

Analysis of soyasaponin content and biosynthesis-related gene expression in young pea (*Pisum sativum* L.) sprouts

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Abstract In legumes, soyasaponins, one of triterpenoid saponins, are major components of secondary metabolites with a more diverse array of bioactive chemicals. Although the biosynthetic pathway of soyasaponins has been largely studied in soybean, the study on the soyasaponin contents and biosynthesis-related gene expression in pea (*Pisum sativum* L.) is poorly understood. Here, we found the accumulation of only soyasaponin Bb component in the sprouts of two Korean domestic pea cultivars (Dachung and Sachul). This pattern was consistent with our observation that increased expression of *PsUGT73P2* and *PsUGT91H4* genes, but not *PsCYP72A69*, could be responsible for biosynthesis of only soyasaponin Bb in pea by examining their gene expression. However, gradual accumulation of soyasaponin Bb at developmental stages was not consistent with the expression of *PsUGT73P2* and *PsUGT91H4*, suggesting that the changes of their protein activities may affect the accumulation patterns of soyasaponin Bb. We also revealed that the increased expression levels of *PsUGT73P2* and *PsUGT91H4* during light to dark transition led to increase of soyasaponin Bb contents. Collectively, our results provided a molecular basis of metabolic engineering for enhancing useful soyasaponin Bb metabolites in Korean domestic pea cultivars.

Keywords Pea, *PsCYP72A69*, *PsUGT73P2*, *PsUGT91H4*, Soyasaponin Bb

Introduction

Legumes in the *Fabaceae* family are the second most cultivated crops worldwide after cereals, and have been considered as important food ingredients of plant-based foodstuffs (Lu et al. 2020). Legumes as primary sources of nutritional components are enriched in proteins, carbohydrates, fibers, vitamins and minerals. In addition, they contain phytochemicals and antioxidants known as bioactive compounds, which include phytoestrogens, isoflavones, alkaloids, phytates, saponins, protease inhibitors, and α -galactosides (Pedrosa et al. 2020). These molecules contain a number of beneficial pharmacological properties, including cardio-protective, anti-carcinogenic, anti-inflammatory, anti-proliferative, anti-angiogenic, and estrogenic properties (Guang et al. 2014; Messina et al. 1994). Although legumes have numerous benefits for human health, there has been a significant decrease in legume consumption over recent years (Hughes et al. 2022). Thus, recent studies aimed to analyze bioactive compounds and enhance them in legumes for development of new legume products (Aguilera et al. 2009; Arribas et al. 2019).

In legumes, young germinated soybean sprouts contain a more diverse range of bioactive chemicals such as isoflavones, phenolic compounds and triterpenoids than their non-germinated counterparts (Choi et al. 2018; Ma et al. 2018; Mora-Escobedo et al. 2009; Wang et al. 2015). Of them, triterpenoid saponins are a main component of secondary metabolites, which are plentifully accumulated in plant specific tissues such as roots. The biosynthetic pathway of soyasaponins, one of triterpenoid saponins, has been extensively studied in soybean (Abe et al. 1993; Moses et al. 2014; Thimmappa

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et al. 2014). Previous reports have revealed that 2,3-oxidosqualane precursor is synthesized through the mevalonate pathway, and converted to soyasaponins through a biosynthetic pathway with at least 14 different enzymes, including one β -amyrin synthase (BAS), three cytochrome P450s (CYP450s), ten uridine 5'-diphosphate (UDP)-glycosyltransferases (UGTs), and one acetyltransferase. Structural diversity of the sugar numbers and types in triterpenoid saponins contributes to a variety of the biological and chemical activities (Bowles et al. 2005).

Because the common pea (*Pisum sativum* L.) plants in the legumes have a tolerance to low temperatures during germination and growth, their cultivation provides a cold season alternative when compared with soybean or bean plants. However, the correlation between soyasaponin contents and biosynthesis-related gene expression in pea have not yet been known. Therefore, the aim of this study was to investigate the accumulation patterns of soyasaponins and the expression levels of three soyasaponin biosynthetic genes in the sprouts of two Korean domestic pea cultivars (Dachung and Sachul).

