

# Saponarin content and biosynthesis-related gene expression in young barley (*Hordeum vulgare* L.) seedlings

HanGyeol Lee · So-Yeun Woo · Ji-Eun Ra · Kwang-Sik Lee · Woo Duck Seo · Jeong Hwan Lee

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**Abstract** Flavonoids are widely distributed secondary metabolites in plants that have a variety biological functions, as well as beneficial biological and pharmacological activities. In barley (*Hordeum vulgare* L.), for example, high levels of saponarin accumulate during primary leaf development. However, the effect of saponarin biosynthetic pathway genes on the accumulation of saponarin in barley is poorly understood. Accordingly, the aim of the present study was to examine the saponarin contents and expression levels of saponarin biosynthetic pathway genes [*chalcone synthase (CHS)*, *chalcone isomerase (CHI)*, and *UDP-Glc:isovitexin 7-O-glucosyltransferase (OGT)*] during early seedling developmental and under several abiotic stress conditions. Interestingly, the upregulation of *HvCHS*, *HvCHI*, and *HvOGT* during early development was associated with saponarin accumulation during later stages. In addition, exposure to abiotic stress conditions (e.g., light/dark transition, drought, and low or high temperature) significantly affected the expression of *HvCHS* and *HvCHI* but failed to affect either *HvOGT* expression or saponarin accumulation. These findings suggested that the expression of *HvOGT*, which encodes an enzyme that catalyzes the final step of saponarin biosynthesis, is required for saponarin accumulation. Taken together, the results of the present study provide a basis for metabolic engineering in barley plants, especially in regards to enhancing the contents of useful secondary metabolites, such as saponarin.

**Keywords** Barley, *Chalcone isomerase*, *Chalcone synthase*, Saponarin, *UDP-Glc:isovitexin 7-O-glucosyltransferase*

## Introduction

Barley (*Hordeum vulgare* L.) is one of the most important crop plants worldwide and has been cultivated since ancient times for a variety of uses, especially as animal feed, human food, and malting substrate (Honsdorf et al. 2014). However, even though the production of other major cereal crops (e.g., maize, rice, and wheat) increases continuously, barley production has declined over the past two decades. Interestingly, though, since multiple studies have reported the beneficial effects of barley leaves on human health can be attributed to potent antioxidant activities (Kamiyama and Shibamoto 2012), barley is regaining research attention as a potential functional food.

Plant secondary metabolites have been used as important source of active traditional medicine for centuries. Flavonoids, as one of the most abundant natural secondary metabolites, are found in a variety of vegetative and reproductive tissues, including leaves, roots, flowers, and fruits (Middleton 1998). Such plant-derived flavonoids possess a variety of beneficial biological and pharmacological properties, including antioxidant, cardio-protective, anti-carcinogenic, anti-inflammatory, anti-proliferative, anti-angiogenic, and estrogenic properties (Seo et al. 2013). Saponarin, for example, is a naturally occurring diglycoside flavone (apigenin6-*C*-glucosyl-7-*O*-glucoside) that is likely responsible for the immunostimulatory, hypocholesterolemic, anti-carcinogenic, anti-inflammatory, anti-microbial, anti-protozoan, molluscicidal, and anti-oxidant properties of primary barley leaves (Cushnie and Lamb 2005; Hertog et al. 1993; Mojzisoava et al. 2006; Moses et al. 2014; Seo et al. 2014).

Because polyphenolic compounds exhibit such a wide

H. G. Lee · J. H. Lee (✉)  
Division of Life Sciences, Jeonbuk National University, 567  
Baekje-daero, Deokjin-gu, Jeonju, Jeollabuk-do 54896, Republic  
of Korea  
e-mail: jhwanlee90@jbnu.ac.kr

S.-Y. Woo · J.-E. Ra · K.-S. Lee · W. D. Seo  
Division of Crop Foundation, National Institute of Crop Science,  
Rural Development Administration, Jeollabuk-do 55365, Republic  
of Korea

range of biological functions in plants, it is reasonable to question whether flavonoids could affect the yield stability of plants exposed to environmental stressors. In barley, genetic information and resources regarding flavonoid biosynthetic pathways are already available. Peukert et al. (2013), for example, partially sequenced the *chalcone synthase* (*CHS*), *phenylalanine ammonia-lyase* (*PAL*), *cinnamate 4-hydroxylase* (*C4H*), *flavanone 3-hydroxylase* (*F3H*), and *dihydroflavonol 4-reductase* (*DFR*) genes of 16 barley genotypes and developed single nucleotide polymorphism (SNP) markers. However, the genes of many other enzymes involved in flavonoid biosynthesis have yet to be identified. For instance, the genes involved in the conversion of naringenin to isovitexin (apigenin6-*C*-glucoside) have not been identified in barley (Marinova et al. 2007). Furthermore, the lack of suitable mutants and the poor characterization of existing mutant lines in barley impede the identification of new genes (Jende-Strid 1993; Reuber et al. 1997).

