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ARTICLE

Biochemical and proteomic analysis of soybean sprouts at different germination temperatures

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Abstract Soybean sprouts are available throughout the year and have gained popularity as a functional food owing to their high nutritional value. In the present study, soybean seeds were germinated at different temperatures and the effects on growth characteristics, nutrient composition, and secondary metabolites were investigated. Sprout qualities such as whole length and hypocotyl length were observed to increase at a higher temperature of germination (25 vs. 20 °C). The total protein content of the sprouts increased, whereas the total fatty acid content decreased upon germination at 25 °C. The total phenolic content was higher in soybean sprouts than in soybean seeds. Additionally, antioxidant activity increased in a temperature-dependent manner. Both DPPH and ABTS activity were higher at 25 °C than at 20 °C. Proteomic analysis was conducted to generate temperature responsive protein profiles of soybean sprouts. Using 2D gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, 33 differentially expressed spots were identified. Further analysis of these spots revealed potential function in protein storage and modification. Upon germination at 25 °C, 16 spots increased significantly, whereas 17 protein spots were observed to decrease. Interestingly, a trypsin inhibitor was highly expressed at 25 °C. Semi-quantitative RT-PCR analysis showed that mRNA expression level of most of genes encoding the identified proteins correlated well with their protein abundance, suggesting their temperature-dependent transcriptional regulation in soybean sprouts. In summary, our results clearly indicate an effect of temperature on growth of and secondary metabolite production in soybean sprouts.

Keywords Antioxidant activity · Phenolic compound · Proteomics · Soybean sprout · Temperature · Two-dimensional electrophoresis

Sung Cheol Koo and Sang Gon Kim have contributed equally to this work.

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Introduction

Soybean (*Glycine max* L. Merr.) is a major source of protein and fatty acids in human and animal nutrition. Soybean has been utilized in the production of a variety of foods such as soybean sprouts, pastes, soymilk, fermented food products, soybean oil, and tofu in East Asian countries (Lee et al. 1999; Shin and Choi 1996). Owing to the high protein, vitamin C, and mineral content, soybean sprouts constitute one of the staple foods in Korea. Soybean sprouts are considered functional foods, and thus, their demand has increased tremendously.

Germination is a process by which the development of a seed into a plant is initiated, and it improves the nutritive

value of legumes due to desirable changes in nutrient availability and quality (Bau et al. 1997; Frias et al. 2005; Granito et al. 2005). Secondary metabolites are beneficial products that aid in the growth and development of plants but are not required for their survival. Germination often leads to considerable changes in secondary compound content (Fernandez-Orozco et al. 2009; Ghiassi Tarzi et al. 2012). It is also known to decrease anti-nutritional factors, increase antioxidant effects, and subsequently increase the functionality of the seeds. (Doblado et al. 2007; Lopez-Amoros et al. 2006; Vidal-Valverde et al. 2002).

Phenolic compounds are secondary metabolites that are synthesized during plant development (Chon 2013; Harborne 1982). Plant phenolics include simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, hydrolysable and condensed tannins, lignans, and lignins. These compounds may act as phytoalexins, antifeedants, attractants for pollinators, contributors to the plant pigmentation, antioxidants, and protective agents against biotic and abiotic stresses (Shahidi and Naczk 2004). Soybean seeds contain many phenolic compounds such as chlorogenic acid, caffeic acid, ferulic acid, and *p*-coumaric acid, which have been reported to display antioxidative effects that are beneficial to human health (Chung et al. 2000; Kim et al. 2005; Hayes et al. 1997). Recently, several studies reported a high correlation between the total phenolic content and antioxidant activity. For example, Chon compared the relation between total phenolic and flavonoid content and antioxidant activity (DPPH, tyrosinase inhibition, and ADH activity) from mung bean seeds and sprouts. They demonstrated the presence of higher total phenolic content and antioxidant activity in sprouts compared to seeds (Chon 2013). This result was in good agreement with that of a previous study on sesame sprouts, wherein a rapid increase in the total phenolic content along with an increase in DPPH was noted (Liu et al. 2011).

Proteomic analysis based on two-dimensional electrophoresis (2-DE) and subsequent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) is a convenient way for effective analysis of protein profiles of soybean seeds during seed filling and seed germination. These methods have been used to evaluate protein expression, modifications, and stability in soybean seeds (Agrawal et al. 2008; Hajduch et al. 2005; Mooney and Thelen 2004; Xu et al. 2011). Proteomic analyses have been widely used to study seed germination where mobilization of nutrient reserves occurs during this complex physiological process (Kim et al. 2008a, b, c; Pawlowski 2007; Yang et al. 2007; Xu et al. 2011). Recently, Han and colleagues (Han et al. 2013) compared the protein profiles of germinating seeds of soybean and rice. They showed that each crop possessed a distinct mechanism for reserve mobilization during seed germination. Despite its nutritional importance, only few proteomics-based studies have been conducted on soybean germinating seeds.

In the present study, we investigated the physiological and proteomic changes that occur during soybean sprouting at different temperatures. For this purpose, the Pungsannamulkong and Pungwon cultivars, developed in Korea, were used. The cultivars were germinated at different temperatures. Biochemical assays were carried out and global protein expression was evaluated using two-dimensional gel electrophoresis (2-DE) analysis combined with MALDI-TOF/TOF and reverse transcriptase- polymerase chain reaction (RT-PCR). This work aimed at elucidation of the complex process that induces a change in protein profiles of germinating soybean seeds.