Materials and Methods

Database Searching and Sequence Analysis

The sequence data of pea soyasaponin biosynthesis-related genes (*PsCYP72A69*, *PsUGT73P2*, and *PsUGT91H4*) were retrieved from the NCBI (<https://www.ncbi.nlm.nih.gov/>) and Plant Bioinformatics facility (<https://urgi.versailles.inra.fr/Species/Pisum/Pea-Genome-project>) databases. The *PsCYP72A69*, *PsUGT73P2*, *PsUGT91H4*, and *PsTubulin* genes used in this study are listed in Table 1.

Plant Materials and the Growth Conditions

Two pea cultivars, Dachung and Sachul, were kindly

provided by the Division of Crop Foundation (National Institute of Crop Science, Rural Development Administration, Jeonju, Korea). The seeds were sterilized in 70% ethanol (EtOH) containing 0.5% Triton X-100 for 15 min, thoroughly washed once in water, soaked in water at 23°C for 24 h, and then transferred to Sunshine Mix 5 (Sungro, Agawam, MA, USA). Germinated peas were prepared by incubation at 23°C for 6, 9, 12, 15, and 18 days in a dark growth chamber. For light to dark treatment, pea seedlings grown for 9 days at 23°C under long-day (LD) conditions (16 h light/8 h dark at a light intensity of 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were transferred to continuous dark or light chambers for 1 and 2 days at 23°C. Seedlings harvested after each treatment were ground to a powder with liquid nitrogen in a mortar and stored at -80°C until use. All experiments were performed in three biological replicates (independently harvested samples).

RNA Expression Analysis

Total RNA was extracted from the harvested samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA) as previously described (Yun et al. 2021), and the quality and quantity of the resulting RNA was checked using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Waltham, MA, USA). To analyze RNA expression, real time-quantitative PCR (RT-qPCR) analysis was performed.

Complementary DNA (cDNA) was synthesized from 5 μg RNA in accordance with the protocol of the ReverTra Ace qPCR RT Master Mix kit (Toyobo, Osaka, Japan), and RT-qPCR analysis was conducted in 96-well plates using a CFX real-time system (Bio-Rad, Hercules, CA, USA), THUNDERBIRD SYBR qPCR mix (Toyobo), and gene-specific RT-qPCR primers listed in Table 1 were designed using QuantPrime (<http://quantprime.mpimp-golm.mpg.de/>). *PsTubulin* gene was used as a stably reference gene. To determine the relative abundance of the transcripts, the data were analyzed using Bio-Rad CFX Manager

Table 1 Genes and oligonucleotides used for RT-qPCR analysis in this study

Gene name	Gene ID	Primers (5' to 3')	Direction
<i>PsCYP72A69</i>	Psat7g228360	TCCCTACTGGAAGCAAAGATGG	F
		TTATGTTTCCACTTTACGTATAATAATTTGAGC	R
<i>PsUGT73P2</i>	Psatoss5882g0200	CCTTTGTTTGCAGAACATTTTTTCAATG	F
		TCAACCTAATAGCATTCCCAATCTCT	R
<i>PsUGT91H4</i>	Psat1g218280	ATAGTGGATGAAGAAGGAAGTAGTTTTAG	F
		TCATTCTTTGTTGGAATTGGAAGC	R
<i>PsTubulin</i>	Xm_051045246.1	GCTCCCAGCAGTACAGGACTCT	F
		CAACCTCCTTGGTACTCATCTTGCC	R

(Bio-Rad). The student's *t*-test or Duncan's test were used to compare mean expression levels.

