Article

Effect of Abiotic Signals on the Accumulation of Saponarin in Barley Leaves in Hydroponics Under Artificial Lights

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ABSTRACT: Functional flavonoid production is a new agenda in the agricultural industry, and young barley leaves (YBL) are one of the highlighted crops due to their health-beneficial flavonoid, saponarin. For the year-round cultivation of a high saponarin content of YBL, abiotic signal effects on the biosynthesis and metabolism in YBL need to be understood clearly. In this research, the effects of reactive oxygen species (ROS)-related abiotic signals, such as light, potassium, and sodium, were investigated on the biosynthetic metabolism in YBL cultivation under artificial lights. A higher quantity of blue-rich white light (6500 K of light temperature) irradiation enhanced ROS levels and the related enzyme activities (APX and CAT), as well as photosynthesis and saponarin amount, while red-rich white light (3000 K of light temperature) increased the photosynthesis only. In addition, 1.0 g L^{-1} K$^+$ treatment in water slightly reduced ROS levels and increased saponarin accumulation in YBL. These blue-rich light and K$^+$ supplemental conditions relatively increased OGT expression and reduced 4-coumaric acid and isovitexin as saponarin precursors. Furthermore, the relative ratio of lutonarin as an oxidized product of saponarin increased in increments of light quantity. Finally, the abiotic conditions for saponarin production were optimized with the mixture solution treatment of 1.0 g L^{-1} Na$^+$ and 1.0 g L^{-1} K$^+$ under 500 PPFD of 6500 K light, and the saponarin amount per leaf was 219.5 μg plant^{-1}; it was comparable amount with that under sunlight condition.

INTRODUCTION

Flavonoids are a significant group of natural products in plants and have several functions, such as resistance to disease and insects, stabilizing metabolism, and maintaining plant homeostasis.[1−4] Recently, the biosynthetic pathway of flavonoids was proposed in plants, and the relations of abiotic signals that would be related to reactive oxygen species (ROS) regulation were reported.[5−9] However, inappropriate abiotic stress would lead to the imbalance of ROS levels, resulting in damaging cell components, breaking the antioxidative system, and finally leading to cell death.[10−13] Furthermore, ROS signal would downregulate aminotransferase expression, resulting in the lack of phenylalanine as a precursor of flavonoids, and then, flavonoid biosynthesis would be decreased.[13−16]

Saponarin was known as a major antioxidative flavonoid in young barley leaves (YBL) and had several nutraceutical functions, such as antidiabetic, antiobesity, hepatoprotective, and anti-inflammatory; thus, the mass production of saponarin in YBL was a challenge in the nutraceutical industry.[2,17−19] For saponarin production, the optimized practice was suggested with cultivar and harvest time, and the highest saponarin concentration was reported in 13-day-old leaves of Hinchalssal cultivar under sunlight.[20−22] However, the concentrations were not over 11 mg g$_{dw}^{-1}$ under artificial light conditions, and the reason was still unknown.[17,23−25]

Recently, the biosynthetic pathway for saponarin was suggested from phenylalanine and/or cinnamic acid, and the genes encoding enzymes for the biosynthesis were reported to be PAL, 4CL, CHS, CHI, OGT, etc. (Figure 1).[2,26−28] However, the metabolic rate-limit steps and their relations with abiotic signals, such as light quality, temperature, and salts, were not fully understood.