Materials and methods

Seed material and sprout growth

The soybean cultivars Pungsannamulkong and Pungwon, developed in Korea for soybean sprouts, were used in this study. Sprouts were germinated according to the method described by Lee et al. (2007). Briefly, 20 g of seeds were placed in plastic bottles with several small holes in the bottom. The bottles were soaked in a water bath for 8 h, and then moved to a growth chamber. Seeds were cultivated in the dark at different temperatures (20 or 25 °C) for 5 days. Water was sprayed on the seed sprouts using an automatic sprayer for 4 min every 4 h.

Growth characteristics

The total weight of soybean sprouts in each plastic bottle was recorded after 5 days of cultivation. Twenty randomly selected sprouts were used to measure sprout characteristics in each of the two replicates. Whole length was measured from root to the base of the cotyledon. Hypocotyl length was measured from the just above the root to the base of cotyledon. Hypocotyl thickness was measured at the beginning of the hypocotyls.

Analysis of total fatty acid and total protein content

Dried seeds and lyophilized sprouts were used for analysis. Fatty acids were extracted by the Soxhlet method (Extraction unit E-816, BUCHI). Extraction was carried out by adding extraction solution (200 mL of *n*-hexane) to 2.0 g of milled seeds and sprouts and incubation for 2 h. After this, the solution was dried off at 105 °C for 1 h and placed in a desiccator at room temperature, following which the total fatty acid content was weighed. Protein content was

determined by Rapid N cube (Elementar Analysensysteme GmbH, Germany) using 0.2 g milled seeds and sprouts. Protein concentrations were multiplied with a factor (6.25). Statistical analyses of each value were performed using the analysis of variance (ANOVA) to determine statistically different values at a significance of $p \leq 0.05$.

Determination of total phenolic compound content

The total phenolic compound content was determined according to Fernandez-Orozco et al. 2009. This is a colorimetric method based on the reaction of Folin–Ciocalteu reagent with hydroxyl groups contained in phenolic compounds. Absorbance of each sample was spectrophotometrically measured at 765 nm (SpectraMax Plus 384; Molecular Devices, LLC, USA). The total phenolic compound content was determined using a standard curve prepared with gallic acid (0–25 μg/mL). Samples were analyzed in triplicate. Results are expressed as milligrams of gallic acid equivalent (GAE) per 100 g of sample.

DPPH assay

The DPPH assay was performed according to the method described by Brand-Williams and co-workers. (Brand-Williams et al. 1995) with some modifications. Absorbance of each sample was measured at 515 nm by using a spectrophotometer. The DPPH activity was determined using a standard curve prepared with Trolox (0–500 μM). Results are expressed as μM of Trolox equivalent per 100 g of sample. Samples were analyzed in triplicate.

ABTS assay

The ABTS assay was performed according to a modified version of the method described by Arnao et al. (2001). Absorbance of each sample was measured at 734 nm using a spectrophotometer. DPPH activity was determined using a standard curve prepared with Trolox (0–500 μM). Results are expressed as μM of Trolox equivalent per 100 g of sample. Samples were analyzed in triplicate.

Protein extraction

Extraction of proteins was performed as previously described (Kim et al. 2008a, b, c). Briefly, soybean seeds and sprouts were powdered using a pestle in liquid nitrogen. Powdered samples were homogenized with 5 mL of Mg/NP-40 buffer [0.5 M Tris-HCI (pH 8.3), 2 % (v/v) NP-40, 20 mM $MgCl_2$, 2 % β-mercaptoethanol], after which they were centrifuged at 12,000×g for 10 min at 4 °C. The supernatant was mixed with an equal volume of water-saturated phenol, and centrifuged at 12,000×g for 10 min at 4 °C. The phenol phase was mixed with four volumes of methanol containing 0.1 M ammonium acetate, and protein was allowed to precipitate at −20 °C for 1 h and collected by centrifugation at 12,000×g for 10 min at 4 °C. The precipitate was washed as described in (Kim et al. 2008a, b, c). Lastly, the pellet was repeatedly rinsed with ice-cold acetone until a white pellet was obtained, which was stored in 80 % acetone at −20 °C until the protein content was measured using a 2-D quant kit (GE Healthcare, Waukesha, WI, USA).

2-DE analysis

2-DE analysis was performed as previously described (Kim et al. 2008a, b, c). Briefly, the IPG (18 cm) strips were rehydrated using a rehydration solution (GE Healthcare, Waukesha, WI, USA) containing equivalent samples (250 μg). IPG focusing was performed at 50 V for 4 h, 100 V for 1 h, 500 V for 1 h, 1,000 V for 1 h, 2,000 V for 1 h, 4,000 V for 2 h, 8,000 V for 5 h, 8,000 V for 9 h, and 50 V for 6 h using the IPGphor II platform (GE Healthcare, Waukesha, WI, USA). Each focused IPG strip was placed into a 20-mL screw-cap tube containing 5 ml equilibration buffer (GE Healthcare, Waukesha, WI, USA). The strips were stirred gently at room temperature for 20 min, followed by a second round of equilibration with equilibration buffer containing 55 mM iodoacetamide (without DTT) under dark conditions for 20 min with gentle agitation. 2-DE was performed using 13 % SDS-polyacrylamide gels, which were stained by colloidal Coomassie Brilliant Blue (CBB) (Kim et al. 2008a, b, c). Images were acquired using a transmissive scanner (PowerLook 1120, UMAX) with a 32-bit pixel depth, 300 dpi resolution, and brightness and contrast set to default. Gel spots were detected automatically using the Image Master 2D Platinum software 6.0 (GE Healthcare, Waukesha, WI, USA). The volume of each spot was then normalized to the average of the volume of spots on the gel.