A variety of genes involved in flavonoid biosynthesis are subject to both transcriptional and post-translational regulation (Lepiniec et al. 2006; Weisskopf et al. 2006). For example, *CHS*, which catalyzes the first step in flavonoid synthesis, is regulated at the levels of transcription, translation, and enzymatic activity (Block et al. 1990; Hartmann et al. 2005; Hartmann et al. 1998; Knogge and Weissenböck 1986), and *CHS* expression is strongly affected by environmental stimuli, such as light and elicitors (Christie and Jenkins 1996; Faktor et al. 1997; Schulze-Lefert et al. 1989; Wingender et al. 1989). In addition, a variety of genes involved in the flavonoid biosynthetic pathway are differentially expressed in a spatio-temporal manner (Hutzler et al. 1998; Schulz and Weissenböck 1986; 1988). This suggests that the regulation of flavonoid biosynthetic pathways is more complicated than expected.

Even though saponarin in the flavonoid compounds of

barley has highly accumulated during primary leaf development (Reuber et al. 1996), the regulation of saponarin biosynthesis has yet to be established. Accordingly, the aim of the present study was to investigate the relationship between saponarin content and the expression levels of three saponarin biosynthetic genes, namely *CHS*, *chalcone isomerase* (*CHI*), and *UDP-Glc:isovitexin 7-O-glucosyltransferase* (*OGT*; hereafter, *HvCHS*, *HvCHI*, and *HvOGT*), in young barley seedlings. The effects of abiotic stressors (e.g., light/dark transition, drought, and low or high temperature) on saponarin content and gene expression were also investigated.

## Materials and Methods

### Database Searching and Sequence Analysis

The sequence data of barley saponarin biosynthesis-related genes (*HvCHS*, *HvCHI*, and *HvOGT*) were collected from the NCBI (<https://www.ncbi.nlm.nih.gov/>) and Phytozome (<https://phytozome.jgi.doe.gov/pz/portal.html>) databases. The *HvCHS*, *HvCHI*, and *HvOGT* genes used in this study are listed in Table 1. For multiple sequence alignments, the nucleotide or amino acid sequences of *HvCHS* and *HvOGT* were performed using a ClustalW (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) with default parameters.

### Plant Materials and the Growth Conditions

Barley (*Hordeum vulgare* L.) seeds were soaked in water for 1 d, germinated in the dark over 2 d, transferred to soil, and then maintained in a growth room (23°C, 16-h photoperiod, light intensity of 120 mmol m<sup>-2</sup> s<sup>-1</sup>). Leaves were collected for analysis over a 12-d period.

**Table 1** Saponarin biosynthesis-related genes analyzed in this study

Gene Name	Transcript name	Chromosome localization	Direction	Designation for this study
<i>Chalcone synthase</i>	HORVU2Hr1G116390.2	Chr2H:737966163..737969220	R	<i>HvCHS1</i>
	HORVU2Hr1G004170.4	Chr2H:9398287..9446222	R	<i>HvCHS2</i>
	HORVU2Hr1G005220.3	Chr2H:11395530..11400280	R	<i>HvCHS3</i>
<i>Chalcone isomerase</i>	HORVU5Hr1G112670.5	Chr5H:639991759..639992903	F	<i>HvCHI</i>
<i>UDP-Glc:isovitexin 7-O-glucosyltransferase</i>	HORVU7Hr1G031800.2	Chr7H:65071583..65073314	R	<i>HvOGT1</i>
	HORVU3Hr1G110110.2	Chr3H:680047819..680049439	R	<i>HvOGT2</i>

For the light/dark transition treatment, 15-d-old barley plants that had been grown under continuous light conditions were transferred to a dark chamber at 23°C for 1 or 2 d. For the drought treatment, 15-d-old barley plants were removed from the soil and air-dried on a filter paper in a growth chamber at 23°C for 1 or 2 d. For the low and high-temperature treatments, 15-d-old barley plants were placed in dark chambers at 4°C and 37°C, respectively, for 1 or 2 d. After harvesting, the samples were immediately frozen in liquid nitrogen for subsequent analysis. All experiments involved three biological replicates (independently harvested samples).