The wavelength of photosynthetically active radiation (PAR) can be divided into long and short wavelength groups; a long wavelength, such as red color and near IR, is essentially used for photosynthesis in chlorophyll, and a short wavelength of PAR, such as blue and UV lights, involves ROS signal generation.[7,29−32] Thus, the quantity balance of long and short wavelengths of PAR could control the photosynthesis rate and the carbon metabolite flow as well as ROS levels in plants, and these might increase the biosynthesis of flavonoidal antioxidants such as saponarin and lutonarin. At the same time, the excess ROS generation could damage cell components and
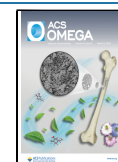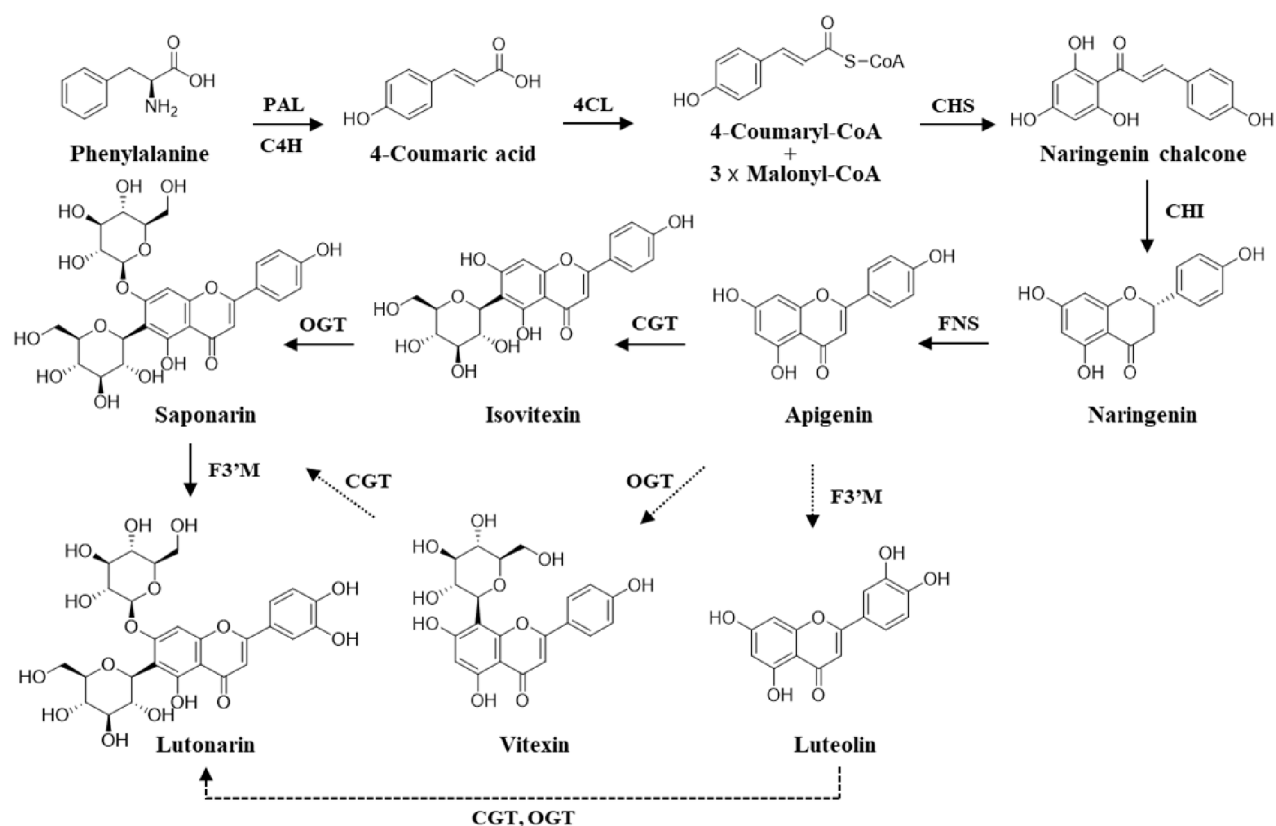
Figure 1. Proposed biosynthetic pathway for saponarin and lutonarin. PAL: phenylalanine ammonia-lyase; C4H: cinnamic acid 4-hydroxylase, 4CL: 4-coumaric acid coenzyme a ligase; CHS: chalcone synthase; CHI: chalcone isomerase; FNS: flavone synthase, CGT: C-glucosyltransferase, OGT: O-glycosyl transferase, F3′M: flavonoid 3′-monooxygenase.