In-gel digestion

In-gel digestion was performed according to the method described by (Kim et al. 2011). CBB-stained protein spots were excised, washed with 50 % (v/v) acetonitrile (ACN) in 0.1 M NH_4HCO_3, and vacuum-dried. The dried gels were treated with 10 mM DTT in 0.1 M NH_4HCO_3 for 45 min at 55 °C, after which the DTT solution was replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3, and samples were incubated for 30 min at room temperature in the dark. Gel pieces were washed with 50 % ACN in 0.1 M NH_4HCO_3 and digested with 12.5 ng/μL trypsin and 25 mM NH_4HCO_3 in 10 μL of digesting solution overnight at 37 °C following which they were air-dried.

MALDI-TOF MS

MALDI-TOF MS analysis was performed as previously described (Kim et al. 2011). Briefly, the α-cyano-4-hydroxycinnamic acid, nitrocellulose, and isopropanol solutions were mixed in a ratio of 100:50:50. Two microliters of this master matrix mixture was added to 2 μL of the prepared peptide sample. One microliter of the resulting peptide and matrix mixture was immediately spotted onto a MALDI plate and incubated for 5 min, followed by washing of the MALDI plate with 0.1 % (v/v) TFA. Gel spots were analyzed using a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA). Parent ion masses were measured in the reflection/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000 %, a guide wire voltage of 0.010 %, and a delay time of 150 ns. Des-Arg1-bradykinin (m/z 904.4681) and angiotensin 1 (m/z 1,296.6853) were used as a two-point internal standard for calibration. Peptides were selected in the mass range of 500–3,000 Da. MoverZ program (http://bioinformatics.genomicsolutions.com) was used for data analysis. Database searches were performed using Mascot (http://www.matrixscience.com). To determine the confidence of identification results, the following criteria were used: more than five matching peptides and a sequence coverage greater than 15 %.

Semi-quantitative RT-PCR

Total RNA amount was extracted from soybean sprouts with Plant Mini RNA Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. cDNA was generated from 1 μg of total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen, Madison, WI, USA). Primers were designed to generate PCR products of 200–700 bp. Soybean actin transcript (Gmax ACT11, Glyma18g52780) was used as a control to normalize the concentration of cDNA in each sample. PCR was carried out with gene-specific primer pairs (supplementary Table 1). PCR products were electrophoretically separated on a 1.2 % agarose gel. Gels were imaged on Syngene Bioimaging System with GeneSnap (Syngene) and bands were quantified with GeneTools software (Syngene).

Results and discussions

Characteristics of soybean sprouts germinated at different temperatures

The effects of temperature on growth characteristics of soybean sprouts of two soybean cultivars, Pungsannamulkong and Pungwon, were determined by germination at different temperatures (20 or 25 °C) under dark conditions for 5 days. The characteristics of the sprouts are listed in Table 1. Hypocotyl thickness was similar after germination at both temperatures. However, different germination temperatures led to significant differences in the total length, hypocotyl length, and sprout yield. The total length and hypocotyl length after germination at 25 °C were about two times longer compared that at 20 °C. Sprout yield was also highly increased at 25 °C compared to 20 °C. Thus, a germination temperature of 25 °C is more effective for sprouting soybean than 20 °C.

Seed germination often exerts an effect on the nutrient composition and secondary metabolite distribution. Total protein was slightly increased and total fatty acid was slightly decreased in soybean sprouts compared to seeds. Additionally, a change in the total protein and fatty acid content could be detected in soybean sprouts grown at different temperatures (Table 1). Total protein was found to be higher at 25 °C compared to 20 °C, whereas total fatty acid was reduced at 25 °C. These results clearly indicate that different temperatures used for soybean sprouting affect the nutrient and metabolite compositions similar to seed germination as observed earlier (Han et al. 2013).

Table 1 Growth characteristics and nutritional composition of soybean sprouts

Variety	Growth temperature	Whole length (cm)	Hypocotyl length (cm)	Hypocotyl thickness (mm)	Sprout yield (%)	Total fatty acid (%)	Total Protein (%)
Pungsannamulkong	Seed	–	–	–	–	15.28 ± 058	37.78 ± 0.71
	20 °C	14.46 ± 0.57	6.93 ± 0.51	2.08 ± 0.23	442 ± 16.51	14.55 ± 0.72	42.48 ± 0.68
	25 °C	30.38 ± 2.82	16.34 ± 1.33	2.25 ± 0.27	764 ± 25.76	13.02 ± 0.33	43.53 ± 0.19
Pungwon	Seed	–	–	–	–	17.92 ± 0.76	33.97 ± 1.02
	20 °C	13.94 ± 0.77	6.96 ± 0.75	2.06 ± 0.25	445 ± 8.39	17.96 ± 0.92	34.98 ± 0.30
	25 °C	27.99 ± 2.61	15.83 ± 1.37	1.94 ± 0.19	724 ± 37.77	15.56 ± 0.22	36.9 ± 1.06