#### Ultra-high performance liquid chromatography (UHPLC) analysis

To prepare barley seedling extracts, 0.5 ~ 1 g dried barley seedlings were extracted in 20 mL 80% methanol (v/v) with shaking at room temperature for 1 d. The saponarin contents of the extracts were measured using UHPLC with a UV detector (Dionex Ultimate 3000; Thermo Scientific, Waltham, MA, USA; Seo et al. 2014). Chromatographic separation was performed using a reversed-phase HPLC column (ACQUITY BEH C18, 2.1 mm × 100 mm, Waters, Milford, MA, USA) at 35°C, with 0.1% TFA in water (A) and acetonitrile (B) as the mobile phases and with a flow rate of 0.5 mL/min. The gradient program was as follows: 0 ~ 3 min, 3% B; 3 ~ 10 min, 3 ~ 15% B; 10 ~ 13 min, 15 ~ 30% B; 13 ~ 15 min, 30 ~ 50% B; 15 ~ 16 min, 50 ~ 90% B; 16 ~ 18 min, 90% B; 18 ~ 20 min, 90 ~ 3% B. The injection volume was 2 µL, and the detection wavelength was 325 nm. The saponarin content of each sample was identified by directly

comparing the retention times to those of saponarin standards (Extrasynthese, Lyon, France). Three technical duplicates were performed for each of the three biological replicates (independently harvested samples). Statistical analyses were performed using SPSS for Windows (version 12.0; SPSS, Seoul, Korea). The student's *t*-test was used to compare mean saponarin contents.

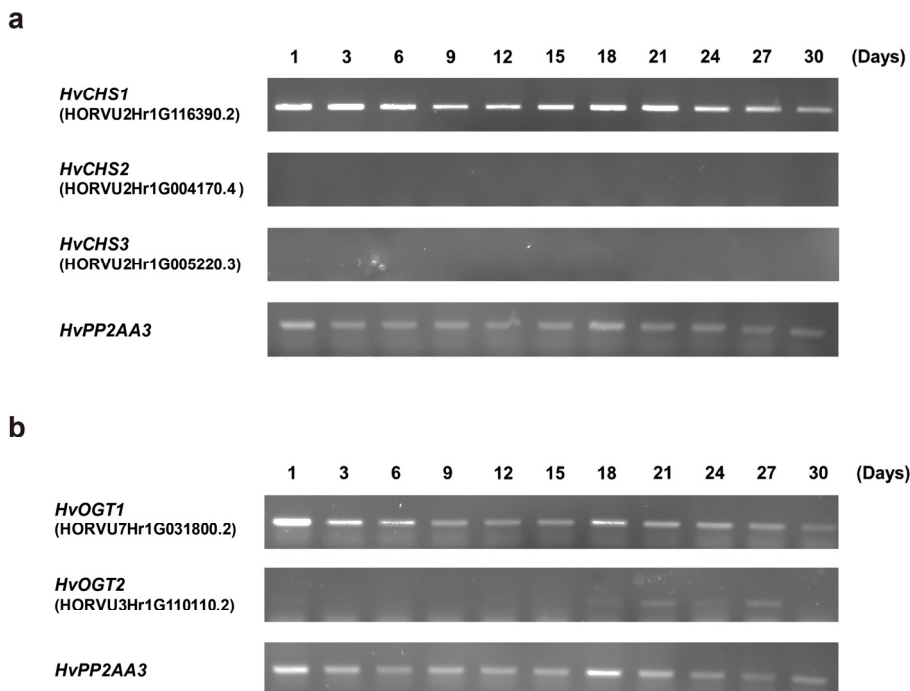
#### RNA Expression Analysis

Total RNA was extracted from the samples using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the quality of the resulting RNA was assessed using a Nanodrop ND-2000 spectrophotometer (Nanodrop Technologies, Waltham, MA, USA). Only high-quality RNA samples (A260/A230 > 2.0 and A260/A280 > 1.8) were used for subsequent experiments. To check RNA expression, Reverse Transcription-polymerase chain reaction (RT-PCR) or real time-quantitative PCR (RT-qPCR) analyses were performed.