Table 1. Saponarin Concentration, Biomass, and Total Saponarin Production in YBL under Different LED Light Quality and Quantity $(p < 0.05)^{a}$

light temperature	light quantity (PPFD)	biomass (mg_{dw} plant^{-1})	saponarin (g kg_{dw}^{-1})	saponarin amount (μg plant^{-1})	saponarin/lutonarin
6500 K	100	10.2[a]	5.0[a]	50.9[a]	65
	300	13.0[b]	6.8[b]	88.1[b]	28
	500	14.6[b]	10.2[cd]	149.7[d]	17
	300 + UVA	11.1[a]	9.1b[c]	101.1[b]	25
	300 + blue	13.4[b]	8.1[c]	108.4[bc]	9
	500 + blue	11.5[a]	11.5[d]	129.0[c]	6
3000 K	100	11.7[a]	4.8[a]	56.3[a]	56
	300	14.3[b]	5.1[a]	72.6[ab]	39
	500	17.1[c]	5.5[a]	94.0[b]	33

[a]Superscript letters indicate the results of Tukey's Test.

suppress the growth of plants and the accumulation of antioxidative metabolites.[33−36] Recently, the effects of light quality and quantity on the growth and saponarin production in YBL were reported under light-emitting diode (LED) light; however, the accumulated saponarin concentrations and the biomass of YBL were lower than under sunlight conditions.[17,23−25] In the reports, the growth inhibition and phytotoxicity were shown over 400 μmol m^{-2} s^{-1} (PPFD) of a white LED light, even though it was significantly lower in light quantity than sunlight.[17] To understand this, the light quality effect should be investigated on ROS generation, the photosynthesis rate, and the metabolites for saponarin. Herein, the light quality effect was investigated with ROS levels on saponarin accumulation and the photosynthetic amount of

YBL, and additionally, the salt effects as ROS regulators were investigated under hydroponic conditions.

■ RESULTS AND DISCUSSION

Light Quality Effect on the Saponarin Concentration in Barley Leaves. The two colors of white LED lights, blue-rich white (6500 K of light temperature) and red-rich white lights (3000 K of light temperature), were irradiated to YBL cultivation under 100−500 PPFD of light quantity, and the light quality effects were investigated on saponarin accumulation in YBL. The photosynthetic biomass under 3000 K of light was higher than that under 6500 K, whereas the saponarin concentration under 6500 K of light was higher than the other. However, extra blue light or UVA irradiation decreased the saponarin amount per leaf due to a reduction in the

Table 2. Saponarin Concentration, Biomass, and Total Saponarin Production in YBL under Different Salt Concentrations ($p <$ 0.05)[a]

treatment	concentration (g L^{-1})	biomass (mg$_{dw}$ plant^{-1})	saponarin (g kg$_{dw}$$^{-1}$)	saponarin amount (μg plant^{-1})	saponarin/lutonarin
Nontreatment	-	14.6^{bc}	10.2^a	149.7^b	17
Na$^+$	1.0	13.9^b	11.3^a	157.3^b	10
	2.0	13.3^b	13.1^b	175.1^c	14
	5.0	11.5^a	9.5^a	109.4^a	20
K$^+$	0.5	15.1^c	10.8^a	162.3^{bc}	15
	1.0	14.7^{bc}	13.6^b	200.0^d	17
	2.0	14.1^b	13.4^b	188.8^c	17
Na$^+$/K$^+$	1.0 + 1.0	15.9^c	13.8^b	219.5^d	17
Na$^+$/K$^+$	2.0 + 1.0	15.1^c	13.5^b	204.5^d	16
Na$^+$/K$^+$	2.0 + 2.0	14.0^b	10.9^a	153.2^b	19