Values are mean ± SD

Total phenolic content and antioxidative properties

The effect of temperature on antioxidant activity from soybean seeds and sprouts was estimated by determining the total content phenolic compounds. Phenolic compounds are secondary metabolites that possess high antioxidant and anti-aging properties (Chon 2013). Our results showed differential total phenolic content between soybean sprouts grown at different temperatures. The total phenolic content in the Pungwon sprouts was higher when grown at 25 °C (1,655 mg GAE/100 g sample) than that when grown at 20 °C (1,420 mg GAE/100 g sample), whereas in Pungsannamulkong sprout, it was higher when grown at 20 °C (1,468 mg GAE/100 g sample) than that when grown at 25 °C (1,211 mg GAE/100 g sample). Furthermore, the total phenolic compound content showed a tendency to increase in soybean sprouts compared to soybean seeds (Fig. 1). Generally, germination process increases the nutritional compounds (free amino acid, carbohydrates, and dietary fiber) and bioactive compounds including phenolics (Doblado et al. 2007; Cevallos-Casals et al. 2010). And several studies reported that the level of phenolic compounds increase in time-dependent manner during germination, suggesting that phenolic compounds could be changed by the light, temperature, and activation of endogenous enzymes during germination (Lopez-Amoros et al. 2006 Troszyńska et al. 2006). Thus, our results are in agreement with those of earlier studies that reported on the seed germination induced accumulation of phenolic compounds in sprouts. More detailed studies of individual phenolic compound could help provide a better understating of the effect of temperature change on secondary metabolite profile in soybean sprouts.

Several studies have shown that the total phenolic content is correlated with antioxidant activity (Chon 2013; Zhou and Yu 2006). To confirm this, the antioxidant activities of soybean seeds and sprouts were determined by DPPH and ABTS assays (free radical scavenging assay). DPPH activity was significantly increased in soybean sprouts of both cultivars in comparison to the activity in seeds (Fig. 2a). This effect was more significant in case of DPPH activity, which was higher in sprouts grown at 25 °C (460 μM Trolox/100 g for Pungsannamulkong and 529 μM Trolox/100 g for Pungwon) than those grown at 20 °C (339 μM Trolox/100 g for Pungsannamulkong and 338 μM Trolox/100 g for Pungwon). ABTS activity also showed a trend similar to DPPH activity (Fig. 2b). Although no significant differences in the DPPH activities between seeds and sprouts grown at 20 °C were observed, the activities in sprouts were higher at 25 °C than at 20 °C (523 vs. 472 μM Trolox/100 g for Pungsannamulkong and 625 vs. 562 μM Trolox/100 g for Pungwon). These results clearly show a high degree of correlation between the total phenolic content and antioxidant activity and suggest that the soybean sprouting temperature changes the secondary metabolites responsible for antioxidant activity.

Comparative 2-DE analysis of soybean sprout proteins at different growth temperatures

Pungsannamulkong and Pungwon cultivars showed only few physiological differences when grown at 20 or 25 °C

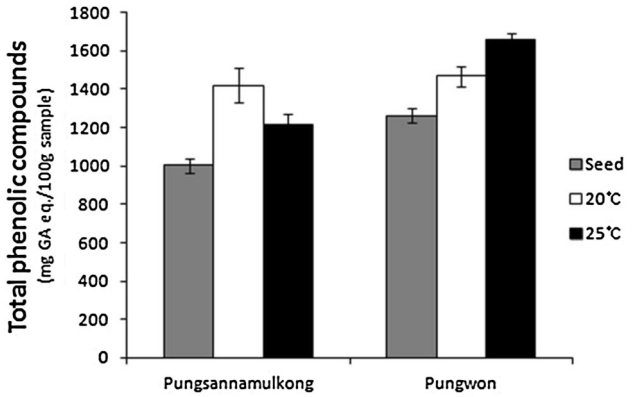

Fig. 1 Total phenolic content in soybean seeds and sprouts. Values indicate mean ± SD

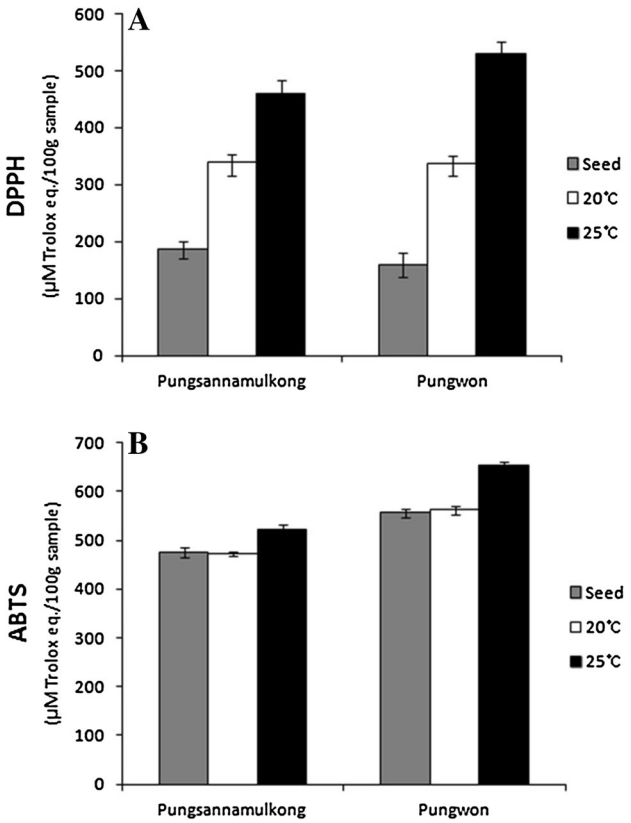

Fig. 2 DPPH (**a**) and ABST (**b**) radical scavenging activity in soybean seeds and sprouts. Values indicate mean ± SD