Complementary DNA (cDNA) was synthesized from 5 µg RNA, following 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 (Table 2) were designed using QuantPrime (<http://quantprime.mpimp-golm.mpg.de/>) and mRNA sequences from the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/>): *HvCHS1* (accession numbers: AK248641, AK354067, and AK357384), *HvCHI* (accession number: AK374952), and *HvOGT1* (accession numbers: AK371730 and AK375231). In accordance with the best-established RT-qPCR practices

**Table 2** Oligonucleotides used for RT-PCR and RT-qPCR analysis in this study

Gene name	Gene ID	Primers (5' to 3')	Direction
<i>HvCHS1</i>	AK248641	TTTCATCATGGACGAGATGC	F
		TGTGGAGGACGACGGTCT	R
		CGCGACACAGACTAACAGGA	R
<i>HvCHS2</i>	AK357384	TTTCATCATGGACGAGATGC	F
		TCAAACGACCACGATCTCAA	R
<i>HvCHS3</i>	AK354067	TTTCATCATGGACGAGATGC	F
		GGAATGCGTAAGCCAAGAGA	R
<i>HvCHI</i>	AK374952	AACTGTGTCGCGTACTGGAA	F
		GGGGAGTGCCTGAAGAGTAT	R
<i>HvOGT1</i>	AK375231	TGTCGAGATAGTTTGTCCGGTGTG	F
		ATCACGCCGTCGGATGGATATCTG	R
<i>HvOGT2</i>	AK371730	CAGCAGGAGGAACCTGGATA	F
		CTGGCACCAAGCAGCAGAT	R
<i>HvPP2AA3</i>	AK251150	ACATCCCTCTGCTGGCTAGT	F
		GCCAGTCGCTTCAAGTTGTT	R



**Fig. 1** Expression levels of *HvCHS1/2/3* and *HvOGT1/2* members during barley developmental stages. (a) Expression levels of *HvCHS1/2/3* members. Barley plants grown at 23°C under long-day (LD) conditions were harvested at 1, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 d, and expression levels were analyzed using RT–PCR. *HvPP2AA3* was used as an internal control. (b) Expression levels of *HvOGT1/2* members

(Janska et al. 2013; Zhang et al. 2018), *HvPP2AA3* (accession number: AK251150) was included as a stably expressed reference gene, and three technical duplicates were performed for each of the three biological replicates. To determine the relative abundance of the transcripts, the data were analyzed using Bio-Rad CFX Manager (Bio-Rad). Statistical analyses were performed using SPSS for Windows (version 12.0; SPSS, Seoul, Korea). The student's *t*-test was used to compare mean expression levels.

## Results and Discussion

### Identification of saponarin biosynthetic pathway genes in barley

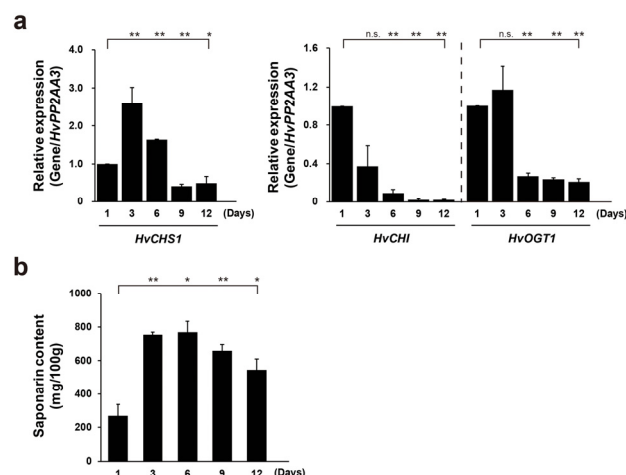
To identify saponarin biosynthesis-related genes in barley, we searched the NCBI and Phytozome databases. The present study showed that three *HvCHSs*, one *HvCHI*, and two *HvOGTs* in barley genome (Table 1). Based on sequence similarity to other plant homologs, we designated the three *HvCHSs* as *HvCHS1* [HORVU2Hr1G116390.2 (AK248641)], *HvCHS2* [HORVU2Hr1G004170.4 (AK357384)], and *HvCHS3* [HORVU2Hr1G005220.3 (AK354067)], and the two *HvOGTs* as *HvOGT1* [HORVU7Hr1G031800.2 (AK375231)] and

*HvOGT2* [HORVU3Hr1G110110.2 (AK371730)].