Ultra-high-performance liquid chromatography-changed aerosol detection (UHPLC-CAD) analysis

To prepare pea extracts, the pea seedlings harvested at different developmental stages or under light/dark transition conditions were dried in an oven at 57°C for 48 h, and then ground into powder using a blender (Electric Mixer SMX-W350, Seoul, Korea). As previously described (Yun et al. 2021), 20 ml of 80% methanol was added to 1 g of the ground samples and the mixtures were agitated for 24 h at 23°C. The supernatants were prepared by centrifugation (7,000 × g) for 5 min and then passed through Donex™ OnGuard™ II RP cartridges (Thermo Fisher Sci., MA, USA) to remove the hydrophobic substance. They were then filtered through a 0.22-μm filter unit. Soyasaponin contents were analyzed with a reverse-phase UHPLC (Dionex Ultimate 3000, Thermo Fisher Sci.) equipped with an Acclaim™ RSLC Polar Advantage II (2.2 μm 120 Å 2.1 × 150 mm) column. Following injection of 2 μL of sample, separated soyasaponin Aa, Ab, Ba, Bb, and Bb' was detected using a CAD (Corona Veo, Thermo Fisher Sci.). The used reagents were UHPLC grade, and soyasaponin Aa, Ab, Ba, Bb, and Bb' (ChemFaces, Wuhan, China) were used as reference substances. The student's *t*-test or Duncan's test were used to compare mean contents.

Results and Discussion

Accumulation patterns of soyasaponin Bb in pea sprouts

We previously found that the accumulation patterns of soyasaponin Aa and Ab were different in the Socheongja and Haepum cultivars (Korean domestic soybean cultivars) (Yun et al. 2021). To investigate the accumulation patterns of soyasaponin contents in pea plants within the legumes, we prepared the sprouts of two Korean domestic pea cultivars (Dachung and Sachul) grown for 15 days at 23°C under darkness conditions, and analyzed their soyasaponin contents using UHPLC-CAD analysis. We found that only soyasaponin Bb was accumulated in two Korean domestic pea cultivars (Fig. 1A). Taken together with a previous data (Yun et al. 2021), this result indicated that different soyasaponin types were exclusively accumulated between soybean and pea sprouts, although they belong to legumes. Our data also suggested that the expression levels of

soyasaponin biosynthetic genes or their protein activities may be different between soybean and pea plants.

Soyasaponin Bb accumulation and biosynthesis-related gene expression at developmental stages in pea sprouts

Based on the soyasaponin biosynthesis pathway identified in soybean plants (Abe et al. 1993; Moses et al. 2014; Thimmappa et al. 2014), we focused on four genes (*CYP72A69*, *UGT73B4*, *UGT73P2*, and *UGT91H4*), which are involved in the transition from soyasapogenol B to soyasaponin A or B types (Fig. 1B). We searched the NCBI and Plant Bioinformatics facility databases to find out four soyasaponin biosynthesis genes in pea, and successfully found the pea genes homologous to *CYP72A69* (*Psat7g228360*), *UGT73P2* (*Psatoss5882g0200*), and *UGT91H4* (*Psat1g218280*), but not *UGT73B4*, from public databases. Thus, we chose *PsCYP72A69*, *PsUGT73P2*, and *PsUGT91H4* for subsequent experiments.

To investigate whether soyasaponin Bb accumulation is affected by the expression changes of soyasaponin biosynthetic genes, we examined the soyasaponin Bb contents and the expression levels of three soyasaponin biosynthetic genes (*PsCYP72A69*, *PsUGT73P2*, and *PsUGT91H4*) at developmental stages in two pea sprouts (Dachung and Sachul) grown for 6, 9, 12, 15, and 18 days at 23°C under darkness conditions. UHPLC-CAD analysis showed that the accumulation patterns of soyasaponin Bb gradually increased from 6 to 18 days in two pea cultivars and their soyasaponin Bb contents were similar (Fig. 2A). However, RT-qPCR analyses revealed that the expression patterns of three soyasaponin biosynthetic genes were different (Fig. 2B). *PsCYP72A69* expression remained unchanged in both pea cultivars, although its expression in Dachung was statistically changed. However, *PsUGT73P2* and *PsUGT91H4* gradually decreased from 6 or 9 to 18 days. Soyasaponin A types' absence in two pea cultivars may be explained by our observation that unchanged expression levels of *PsCYP72A69* in them. Furthermore, gradual accumulation of soyasaponin Bb may be resulted from the changes of *PsUGT73P2* and *PsUGT91H4* activities.