(Table 1). However, a significant increase in the antioxidant activity of Pungwon could be observed when grown at 25 °C compared to 20 °C. To investigate the reason behind this phenomenon, a 2-DE analysis was carried out using Pungwon sprouts germinated in dark at 20 or 25 °C for 5 days. Total proteins were extracted by phenol extraction method and 500 μg of total protein was loaded onto 18 cm neutral IPG strips (pH 4–7) to optimize the resolution on 2-DE gels. Proteins that were overexpressed or decreased when two different growth temperatures were used, were analyzed. Thirty-three proteins were found to be differentially expressed at 25 °C compared to 20 °C

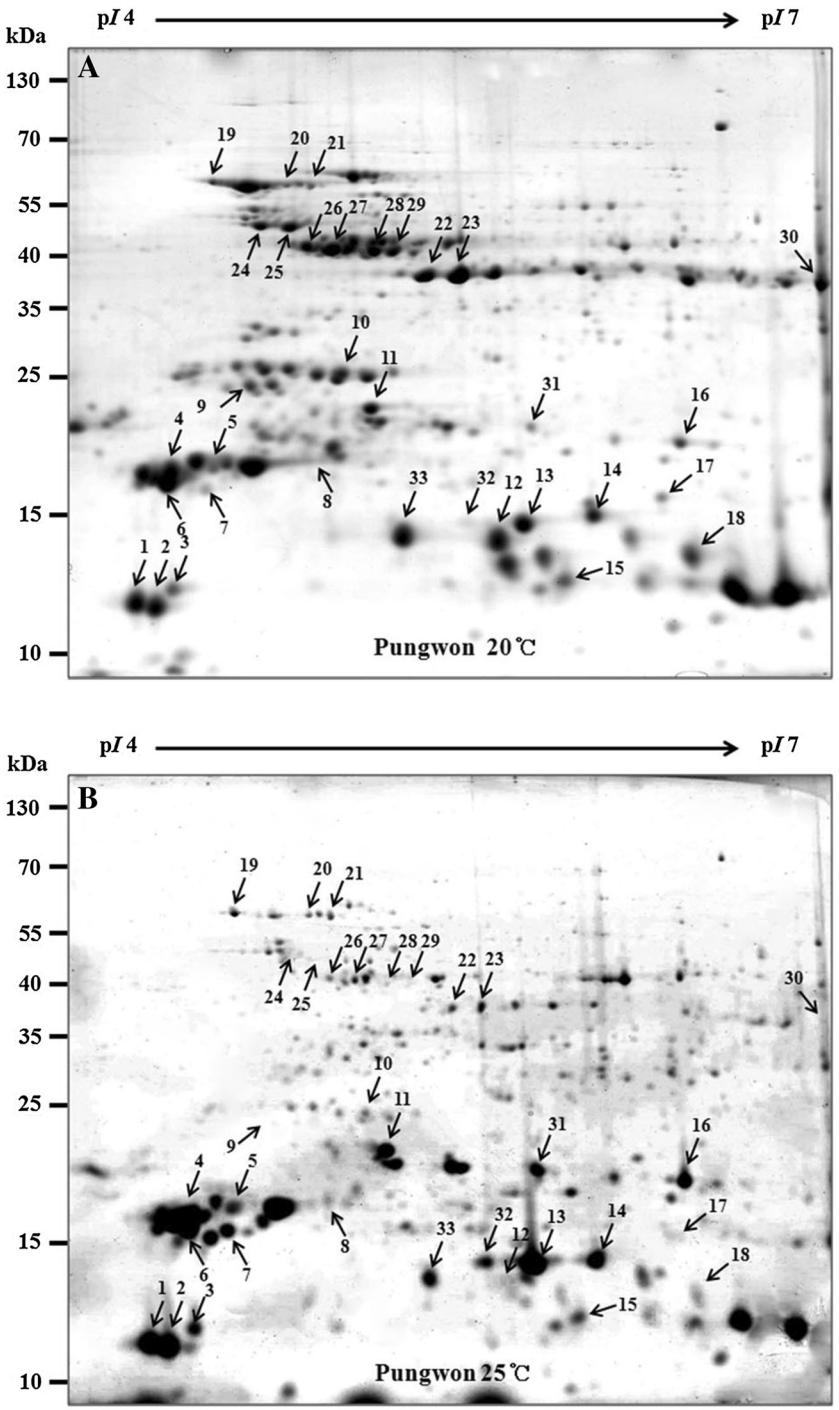

Fig. 3 2-DE gel analysis of proteins extracted from soybean sprouts. Proteins were separated in the first dimension using an IPG strip pH 4.0–7.0 and in the second dimension on a 12 % SDS-PAGE. **a** Representative 2-DE gel of proteins from Pungwon sprouts germinated at 20 °C. **b** Representative 2-DE gel of proteins from Pungwon sprouts germinated at 25 °C. Distinct protein spots detected on the 2-DE gels are marked by *arrows*

