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Soyisoflavone diversity in wild soybeans (*Glycine soja* Sieb. & Zucc.) from the main centres of diversity

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ABSTRACT

Wild soybeans are naturally found in China, and the Korean Peninsula, Japanese archipelago and Russian Far East, which are considered their centres of diversity. The presence of higher amounts of secondary metabolites in wild soybeans makes them the ideal choice to increase soyisoflavone content in cultivated soybeans through modern breeding and biotechnological techniques. Such a superiority calls for soyisoflavone profiling of germplasm resources. The soyisoflavone diversity of 298 wild soybean accessions collected from main centres of diversity, i.e. China, South Korea, Japan, and the Russian Far East were subjected to high-performance liquid chromatography. The profiles of six soyisoflavone components from whole seeds were quantified. The analysis of variance, principle component analysis, and Pearson's correlation analysis clearly revealed that the 298 wild soybean accessions studied had low variation in terms of the six soyisoflavone components, i.e. daidzin, glycitin, genistin, malonyl-daidzin, malonyl glycitin, and malonyl genistin. The accessions with higher soyisoflavone contents are good candidates for future breeding strategies.

1. Introduction

Soybean seeds contains several classes of biologically active compounds that are responsible for numerous beneficial health effects. Epidemiological data have supported that the low incidence of cardiovascular and cancer diseases in Asian populations can be attributed to higher soy-based food consumption; with isoflavones being implicated in such health benefits (He and Chen, 2013). Isoflavones are a distinct group of naturally occurring secondary metabolites that are structurally similar to estrogens and have weak estrogen-like effects, which is the reason they are classified as phytoestrogens. They are present in almost all the members of the Leguminosae family, but they are also occasionally found in other angiosperm families (Raynaud et al., 2005; Mackova et al., 2006; Lapcik, 2007; Anderson and Wolf, 1995). Being a prominent member of the Leguminosae family, soybeans are one of the few common sources of dietary isoflavones in human nutrition. Soyisoflavones are major phenolic metabolites accumulated in soybean seeds, making soybeans a functional food (Li et al., 2016). Soyisoflavones exist in 12 different chemical forms and have been classified into two major groups based on conjugate functional groups, i.e. malonyl and acetyl glycosides, and four major groups, i.e. aglycones, glycosides, malonyl glycosides, and acetyl glycosides (Lee et al., 2004). Collectively, these phenolic metabolites have been associated with beneficial health effects, such as anticancer, antiestrogenic, and antioxidant activities, cardiovascular disease prevention, and improvement of bone health (Farina et al., 2006; Gil-Izquierdo et al., 2012; McCue and Kalidas, 2004). Such characteristics have significantly shifted the focus of soybean research towards a new dimension, i.e. functional soy foods. This has resulted in the escalated usage of soy-based disease preventing functional foods during last two decades (Cvejic et al., 2011).

Soyisoflavones are also involved in ontogenic tolerance to various biotic and abiotic stresses (Cheng et al., 2015). The stress responsive characteristics, coupled with health benefits, support

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the development of soybeans with higher isoflavone contents. The amount of total isoflavones in soybean have been implicated with genotype, the environment (particularly temperature), and the geographical location (Tsukamoto et al., 1995; Zhang et al., 2012). However, it was recently reported that genetic factors play a more significant role in soy isoflavone biosynthesis compared to that of other factors, i.e. geographic location and/or environment (Liu et al., 2017). To improve soy isoflavone contents, a logical choice would involve isoflavone profiling of wild soybeans (*Glycine soja* Sieb & Zucc.) that contain relatively high amounts of soy isoflavone as compared to cultivated soybeans (Li et al., 2016). Humans might have unintendedly selected soybeans with lower soy isoflavone contents during the domestication process. Hardened seed coats (an unwanted characteristic) has been reported to be associated with higher levels of phenolic compounds. Similarly, it is possible that soyisoflavone content and accumulation are linked with unfavourable traits, similar to the browning of mature pods being associated with higher antioxidant contents (Zhou et al., 2010; Qi et al., 2014; Li et al., 2016).

To explore the genetics of soyisoflavones, investigation of wild soybean accessions as a potential genetic resource would be promising (Nawaz et al., 2017; Tsukamoto et al., 2017; Munoz et al., 2017). Whole-genome sequencing has illustrated that wild soybeans maintain higher genetic diversity, whereas cultivated soybeans have lost almost half of their genetic diversity during the process of domestication and improvement (i.e. genetic diversity of wild soybeans was 2.94×10^{-3} compared to that of improved cultivars, $1.05 \times 10^{-}3$; Zhou et al., 2015). Exploring the variation in soy isoflavone profiles of wild soybeans from the primary centres of diversity, i.e. China, Korea, Japan, and the Russian Far East, could yield promising information that would aid ongoing breeding programs conducted to improve soy isoflavone content. Although soy isoflavone profiles of wild soybeans have been previously reported, neither the soy isoflavone content nor composition profiling studies focused on all representative natural habitats of wild soybeans (Devi et al., 2009; Genovese et al., 2005, 2006; Lee et al., 2003, 2008; Wang and Murphy, 1994). Large scale wild soybean germplasms remain poorly investigated in this regard. In this study, we selected wild soybeans among those available at CWLGC (Chung's Wild Legume Germplasm Center, Chonnam National University, Yeosu, South Korea) belonging to natural habitats from China, South Korea, Japan, and the Russian Far East, and explored the content and composition of soyisoflavones, i.e. daidzein, glycitin, genistin, malonyl-daidzein, malonyl-glycitin, and malonyl-genistin. The generated information will be very useful to the broader community of researchers working on health effects of soy foods, and improvement of soy isoflavone content through conventional breeding approaches, as well as modern genomics and genetic engineering tools.