Effect of light to dark transition on soyasaponin biosynthesis and accumulation

Because light is important for inducing or regulating plant metabolism, two pea cultivar seedlings (Dachung and Sachul) grown for 9 days at 23°C under LD conditions were transferred to continuous dark or light chambers for

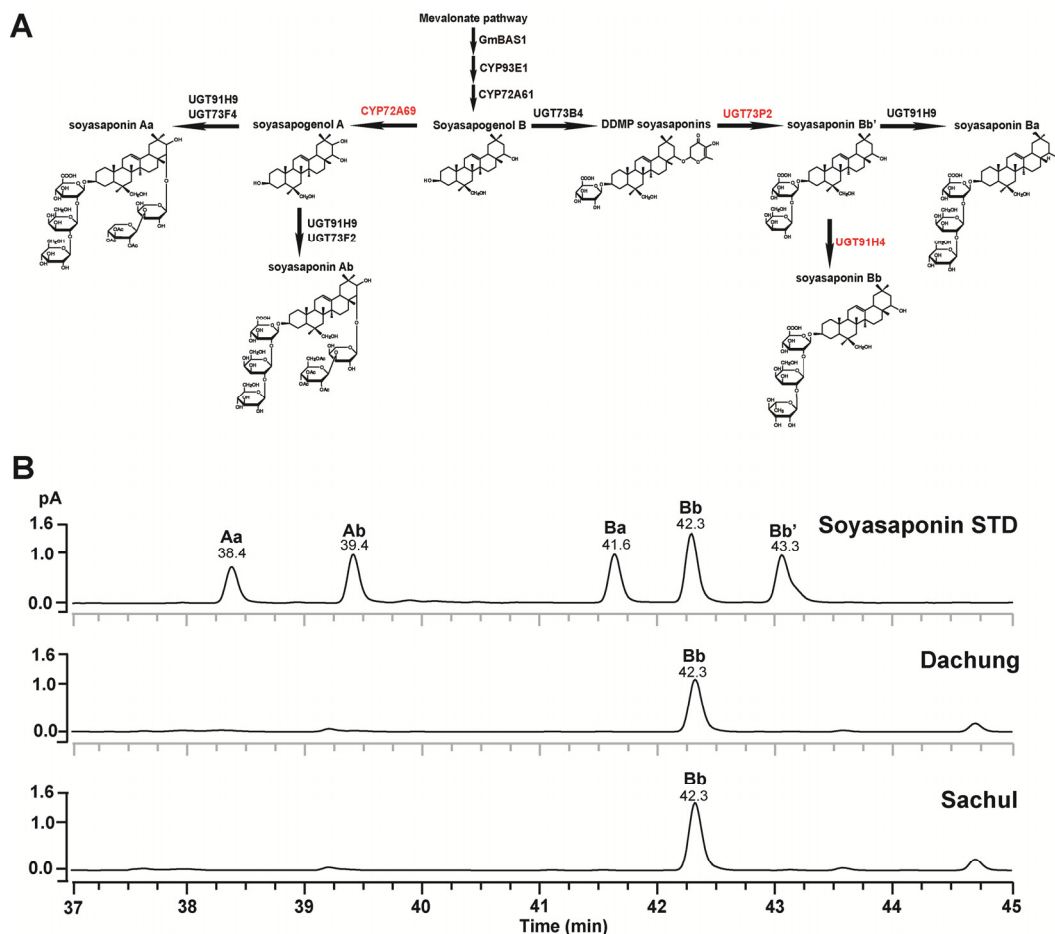


Fig. 1 Accumulation of only soyasaponin Bb in young pea sprouts. (A) Detection of only soyasaponin Bb in two Korean domestic pea cultivars (Dachung and Sachul). Two pea cultivars were grown for 15 days at 23°C under darkness conditions. The accumulation of four different soyasaponin types (Aa, Ab, Ba, Bb, and Bb') was determined by UHPLC-CAD analysis. (B) Soyasaponin biosynthesis pathway identified in soybean. The genes (*CYP72A69*, *UGT73P2*, and *UGT91H4*) used in this study are shown in red. This pathway was modified from our previous report (Yun et al. 2021)