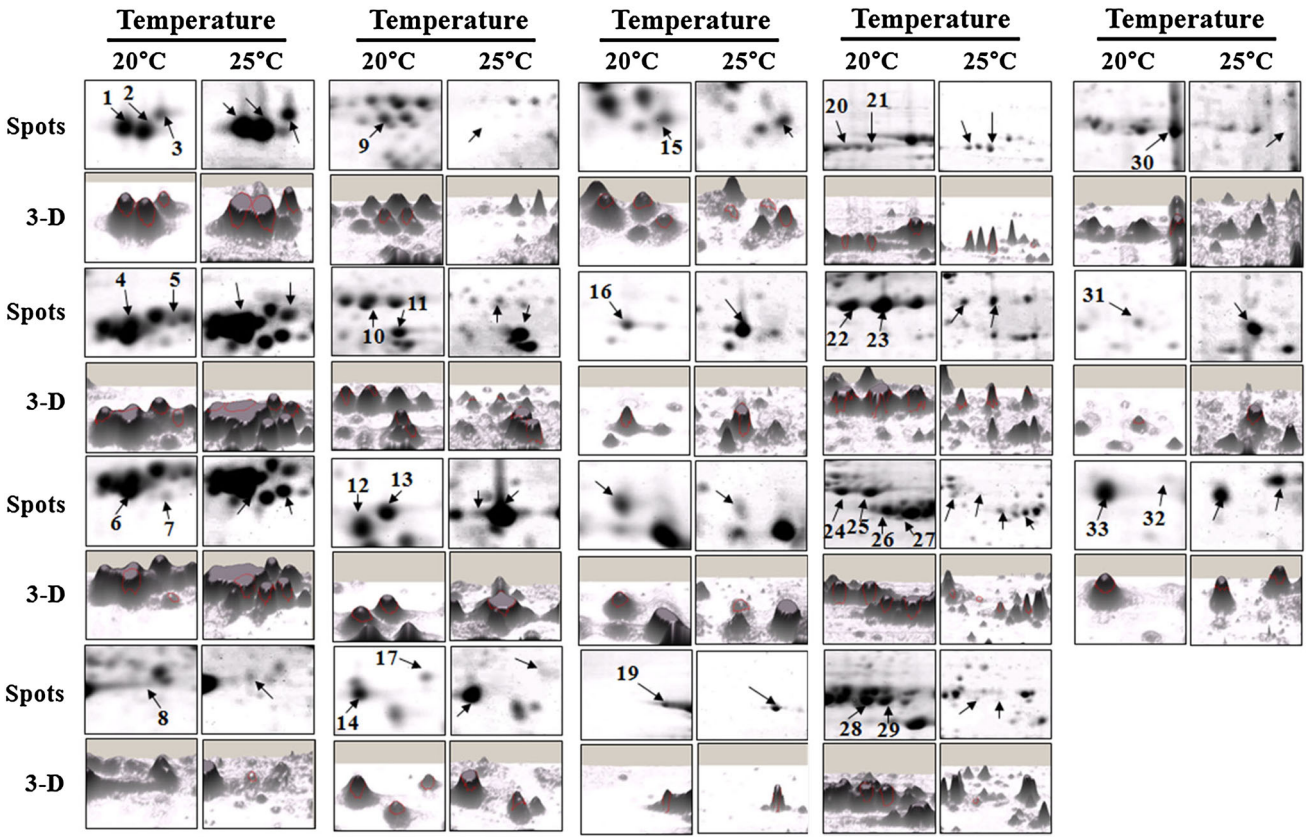

Fig. 4 Enlarged images showing 33 differentially expressed protein spots in soybean sprouts germinated under different temperatures. The 3-D image was generated by ImageMaster software

(Figs. 3, 4). Among these, 16 protein spots were markedly increased at the 25 °C condition, whereas 17 were decreased. These results suggested that, in contrast to the lower temperature (20 °C), a higher temperature during soybean germination (25 °C) resulted in a higher metabolic change.

Identification of soybean sprout proteins responsive to temperature change

To gain a better understanding of proteins responsive to temperature during germination of soybean sprouts, we analyzed the differentially expressed proteins using MALDI-TOF and identified them by database searches using Mascot and UniProt. Among the identified proteins, 15 were storage proteins, two responsible for protein modification (spots 1, 2) and 15 were unknown proteins (Table 2). Primary storage proteins were most abundant during soybean sprouting. Two major storage proteins, β-conglycinin and glycinin, account for about 70–80 % of the total proteins, and are largely responsible for the nutritional and physicochemical properties of soybean (Thanh and Shibasaki 1976). In this study, 14 β-conglycinins (spots 4, 5, 13, 14, 17, 22, 24, 25, 26, 27, 28, 29, 30, and 31) storage proteins could be identified. The β-conglycinin is a trimeric glycoprotein consisting of α-, ά-, and β-subunits. Among these, only the α-subunit induces allergenic reactions (Thanh and Shibasaki 1976). Three α-subunits (spots 25, 26, and 29) of β-conglycinin were decreased at 25 °C compared to 20 °C during soybean sprouting, whereas other β-conglycinins were either up- or down-regulated at different temperatures (Fig. 5). These results suggest that allergenic reactions induced by these proteins may be reduced at a relatively high temperature (25 °C).

Interestingly, trypsin inhibitor A (spots 1 and 2) were highly expressed at 25 °C. The Kuntiz-type trypsin inhibitors could be identified as secreted proteins in soybean (Brechenmacher et al. 2009) and are reported to play an important role in resistance against plant pathogens (Major and Constabel 2008). Trypsin inhibitors were also differentially accumulated in soybean seedlings in response to flooding (Hashiguchi et al. 2009). Recently, a trypsin inhibitor isolated from soybean and potato was reported to play an antioxidative role (Hou et al. 2001; Gu et al. 2014).

