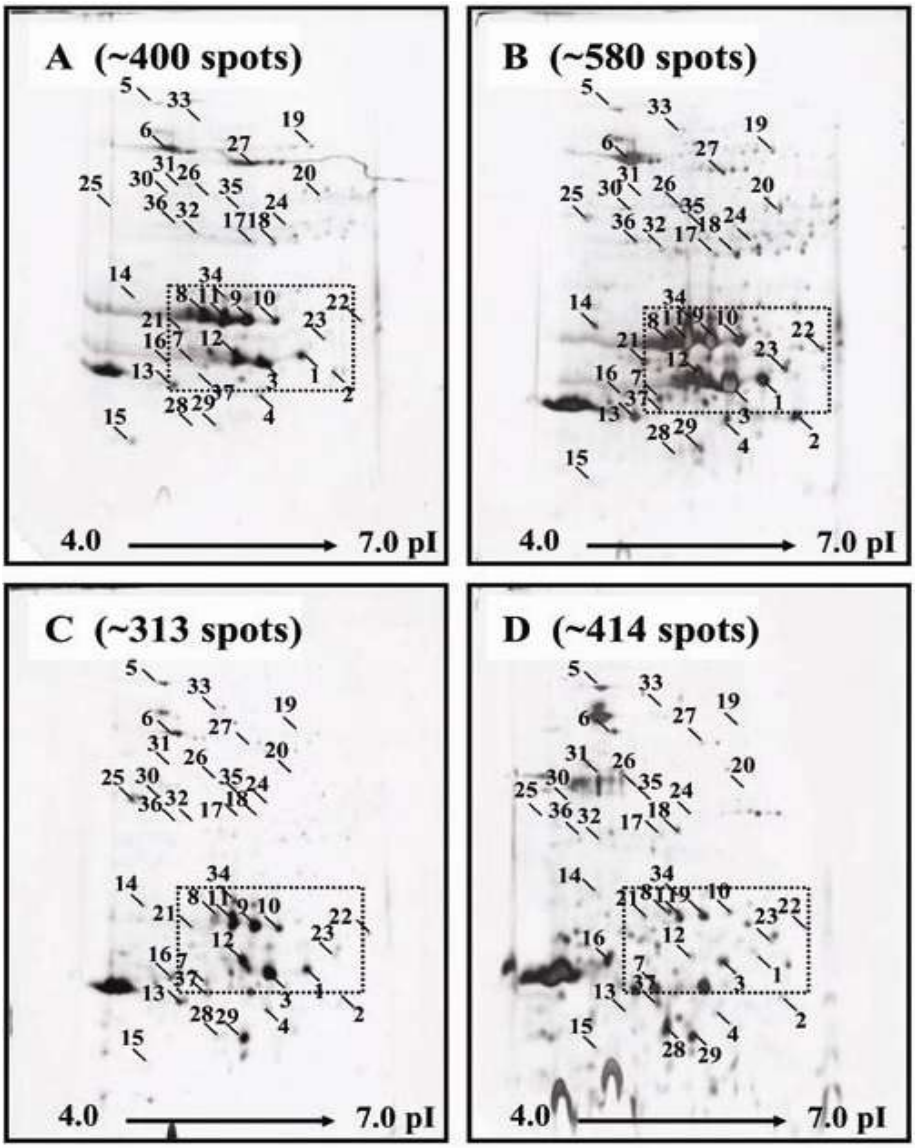

Figure 3. Typical 2-DE profiles of the main root of ginseng from four different geographic regions obtained using the cup-loading technique. (A) North gyeonggi; (B) Gochang; (C) Geumsan; (D) Kanghwa. All gels were loaded with 65 μg ginseng proteins and separated in the second dimension by SDS-PAGE using 10% (w/v) Tris-Tricine SDS gels, followed by silver staining. Protein spots with masses ~17 to 40 kDa and pI values ~5.0 to 6.0 that were differentially expressed among regional ginsengs isolates are boxed. Spots numbered 1 to 37 represent proteins exhibiting geographic region-specific differential expression. These proteins are listed in Table 1.