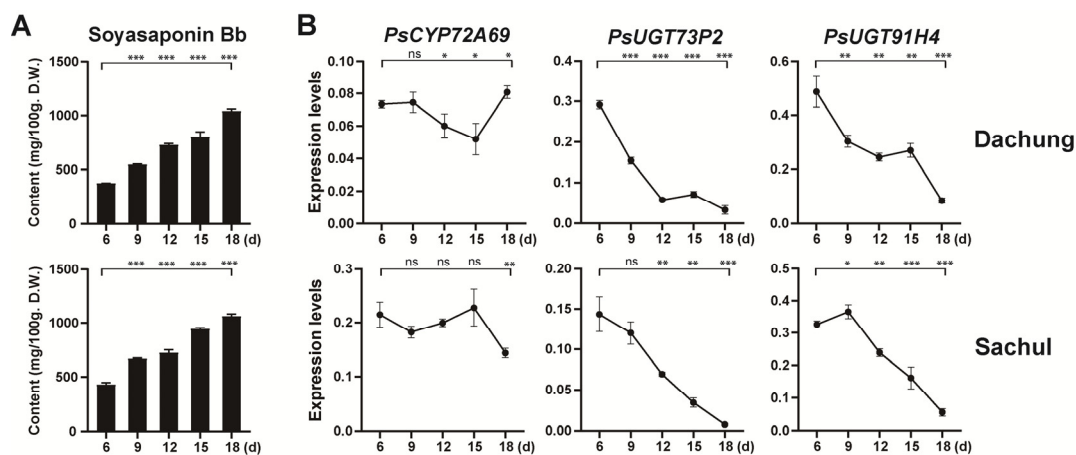


Fig. 2 Expression of *PsUGT73P2* and *PsUGT91H4* in young pea sprouts at developmental stages affects accumulation of soyasaponin Bb contents. (A) Soyasaponin Bb contents of two Korean domestic pea cultivars (Dachung and Sachul). Two pea cultivars (Dachung and Sachul) were grown for 6, 9, 12, 15, and 18 days at 23°C under darkness conditions. The accumulation of soyasaponin Bb was determined by UHPLC-CAD analysis. The error bars indicate the standard error of the mean (SEM) of three biological replicates. (B) Expression levels of three soyasaponin biosynthetic genes. *PsCYP72A69*, *PsUGT73P2*, and *PsUGT91H4* expression was determined by RT-qPCR analysis. *PsTubulin* was used as a reference gene. Asterisks indicate significant differences, when compared to content or the expression level at Day 6 (Student's t-test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). ns, not significant