Table 2 List of proteins differentially expressed in soybean sprouts and identified by MALDI-TOF MS analysis

Spot no.	AC[a]	Protein name	Score	Expect	MP[b]	Theor. MW[c]	Theor.pI[d]	SC (%)[e]	Source	GO term
1	ITRA_SOYBN	Trypsin inhibitor A	98	8.00E−05	13	24,275	4.99	29	Glycine max	Serine-type endopeptidase inhibitor activity
2	ITRA_SOYBN	Trypsin inhibitor A	75	0.018	14	24,275	4.99	32	Glycine max	Serine-type endopeptidase inhibitor activity
3	C6SWW4	Uncharacterized protein	105	6.30E−05	9	22,718	4.85	39	Glycine max	Endopeptidase inhibitor activity
4	GLCB_SOYBN	Beta-conglycinin, beta chain	79	0.0,073	9	50,578	5.88	19	Glycine max	Nutrient reservoir activity
5	GLCB_SOYBN	Beta-conglycinin, beta chain	70	0.057	9	50,578	5.88	16	Glycine max	Nutrient reservoir activity
6	B6EBD5	SGT1-2	60	2.2	10	40,812	5.18	23	Glycine max	None
7	K7KT39	Uncharacterized protein	34	7.10E+02	5	7,398	5.17	72	Glycine max	None
8	K7MAJ1	Uncharacterized protein	49	23	4	7,387	5.69	83	Glycine max	None
9	K7LI10	Uncharacterized protein	34	7.60E+02	9	72,269	6.56	17	Glycine max	None
10	K7KHZ7	Uncharacterized protein	43	1.10E+02	4	11,627	4.81	18	Glycine max	Zinc ion binding
11	K7MAX3	Uncharacterized protein	49	23	17	188,585	6.48	11	Glycine max	None
12	C6TDW0	Putative uncharacterized protein	61	1.7	9	20,774	9.25	54	Glycine max	Sequence-specific DNA binding transcription factor activity
13	GLCB_SOYBN	Beta-conglycinin, beta chain	62	0.37	9	50,578	5.88	31	Glycine max	Nutrient reservoir activity
14	GLCB_SOYBN	Beta-conglycinin, beta chain	65	0.18	10	50,578	5.88	32	Glycine max	Nutrient reservoir activity
15	K7MXA1	Uncharacterized protein (Fragment)	54	7.3	6	12,584			Glycine max	Structural molecule activity
16	VSPB_SOYBN	Stem 31 kDa glycoprotein	128	8.60E−08	15	29,433	6.72	61	Glycine max	Acid phosphatase activity, nutrient reservoir activity
17	O22121	Beta subunit of beta conglycinin (Fragment)	72	0.13	11	47,947	5.67	27	Glycine max	Nutrient reservoir activity
18	I1MIJ9	Uncharacterized protein	75	0.066	18	142,843	6.02	21	Glycine max	ADP binding, defense response
19	I1MJU7	Uncharacterized protein	104	8.00E−05	17	73,880	5.2	25	Glycine max	ATP binding, protein folding
20	I1JPC5	Uncharacterized protein	121	1.60E−06	17	67,939	4.98	39	Glycine max	ATP binding, nucleotide binding
21	I1KPN3	Uncharacterized protein	135	6.30E−08	20	73,660	5.08	33	Glycine max	ATP binding, nucleotide binding
22	O22121	Beta subunit of beta conglycinin (Fragment)	128	3.20E−07	17	47,947	5.67	43	Glycine max	Nutrient reservoir activity
23	K7KDG0	Uncharacterized protein	27	4.20E+03	3	8,902			Glycine max	None
24	GLCA_SOYBN	Beta-conglycinin, alpha chain	84	0.0019	14	70,535	5.07	18	Glycine max	Nutrient reservoir activity
25	Q9FZP9	Alpha' subunit of beta-conglycinin (Fragment)	61	1.5	10	65,160	5.23	14	Glycine max	Nutrient reservoir activity
26	Q9FZP9	Alpha' subunit of beta-conglycinin (Fragment)	91	0.0017	14	65,160	5.23	21	Glycine max	Nutrient reservoir activity
27	Q0MUU5	Beta-conglycinin alpha'-subunit	78	0.035	13	70,130	5.43	27	Glycine max	Nutrient reservoir activity
28	GLCA_SOYBN	Beta-conglycinin, alpha chain	90	0.00051	13	70,535	5.07	19	Glycine max	Nutrient reservoir activity
29	O22120	Alpha subunit of beta conglycinin (Fragment)	133	1.00E−07	18	63,184	4.92	32	Glycine max	Nutrient reservoir activity
30	GLCA_SOYBN	Beta-conglycinin, alpha chain	118	8.60E−07	16	70,535	5.07	28	Glycine max	Nutrient reservoir activity

Table 2 continued

Spot no.	AC[a]	Protein name	Score	Expect	MP[b]	Theor. MW[c]	Theor.pI[d]	SC (%)[e]	Source	GO term
31	C6SWV3	Uncharacterized protein	85	0.0069	13	27,847	5.72	49	Glycine max	None
32	GLCB_SOYBN	Beta-conglycinin, beta chain	71	0.042	12	50,578	5.88	25	Glycine max	Nutrient reservoir activity
33	I1N887	Uncharacterized protein	56	5.3	10	34,958	7.72	47	Glycine max	Chaperone