[a]Superscript letters indicated the result of Tukey's Test.
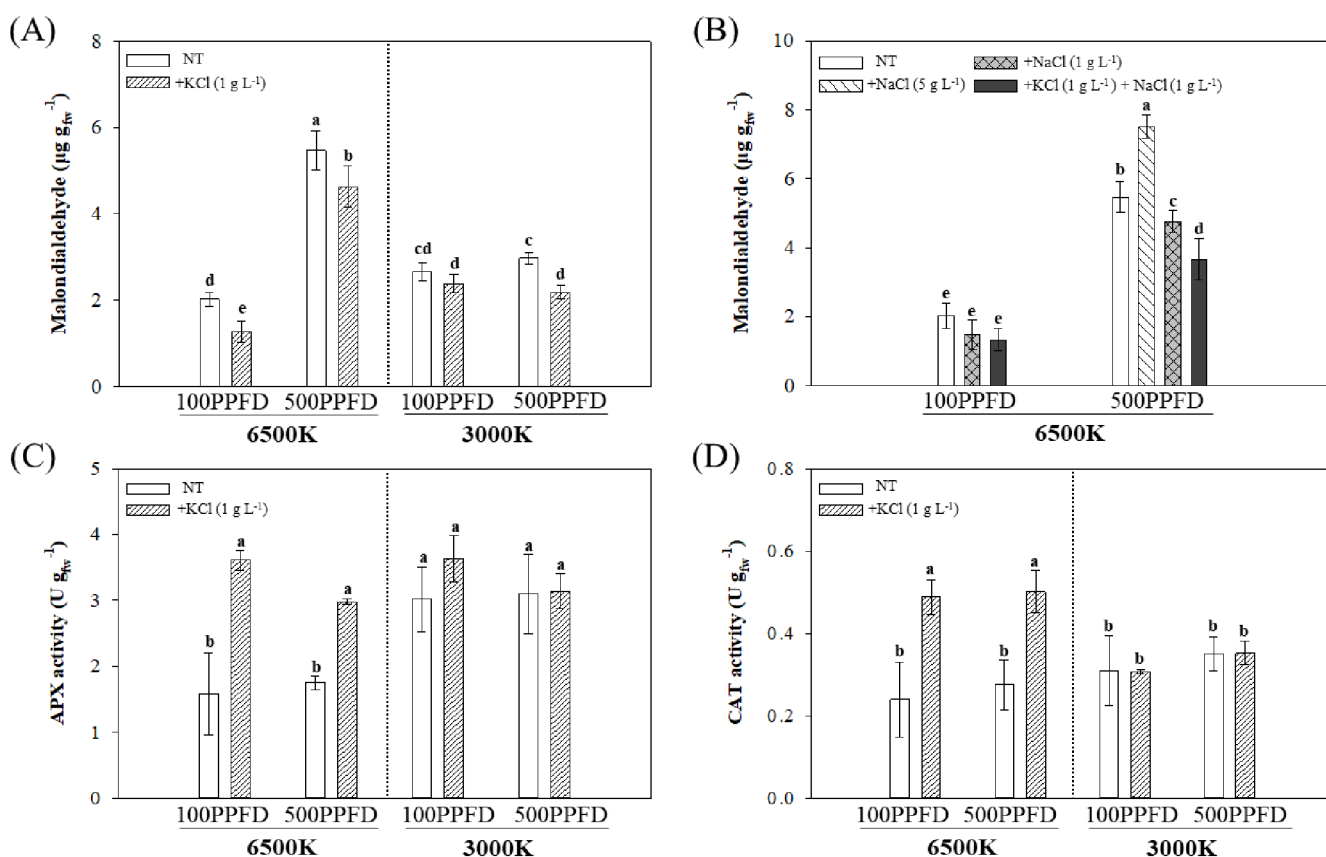
Figure 2. APX, CAT, and MDA contents by light quality, light quantity, and K$^+$/Na$^+$ salt treatment. MDA contents under different light conditions (A) and on salts (B), APX activity (C), and CAT activity (D) under different light conditions.