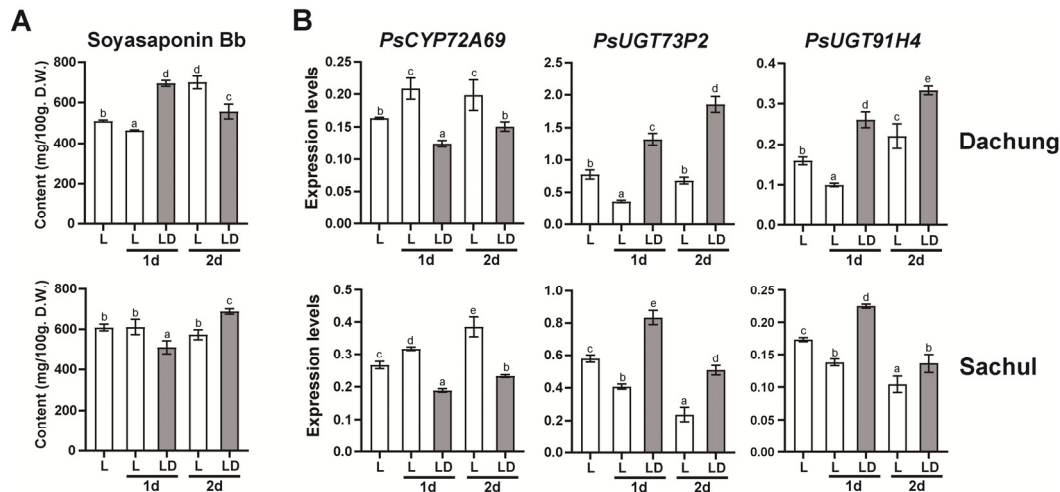


Fig. 3 Increased expression of *PsUGT73P2* and *PsUGT91H4* during light to dark transition in young pea sprouts leads to increased soyasaponin Bb contents. (A) Soyasaponin Bb contents of two Korean domestic pea cultivars (Dachung and Sachul). The seedlings of two pea cultivars were grown for 9 days at 23°C under long-day (LD) conditions. Pea seedlings were subsequently incubated for 1 and 2 days under continuous dark (D) or light (L) conditions. Pea seedlings incubated for 9 days at 23°C under LD conditions were used as a control. The error bars indicate the SEM of three biological replicates. Different lower cases letters indicate significant difference ($P < 0.05$) according to Duncan's test. (B) RT-qPCR analyses of three soyasaponin biosynthetic genes. *PsTubulin* was served as a reference gene.

1 and 2 days, and then soyasaponin Bb contents and three soyasaponin biosynthetic genes were measured. We found that the accumulation patterns of soyasaponin Bb increased for 1 and 2 days in Dachung and Sachul cultivars, respectively (Fig. 3A). Furthermore, the light to dark transition differentially affected the expression levels of three soyasaponin biosynthetic genes. The expression of *PsCYP72A69* decreased in both pea cultivars, whereas *PsUGT73P2* and *PsUGT91H4* increased (Fig. 3B). These results indicated that the increased expression of *PsUGT73P2* and *PsUGT91H4* during light to dark transition affected soyasaponin Bb contents.

Conclusion

Soyasaponins, one of the triterpenoid saponins, are used as medicinal and food ingredients, and also served as plant secondary metabolites made against biotic and abiotic stress conditions. Accumulations of soyasaponins, and the polymorphisms and the expression levels of their biosynthetic genes have been extensively studied in soybean plants. However, the study on the soyasaponin contents and their biosynthetic gene expression in pea have not yet been known. Here, we found that only soyasaponin Bb was exclusively accumulated in the sprouts of two Korean pea cultivars (Dachung and Sachul), compared with our previous study in soybean sprouts (Fig. 1A) (Yun et al. 2021). Furthermore, these accumulation patterns of soyasaponin

Bb were correlated with differential expression patterns between *PsCYP72A69* and *PsUGT73P2* or *PsUGT91H4* (Fig. 2). Lastly, the expression of *PsUGT73P2* and *PsUGT91H4* during light to dark transition was increased, thereby inducing increase of soyasaponin Bb contents (Fig. 3). Based on the our observation that gradual increase of soyasaponin Bb accumulation at developmental stages was not correlated with the changes of *PsUGT73P2* and *PsUGT91H4* expression, we could not exclude the possibility that their protein activities may affect the change of soyasaponin Bb contents. Thus, further study on the corresponding proteins' activities at the post-transcriptional level is needed.

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Conflict of interest

The authors declare that they have no conflict of interest.

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