that corresponded to heat-shock proteins and four spots (spots 3, 4, 12 and 37) that corresponded to transcription and translation were identified. The most intense spot (spot 6 in Figure 3) was identified as heat-shock protein 70 (Hsp70), consistent with the reported prominent role of Hsp70 in ginseng root growth (Sung et al., 2001). This protein is also involved in cell rescue and defense against a number of environmental stress conditions, including heat, cold and drought, as well as chemical and other stresses (Guy and Li, 1998; Lin et al., 2001). Two major spots (spots 3 and 12) are transcription-related proteins that are thought to play a role in regulating ginseng responses to environmental stress. In *Arabidopsis*, transcription factor proteins are induced or repressed under different stress conditions, indicating a role in plant stress responses (Shinozaki et al., 2000). Histidyl-tRNA synthetase (spot 10), a protein involved in nucleotide metabolism, was also observed. In addition to its role in

Table 1. Characteristics of the 37 proteins consistently present in ginseng samples from four different ginseng's sites of area.

Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Stress response	6	cpHSC70-2 (Heat shok protein 70-7): ATP binding	5.0	77.06	gi | 15240578	27.4
	30	High molecular weight heat shock protein	5.0	71.57	gi | 6969976	32.8
Transcription and translation	3	ANAC087: transcription factor [*Arabidopsis thaliana*]	5.7	38.45	gi | 42573407	29
	4	ZCW32: DNA binding/transcription factor [*A. thaliana*]	6.2	29.03	gi | 18406408	28
	12	Protein: ORG2; DNA binding/transcription factor [*A. thaliana*]	5.63	28.76	gi | 152301	30
	37	Transcription factor [*A. thaliana*]	6.22	33.97	gi | 15221262	20
Nucleotide metabolism	10	Putative histidyl tRNA synthetase [*A. thaliana*]	5.71	21.98	gi | 110739016	58
Plant hormone response	35	S-adenosyl-L-homocystein hydrolase	5.8	54.05	gi | 71000473	33.1
Signal transduction	8	Putative protein kinase [*A. thaliana*]	5.74	66.00	gi | 9802793	21
	18	Protein serine/threonine kinase-like protein [*A. thaliana*]	5.69	67.78	gi | 8953410	25
	26	Kinase [*A. thaliana*]	79.5	6.10	gi | 22329045	20
Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Protein degradation	29	Hydrolase [*A. thaliana*]	5.27	23.78	gi | 15221055	56
Protein destination and storage	14	Endopeptidase/peptidase/threonine endopeptidase [*A. thaliana*]	4.70	26.05	gi | 15231824	37
Unassigned	1	Unknown protein [*A. thaliana*]	6.04	21.09	gi | 79479073	32
	2	Unknown protein [*A. thaliana*]	5.39	12.73	gi | 8404455	64
	3	Unknown protein [*A. thaliana*]	4.85	84.25	gi | 18402909	58
	9	Os02g0821900:Putative uncharacterized protein [*Oryza sativa* (japonica cultivar-group)]	5.77	28.40	gi | 110743760	43
	11	Os02g0821900 [*O. sativa* (japonica cultivar-group)]	5.43	32.27	gi | 115449795	44
	13	Putative protein (fragment) [*A. thaliana*]	4.12	11.95	gi | 5262206	74
	16	Hypothetical protein [*O. sativa* (japonica cultivar-group)]	5.98	22.18	gi | 54291450	66
	20	TH65 protein [*A. thaliana*]	6.48	73.60	gi | 110741724	15
	22	mRNA cleavage factor subunit-like protein [*A. thaliana*]	7.64	21.48	gi | 4914406	41
	24	Sulfotransferase [*A. thaliana*]	5.97	37.60	gi | 15230602	30
	27	Unknown protein [*O. sativa* (japonica cultivar-group)]	6.04	53.29	gi | 56783943	75
Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Unassigned	28	Unknown protein [*A. thaliana*]	5.72	27.95	gi | 15221706	39
	34	Protein phosphatase 2C-like protein [*O. sativa* (japonica cultivar-group)]	4.88	24.40	gi | 42409501	41

Table 2. Characteristics of the six proteins differentially expressed in ginseng samples from Kwangwha