We investigated the temporal expression patterns of three *HvCHSs* and two *HvOGTs* during the developmental stages. Only *HvCHS1* transcripts, but not *HvCHS2* and *HvCHS3* transcripts, were expressed in all developmental stages (Fig. 1a). *HvOGT1* transcripts were expressed in all stages, whereas *HvOGT2* transcripts were detected in later stages (from 18 to 27 d) (Fig. 1b). Furthermore, higher levels of *HvCHS1* and *HvOGT1* expression were seen in early stages (from 1 to 6 d). Because higher accumulation of saponarin is found in earlier developmental stages (Reuber et al. 1996), we chose *HvCHS1*, *HvCHI*, and *HvOGT1* for subsequent experiments.

### Saponarin biosynthesis and accumulation in barley seedlings

Because saponarin accumulates to high levels as the major flavonoid compound during primary barley leaf development (Reuber et al. 1996), the expression of saponarin biosynthetic pathway genes in young barley seedlings were examined in detail. Barely plants were grown at 23°C under LD conditions and harvested after 1, 3, 6, 9, or 12 d. Expression analysis revealed that *HvCHS1* expression increased significantly at Day 3 and then decreased to Day 12, whereas *HvCHI* and *HvOGT1* expression peaked on either Day 1



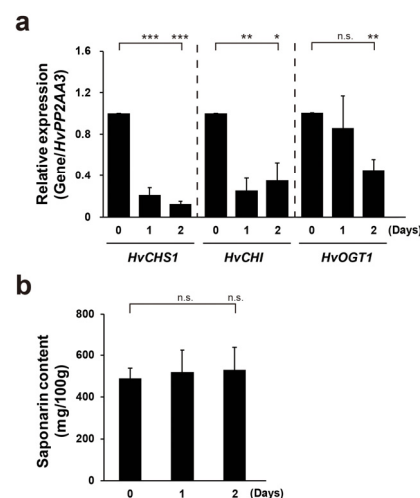
**Fig. 2** Saponarin content and related gene expression in young barley seedlings. (a) Expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1*. Barley plants grown at 23 °C under LD conditions were harvested at 1, 3, 6, 9 and 12 d, and expression levels of *HvCHS1*, *HvCHI* and *HvOGT1* in each sample were analyzed using RT–qPCR. The expression levels of each gene at Day 1 were set to 1. Values and error bars indicate mean  $\pm$  SEM values ( $n = 3$ ). Asterisks indicate significant differences, when compared to the expression level at Day 1 (Student’s *t*-test, \* $P < 0.05$ , \*\* $P < 0.01$ ). n.s., not significant. (b) Saponarin content of young barley seedlings. The accumulation of saponarin was determined by UHPLC analysis

or Day 3 days and then decreased rapidly to Day 12 (Fig. 2a). These results are consistent with previous reports that *CHS* and *CHI* expression are developmentally regulated (Schulz and Weissenböck 1988) and that *CHS* protein is present in young rice leaves but not in old leaves (Reddy et al. 1996).

The saponarin contents of young barley seedling extracts were also investigated. UHPLC analysis revealed that saponarin content highly accumulated at 3 and 6 d and slightly reduced until 9 and 12 d (Fig. 2b). These results indicated that higher expression of three saponarin biosynthetic genes at 1 or 3 d caused high accumulation of saponarin during early developmental stages of barley seedlings.

#### Effect of photoperiod on saponarin biosynthesis and accumulation

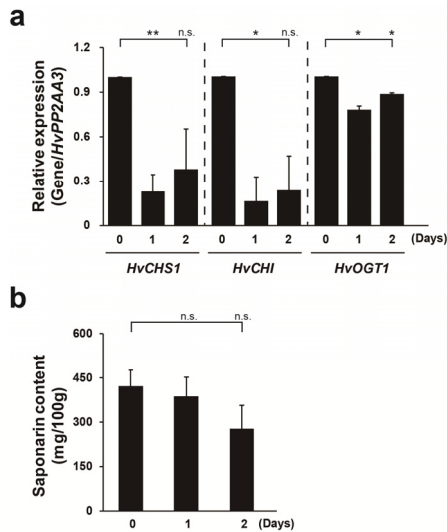
Because light/dark conditions have been reported to affect *CHS* and *CHI* expression (Dao et al. 2011; Wang et al. 2012) and because light is important for inducing or regulating plant metabolism (Yang et al. 2018), 15-d-old barley seedlings were transferred to a dark chamber for 1 or 2 d, and then *HvCHS1*, *HvCHI*, and *HvOGT1* expression were measured. Expression analysis revealed that *HvCHS1* and *HvCHI* expression decreased dramatically after the light/dark transition, whereas *HvOGT1* expression declined after 2 d under