photosynthetic biomass or saponarin concentration. Conclusively, the light color for better saponarin production per leaf was suggested for better conditions under 6500 K than 3000 K of light. In addition, the saponarin concentration and the photosynthetic biomass of YBL were increased depending on the light quantity increment both under 6500 K and 3000 K conditions, and the saponarin amount per leaf was increased to 149.7 and 94.0 μg plant^{-1} under 500 PPFD, respectively (Table 1). Interestingly, the relative saponarin concentration was decreased in comparison with that of the oxidized product, lutonarin, in a higher light quantity. This result would be explained by the blue light effect,[16,17] and the saponarin accumulation and the YBL growth were expected to relate to ROS in plant cells.

Salt Treatment Effect on the Saponarin Content in Barley Sprouts. Na$^+$ and K$^+$ salts were known as ROS regulation factors in plants,[12,15,37−39] and these might affect the saponarin biosynthesis in YBL. Thus, Na$^+$ as a ROS stimulator was used in the hydroponics, and the YBL growth and saponarin contents were measured under 6500 K light conditions. A total of 2.0−5.0 g L^{-1} of NaCl treatment showed a significant increase in saponarin concentration, whereas the biomass was decreased. The saponarin amount per leaf was up to 175 μg plant^{-1} (Table 2). On the other hand, K$^+$ as a ROS stress reducer treatment increased both the biomass and saponarin concentration, and the saponarin amount per leaf was up to 200 μg plant^{-1} of saponarin in YBL. In addition, the Na$^+$ and K$^+$ salt mixture treatment showed the highest saponarin production (219 μg plant^{-1}) under the 500 PPFD

light condition, and this was a 46% increment of saponarin amount from the nonsalt treatment. However, it was negatively affected that the salt concentration was over 2.0 g L^{-1} with respect to the photosynthetic biomass and saponarin concentration.

ROS Level and the Biosynthetic Gene Expression for Saponarin in YBL. The present study investigated the impact of light irradiation and salt treatment on hydroponics as abiotic signals, focusing on the ROS levels in YBL. The levels of malonyldialdehyde (MDA) and saponarin under various light conditions, both with and without salt treatments, were compared. In the comparison of the light quantity effect on MDA in YBL, the findings indicated that the application of a high quantity of light, such as 500 PPFD resulted in increased MDA levels compared to 100 PPFD under 6500 K light (Figure 2). However, no significant difference in MDA was observed between the light quantities at 3000K light, and these results could be attributed to the blue light irradiation effect. Similarly, saponarin concentration and protein activity of the ROS-related proteins ascorbate peroxidase (APX) and catalase (CAT) showed no significant difference in various light quantity conditions under 3000 K (Table 1 and Figure 2). In addition, K^+ treatment reduced MDA in YBL by the increment of ROS-related protein activities under 6500 K conditions, and the growth and saponarin concentrations were increased significantly, while MDA level and the APX and CAT activities were not changed under 3000K light (Figure 2).

In this study, we determined whether the increase in saponarin due to changes in light and potassium conditions was due to an increase in the expression of related genes. In a recent report, genetic information on saponarin biosynthesis is very limited in YBL, and only CHS, CHI, and OGT genes were experimentally identified in young barley seedlings.[28] Thus, we determined the transcription levels of these three genes, including CHS, CHI, and OGT, under a different light (at 500 PPFD compared to 100 PPFD under 6500 K light irradiation) and potassium (with/without 1.0 g L^{-1}) conditions (Figure S1). It was observed that CHS1 exhibits an increase in expression, surpassing at least a 3-fold increase, in response to alterations in the external environment, such as increases in light intensity and potassium ion concentrations. Additionally, CHI demonstrated a similar pattern of heightened expression exceeding 3-fold when subjected to light and potassium ion stimulation, aligning with the behavior observed in CHS1. Interestingly, when exposed to heightened light intensity, all three tested CHS isozymes displayed an elevation in expression levels. In contrast, only CHS1 exhibited overexpression in response to potassium ion stimulation. This discrepancy suggests the different transcriptional response mechanisms for light and potassium ion stimulation in YBL. In the case of OGT, an upregulation in expression was observed, with a relatively greater sensitivity to changes in the light environment compared to alterations in the potassium environment. Certainly, the overexpression of both OGT isozymes was exclusively observed in response to changes in the light environment. Conclusively, these findings imply that the simultaneous increase in the expression of CHS1 and CHI genes might have contributed to increased saponarin biosynthesis. However, the response mechanisms to light and potassium ion stimulation seem to be divergent.