[a] Accession number
[b] Number of matched peptides
[c] Theoretical molecular weight
[d] Theoretical isoelectric point
[e] Sequence coverage (%)

Further, it was also reported to trigger inflammatory responses, resulting in increased antioxidative activities in mice. However, in this study, we could not identify any antioxidant enzymes (glutathione peroxidase, catalase, or superoxide dismutase) and phenolic compound-related enzymes such as phenol biosynthesizing enzymes (phenylalanine ammonia lyase) and phenol catabolizing enzymes (hydrolase and polyphenoloxidase) that were differentially modulated. An abundant protein depletion method might be needed for clarifying antioxidant and phenolic enzymes during soybean sprouting. Fifteen uncharacterized proteins were identified in this study using MALDI-TOF and MASCOT. These proteins were analyzed further using GO terminology. Nine uncharacterized proteins could then be identified one of which possesses endopeptidase inhibitor activity (spot 3), and another is a zinc ion binding protein (spot 10). Other activities include DNA-binding transcription factor activity (spot 12), structural molecular activity (spot 15), ADP binding (spot 18), ATP binding (spots 19–21), and chaperone activity (spot 33) (Table 2). Among these spots, spot 3, 12, 15, and 19 increased at 25 °C. Further analysis of these proteins is needed to more comprehensively understand the basis for temperature-dependent differential expression of these proteins.

Differential expression of selected proteins corresponding to transcript levels

The mRNA expression levels of seven randomly selected genes encoding proteins identified in this study, including trypsin inhibitor A (ITRA_SOYBN), β-conglycinin (GLCB_SOYBN), and uncharacterized proteins (C6SWW4, C6TDW0, K7MXA1, I1JPC5, and I1N887) were analyzed using semi-quantitative RT-PCR in order to determine whether the changes in protein abundance were regulated at their transcriptional level. The mRNA expression levels of most of these genes, except for ITRA_SOYBN, showed a similar tendency concerning protein abundance (Fig. 6). This was suggestive of their temperature-dependent transcriptional regulation in soybean sprouts. Contrastingly, ITRA_SOYBN was up-regulated at the translational level but down-regulated at the transcriptional level due to the temperature change. This discrepancy might be explained by mRNA stability, post-translational modifications, or protein degradation under different temperatures. A comprehensive analysis of the identified proteins would provide a better understanding of the global effects of temperature change in soybean sprouts. Furthermore, it would be worth to compare protein profile to metabolite profile of phenolic compounds in soybean sprouts. This comparative analysis might be very helpful to elucidate a regulatory mechanism of antioxidant activity responsive to temperature change during germination in soybean.

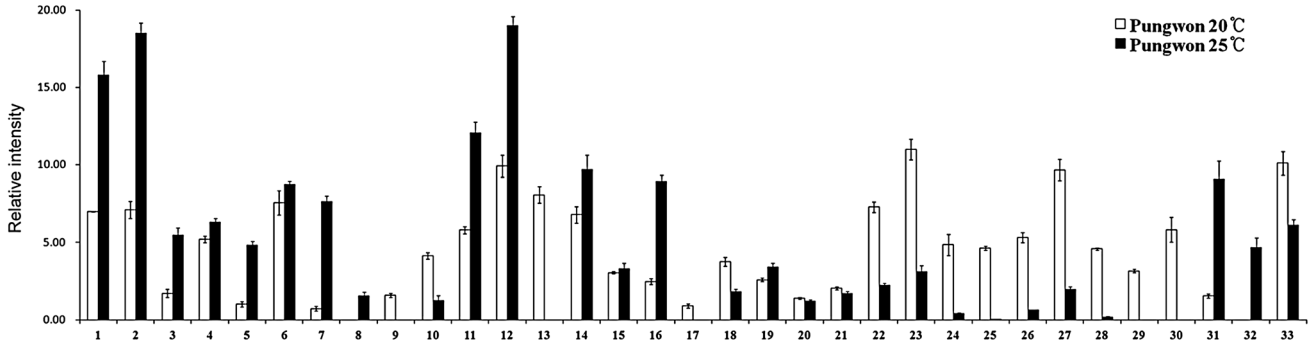

Fig. 5 Quantitative analysis of 33 differentially expressed protein spots. The relative intensity of protein spots was measured by ImageMaster software

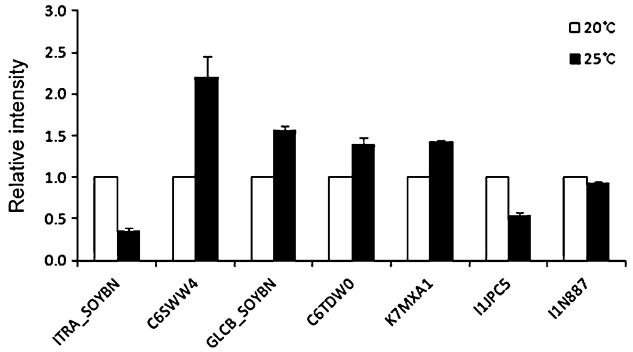

Fig. 6 Gene expression analysis by semi-quantitative RT-PCR performed with gene-specific primers. The relative gene expression was evaluated using GeneTools software (Syngene) and Gmax ACT11 (Glyma18g52780) was used as a control

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