**Fig. 3** Effect of light/dark transition on the saponarin content and related gene expression of young barley seedlings. (a) Expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1*. Fifteen-d-old barley plants grown at 23°C under continuous light conditions were transferred to dark conditions for 1 or 2 d, and the expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1* in each sample were analyzed using RT–qPCR. The expression levels of each gene at Day 0 were set to 1. Values and error bars indicate mean  $\pm$  SEM values ( $n = 3$ ). Asterisks indicate significant differences, when compared to the expression level at Day 0 (Student’s *t*-test, \* $P < 0.05$ , \*\* $P < 0.01$ ). n.s., not significant. (b) Saponarin content of light-stressed barley seedlings. The accumulation of saponarin was determined by UHPLC analysis.

dark conditions (Fig. 3a). Many studies have reported that *CHS* and *CHI* promoters contain the CACGTG motif (i.e., G-box), which is associated with important light responses (Kaulen et al. 1986; Schulze-Lefert et al. 1989; Staiger et al. 1989), and other regulatory domains, such as the Box I, Box II, Box III, Box IV, or H-box (CCTACC) domains, which are also found in the *CHS* promoter region, are also involved in light responses (Block et al. 1990; Lawton et al. 1990; Weissshaar et al. 1991). This result was consistent with the aforementioned findings.

The effect of light/dark transition on the saponarin contents of young barley seedling extracts was also investigated. However, the treatment had no effect on saponarin content (Fig. 3b). Given the finding that *in vitro* translation products of oat *CHS* mRNA are highly found at the dark or light phase (Knogge and Weissenböck 1986), it was expected that neither light nor dark would directly influence the translation of certain flavonoid biosynthetic genes, even if light exposure affects the expression of some flavonoid biosynthetic genes. These results indicate that the reduced *HvCHS1*, *HvCHI*, and *HvOGT1* expression of the light-to-dark transitioned barley seedlings did not affect saponarin content, which suggests that *HvCHS1*, *HvCHI*, and *HvOGT1* translation still occurs in darkness.

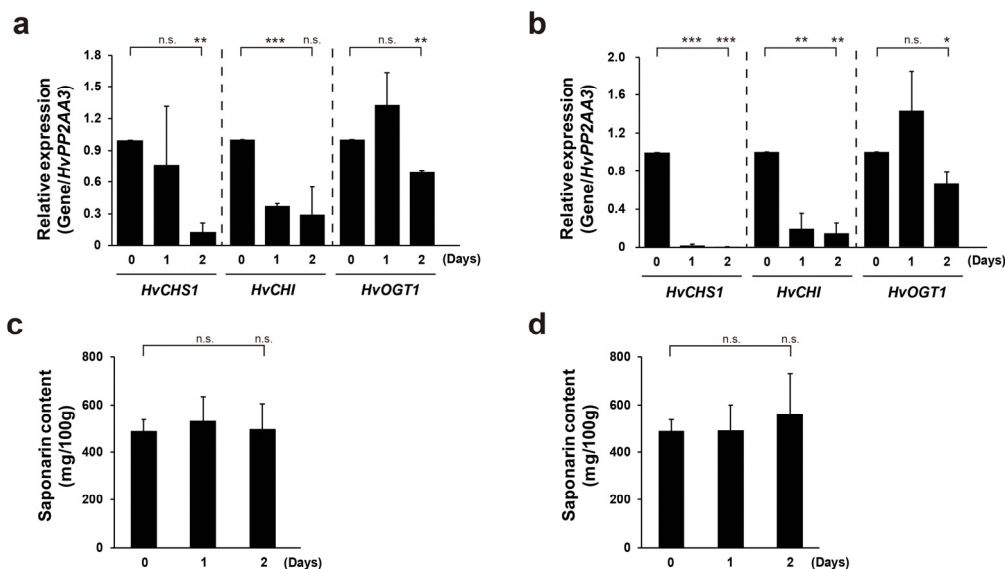


**Fig. 4** Effect of drought on the saponarin content and related gene expression of young barley seedlings. (a) Expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1*. Fifteen-d-old barley plants grown at 23°C under long day (LD) conditions were subjected to dehydration conditions for 1 or 2 d, and the expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1* in each sample were analyzed using RT-qPCR. The expression levels of each gene at Day 0 were set to 1. Values and error bars indicate mean  $\pm$  SEM values ( $n = 3$ ). Asterisks indicate significant differences, when compared to the expression level at Day 0 (Student's  $t$ -test, \* $P < 0.05$ , \*\* $P < 0.01$ ). n.s., not significant. (b) Saponarin content of drought-stressed barley seedlings. The accumulation of saponarin was determined by UHPLC analysis