Analysis of the Metabolites for Saponarin Biosynthesis in YBL. As shown in Figure 1, saponarin could be biologically synthesized from phenylalanine, p-coumaric acid, naringenin-chalcone, naringenin, and isovitexin and degraded to lutonarin. However, the related gene expression information may not directly correlate with accumulated metabolites, although a comparison of gene expression may give useful information on the metabolite flow. Thus, quantitative analysis of the metabolites was needed to understand saponarin metabolism. Generally acceptable precursors of saponarin were phenylalanine, 4-coumaric acid, naringenin-chalcone, naringenin, apigenin, and isovitexin, and luteolin and lutonarin were the oxidized products of apigenin and saponarin, respectively (Figure 1). From the analysis, naringenin-chalcone as the product of CHS and naringenin as the product of CHI were not detected as well as apigenin and its oxidized metabolite, lutonarin. Thus, these biosynthetic reaction steps were not considered to be rate-limiting steps for saponarin synthesis. Under the high ROS-stimulated light conditions, such as 6500 K and 500 PPFD, isovitexin as a precursor of saponarin was decreased, whereas saponarin and lutonarin were increased relatively (Table 3). It was well matched with

Table 3. Relative Amounts of the Accumulated Metabolites under Different Light Quality, Light Quantity, and K^+ Treatment Conditions

	light quality (6500 K/3000 K)		light quantity (500 PPFD/100-PPFD)		potassium[a] (treatment/nontreatment)	
	100 PPFD	500 PPFD	3000 K	6500 K	100 PPFD	500 PPFD
phenylalanine	1.2	1.0	0.9	0.6	1.0	1.3
4-coumaric acid	1.2	0.6	1.2	0.6	1.9	2.2
naringenin-chalcone	ND[b]	ND	ND	ND	ND	ND
naringenin	ND	ND	ND	ND	ND	ND
apigenin	ND	ND	ND	ND	ND	ND
luteolin	ND	ND	ND	ND	ND	ND
isovitexin	0.6	0.7	0.8	0.9	3.0	1.0
saponarin	1.2	1.8	1.2	1.7	1.4	1.1
lutonarin	1.2	2.3	1.7	3.2	0.6	1.8

[a]The light temperature was 6500 K. [b]ND means no detection.

the increase in the level of the level of the expression of the OGT, indicating that the OGT governs one of the decisive regulatory phases rather than CHS and CHI in saponarin synthesis. In addition, 4-coumaric acid decreased 0.6-fold under a higher light quantity of 6500 K, but increased 1.9- to 2.2-fold under K^+ treatment conditions compared to the nontreatment. Thus, 4CL for 4-coumaroyl CoA was considered as another additional rate-limiting step.

Optimization of Light and Salt Conditions for Saponarin Production in YBL. The response surface plots were utilized to optimize the light and salt conditions (Table S1), and the hydroponic condition suggested that the cultivation solution was a mixture of 1.0 g L^{-1} NaCl and 1.0 g L^{-1} KCl, and the light condition was 500 PPFD of 6500 K light; the saponarin amount under the condition closely approached the level under the sunlight condition (Figure 3). K^+ or Na^+ single salt treatment or a higher salt condition decreased saponarin production in YBL by the loss of saponarin concentration and photosynthetic biomass.