2. Materials and methods

2.1. Plant material and sample preparation

Two-hundred and ninety-eight wild soybean germplasm accessions covering all the natural geographic habitats were carefully selected from the CWLGC, South Korea (Supplementary Table 1). Accessions were grown in an off-campus research farm (35.1800°N, 128.1076°E) during the growing season of 2016. Initially, six seeds of each accession were grown in commercial soil in 72 cell plug trays (each cell with $3.8 \times 3.8 \times 5.7$ cm size). Standard agronomic practices were observed with a row-to-row and plant-to-plant distance of 2 m to avoid seed mixing. Upon maturity, pods were harvested separately from each accession, thrashed, and further processed for isoflavone profiling. Briefly, 10 dried seeds of each accession were ground with a pestle and mortar. Soyisoflavones were extracted from fine ground seed powder with a 10-fold volume of aqueous methanol (Mt-OH) for 1 h at room temperature after pulverizing twice in a multi-bead shaker for 15 s. The extract was centrifuged at 12,000 rpm for 5 min and the supernatant was collected to use for HPLC analysis.

2.2. Isoflavone analysis by HPLC

HPLC analysis was conducted by following the method of Lee et al. (2008) on a HPLC-photodiode array detector and analyser system (LC9A, Shimadzu Corporation, Kyoto, Japan). A Develosil C-30 UG-3 analytical HPLC column (I.D. 2.0×150 mm) was used for quantitative analyses. We identified and quantified soyisoflavones by UV at a wavelength of 254 nm and adjusted the solvent flow rate to 0.15 mL/min with an injection volume of 20 μL. A mobile phase HPLC gradient was used: 0 min, 85% A/15% B; 0–30 min, 55% A/45% B, with washing 0% A/100% B, 30–35 min, and recycling to the initial condition 85% A/15% B 15 min, for a total analysis time of 60 min. Solvent A consisted of acetonitrile containing 0.1% 20 (v/v) formic acid, and solvent B was a 0.1% formic acid solution. The genuine standard of daidzin (Nakalaiteque, Japan) was dissolved with dimethylsulfoxide (DMSO) to prepare a 1 mg/mL solution and was further used to establish the calibration curve for the six crude soyisoflavones, i.e. daidzin, glycitin, genistin, malonyl daidzin, malonyl glycitin, and malonyl genistin. We used the crude soyisoflavone solution as a standard for HPLC identification and quantification after obtaining their high linearity. We then identified each of the six soyisoflavones by their retention times and the concentrations were measured by comparing their peaks with that of the standard.

2.3. Statistical analysis

Quantitative data of each soyisoflavone and total soyisoflavones (in this report the total soyisoflavone refers to the sum of the three common glycosides and three malonyl glycosides) were subjected to statistical analysis in SPSS (Chicago, IL, USA). Analysis of variance (ANOVA) and t-tests, with a significance level of $P < .05$, were computed for all countries and the six soyisoflavone components to evaluate the similarities and differences among wild soybean accessions from the four different countries. We employed Microsoft Excel (2013) for frequency distribution of total soyisoflavones. Principle component analysis (PCA) was also conducted with the selected wild soybean accessions. Pearson's correlation analysis was performed using the online tool Heatmapper (http://www.heatmapper.ca/; Babicki et al., 2016), which generated an output as a heatmap. We also generated heatmaps of total soyisoflavone content of each wild soybean accessions for each country.

3. Results and discussion

Because we knew all crop plants had a long domestication history, wild ancestral species are the primary sources of diversity to be explored and employed in the process of crop improvement (Brozynska et al., 2016). Wild soybeans contain higher antioxidant (soyisoflavones) content compared to cultivated soybeans (Li et al., 2016). This makes wild soybeans an ideal study material to investigate soyisoflavone content for improving health benefits of soy-based food products. The core collection at CWLGC was explored to determine the content of six soyisoflavones and total soyisoflavones in mature seeds. Identification and peak assignment of soyisoflavones in mature wild soybean seeds was accomplished by comparing their retention time with those of the standard (Fig. 1). We divided the accessions in four groups based on their place of