#### Effect of abiotic stress on saponarin biosynthesis and accumulation

Because drought and temperature are the main environmental factors that affect the production of secondary metabolites, such as flavonoids, which are produced to cope with the oxidative stress generated by abiotic stresses (Yang et al. 2018), 15-d-old barley seedlings were exposed to dehydration conditions for 1 or 2 d, and then *HvCHS1*, *HvCHI*, and *HvOGT1* expression were measured. Expression analysis revealed that *HvCHS1* and *HvCHI* expression decreased dramatically, whereas *HvOGT1* expression was unaffected (Fig. 4a). The saponarin content of the dehydrated barley seedlings was slightly lower at Day 2, although not significantly (Fig. 4b).

To investigate whether low or high temperatures affect saponarin content or saponarin biosynthetic gene expression, 15-d-old barley seedlings were transferred to low- or high-temperature conditions for 1 or 2 d, and *HvCHS1*, *HvCHI*, and *HvOGT1* were measured. The expression patterns of *HvCHS1*, *HvCHI*, and *HvOGT1* were affected similarly by low- and high-temperature conditions. More specifically, *HvCHS1* and *HvCHI* expression declined dramatically, whereas *HvOGT1* expression remained stable (Fig. 5a, b). However, neither low- nor high-temperature conditions significantly affected saponarin content (Fig. 5c, d). These



**Fig. 5** Effect of low and high temperature on the saponarin content and related gene expression of young barley seedlings. (a and b) Expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1*. Fifteen-d-old barley plants grown at 23°C under long day (LD) conditions were transferred to low (4°C, a) or high (37°C, b) temperature conditions for 1 or 2 d, and the expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1* in each sample were analyzed using RT-qPCR. The expression levels of each gene at Day 0 were set to 1. Values and error bars indicate mean  $\pm$  SEM values ( $n = 3$ ). Asterisks indicate significant differences, when compared to the expression level at Day 0 (Student's  $t$ -test, \* $P < 0.05$ , \*\* $P < 0.01$ ). n.s., not significant. (c and d) Saponarin content of low (4°C, c) and high (37°C, d) temperature-stressed barley seedlings. The accumulation of saponarin was determined by UHPLC analysis.

results indicate that the reduced expression levels of *HvCHS1* and *HvCHI*, but not *HvOGT1*, were not correlated with saponarin accumulation under abiotic stress conditions.

Saponarin is synthesized from isovitexin (apigenin6-*C*-glucoside) after the addition of glucose in the 7-*O* position catalyzed by HvOGT as a soluble UDP-Glc:flavone glucosyltransferase (Blume et al. 1979). Because HvOGT catalyzes the final step of saponarin synthesis (Marinova et al. 2007), the stable expression of *HvOGT1* under abiotic stress conditions, such as drought and extreme temperature (Figs. 4 and 5), could maintain the level of saponarin content without significant change.

## Conclusions

Flavonoids, such as saponarin, are used as medicinal ingredients and food additives and also served as plant secondary metabolites that provide protection against biotic and abiotic stresses. Even though saponarin mainly accumulates during early stages of barley leaf development, the correlation between saponarin content and the expression of saponarin biosynthetic pathway genes is not well understood. In the present study, the upregulation of *HvCHS1*, *HvCHI*, and *HvOGT1* during early stages was associated with saponarin accumulation at later stages. In addition, exposure to abiotic stresses significantly affected the expression of *HvCHS1* and *HvCHI* but failed to affect either *HvOGT1* expression or saponarin accumulation. Even though the expression levels of *HvCHS1*, *HvCHI*, and *HvOGT1* under abiotic stress were not correlated with saponarin content, the results of the present study may provide a potential and profitable way to increase the accumulation of bioactive compounds, such as saponarin, in young barley seedlings.

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