■ **CONCLUSION**

Saponarin, a well-known health-beneficial flavonoid found in young barley leaves, has drawn interest for its production in
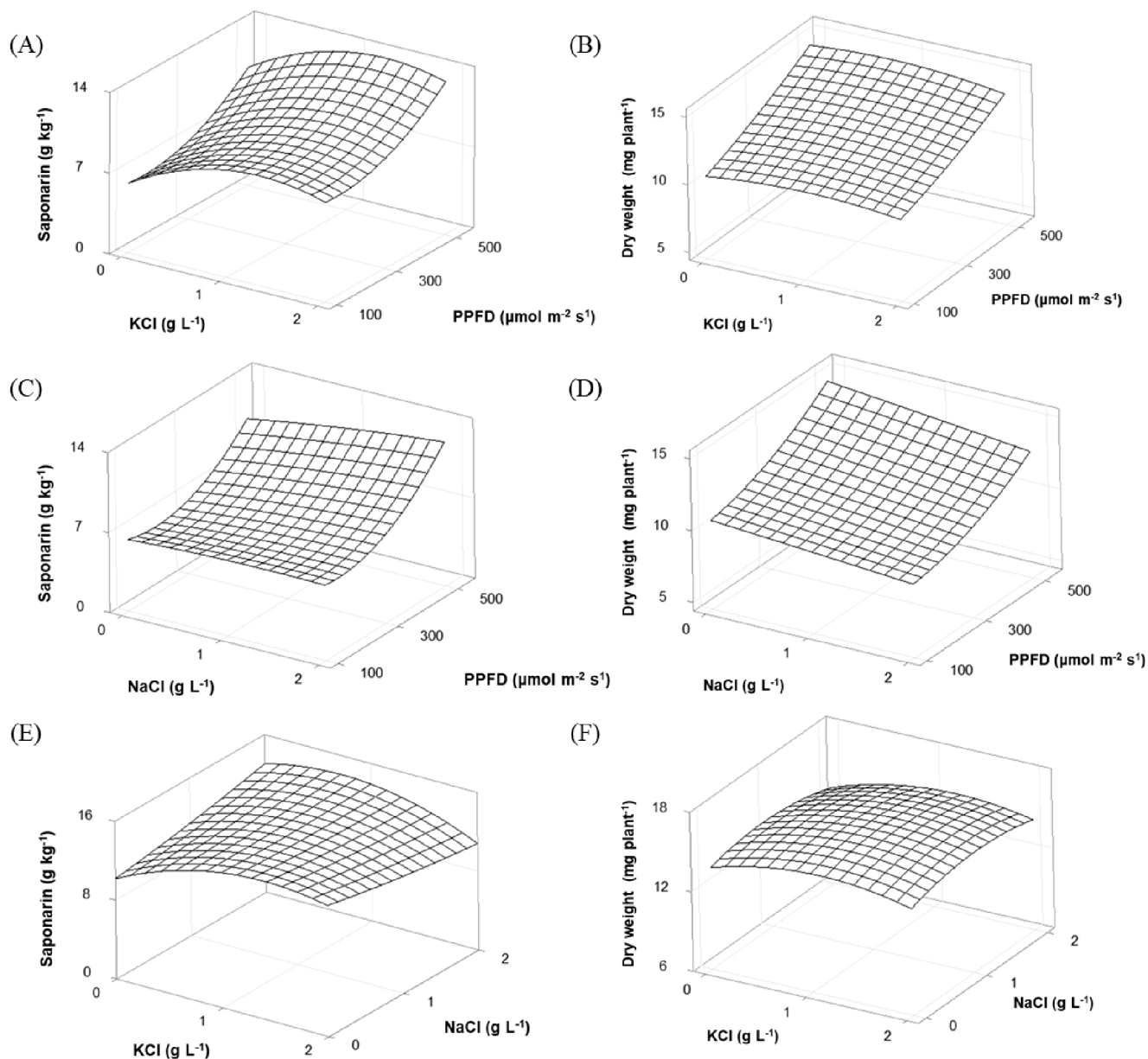
Figure 3. Effects of light quantity and Na$^+$/K$^+$ treatment on saponarin and photosynthetic biomass of YBL in three-dimensional response surface plots. K$^+$ and light quantity effects on saponarin (A) and biomass (B), Na$^+$ and light quantity effects on saponarin (C) and biomass (D), and K$^+$ and Na$^+$ effects on saponarin (E) and biomass (F).