Functional category	Spot number	Protein identification	pI	MW (KDa)	Accession number	% Sequence coverage
Transcription	2	Transcription factor [*A. thaliana*]	6.22	33.97	gi | 15221262	20
	6	ANAC087 [*A. thaliana*]	5.70	38.44	gi | 42573407	33
Unassigned	1	Hypothetical protein [*O. sativa* (japonica cultivar-group)]	5.98	22.18	gi | 54291450	66
	3	Os10g0486900 [*O. sativa* (japonica cultivar-group)]	4.66	26.13	gi | 115482622	55
	4	Rid2 protein [*O. sativa* (japonica cultivar-group)]	5.13	30.99	gi | 77539080	69
	5	Unknown protein [*A. thaliana*]	5.72	27.95	gi | 15221706	39

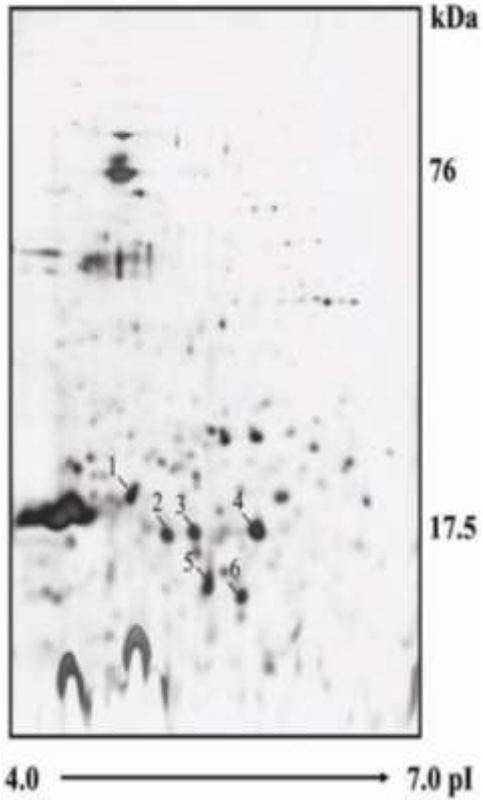

Figure 4. 2-DE of the main root from Kwanghwa ginseng. Note that protein spots 1 to 6 were common to all ginseng sample 2-DEs; however, these spots were significantly over-expressed in Kwanghwa ginseng. These proteins are listed in Table 2.

deciphering the genetic code during protein synthesis, this protein is reported to function in many other cellular processes that lead to stress responses, apoptosis and embryo development (Szymanski et al., 2000). In addition, S-adenosyl-L-homocysteine hydrolase (spot 35) was also identified, which is involved in plant hormone responses. This enzyme is responsible for maintaining active methylation for multiple lipid, protein and nucleic acid metabolic pathways (Tanaka et al., 1996). Several enzymes (spots 8, 18 and 26) involved in regulating a variety of cell functions, including proliferation, gene expression, cell-cycle progression, differentiation, cytoskeletal organization, cell migration and apoptosis, were also expressed; these proteins may be related to signal transduction. Further, a glycoside hydrolase (spot 29) was identified. The hydrolases are involved in degradation of glycoproteins and starch, and have various functions in plant defense and signaling (Minic and Jouanin, 2006). Interestingly, an endopeptidase (spot 14) was observed, which is involved in protein targeting and storage. This enzyme plays a key role in proteolytic processes that are associated with plant programmed cell death (Beers et al., 2000).

Finally, 13 proteins lacking good functional annotations were observed. The proteomic analysis further revealed six proteins that were specifically over-expressed in Kwanghwa ginseng (Figure 4 and Table 2). Two identified proteins (spots 2 and 6) are involved in transcription and three are unknown proteins.

Conclusion

In this study, a proteomic approach was used to analyze the protein expression profiles of ginsengs collected from four different regions of Korea: North gyeonggi, Gochang, Geumsan and Kanghwa. The proteomes of the different Korean ginseng isolates were different and could be used as distinguishing factors. The results presented here clearly showed intra-specific differences in the protein composition of ginseng plants collected from the different geographic regions. This probably reflects innate individual variation in protein synthesis, because genetic variation may be caused by local environmental conditions, geographic separation, nutritional status and time. Several common and region-specific protein spots were also identified in the 2-DE maps of different ginseng isolates. Ginseng proteomic data can be used as reference maps for comparative analysis of 2-DEs of

ginseng from different geographic regions.

Conflict of interest

The authors have not declared any conflict of interest.

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