hydroponic environments under artificial light conditions. This study investigated the effects of ROS-mediated abiotic signals, such as light intensity, blue light, and K$^+$, on saponarin accumulation and photosynthetic biomass. The findings indicated that these abiotic signals increased ROS levels in plants and stimulated the expression of biosynthetic genes responsible for saponarin production such as CHS, CHI, and OGT. Notably, the OGT was identified as a potential rate-limiting enzyme for saponarin synthesis. In addition, K$^+$ treatment slightly reduced ROS in cells while enhancing APX and CAT activities, as well as OGT expression. Consequently, these abiotic factors would affect saponarin synthesis and accumulation in plants. In addition, the optimal hydroponic conditions for achieving high saponarin levels in barley leaves were suggested as a mixed solution consisting of 1.0 g L^{-1} KCl and 1.0 g L^{-1} NaCl under 500 PPFD of 6500 K light.

■ MATERIALS AND METHODS

Chemicals and Reagents. Analytical standards of saponarin were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Reagent grade formic acid, NaCl, KCl, hydrogen chloride, hydrogen peroxide, ascorbic acid, 1,1,3,3-tetramethoxypropane (TMP), trichloroacetic acid (TCA), and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich. Methanol, acetonitrile, and water used were of high-performance liquid chromatography (HPLC) grade and obtained from Honeywell Burdick & Jackson (Charlotte, NC, USA).

Growth Conditions for YBL. Barley (*Hordeum vulgare* L.) was obtained from the National Institute of Crop Science (RDA, Wanju, Republic of Korea) and grown using hydroponics at 60% relative humidity and 20−22 °C. Cultivar of the barley was Keunalbori-1. The seeds (50 g) were primed by

soaking in water for 1−2 days and hydroponically cultivated under the controlled LED light (12 h in a day). The leaves were harvested from the sawed sample after 10 days. The hydroponic solutions were prepared with distilled water, NaCl, and KCl. The harvested leaves were dried in a drying chamber (PS-1000A, Shinil Tech, Kimhae, Republic of Korea) at 45 °C for 48 h and then stored at −20 °C until the analysis.

Light Conditions. The light irradiation effects were experimented with 6500 K and 3000 K light temperatures (Kkumi-Lighting Co., Namyangju, Republic of Korea). The light spectrum was measured with Sherpa Eye (Sherpa Space Co., Daejeon, Republic of Korea) and presented in Figure S2. The light quantity was controlled at 100, 300, and 500 PPFD, and the quantity was measured with a Portable Luxmeter (HD2102.1, Delta OHM S.r.l., Selvazzano Dentro, Italy). The blue light LED (Zhongshan Jinsung Electronic, Zhongshan, China) and UVA lamp (Hansung Ultraviolet Co. Ltd. Seongnam, Republic of Korea) were used as additional light sources.[40] The additional blue light and UVA were irradiated to 10% of the total PPFD and 2.5 W m^{-2}, respectively.

Sample Preparation for the Saponarin and Its Precursor Analysis in YBL. The analytical method of extracting saponarin and its metabolites from YBL was performed using the method described by Oh et al.[16] Briefly, 1.0 g of homogenized sample was added to 30 mL of 60% methanol and shaken for 4 h at room temperature. The extract was then centrifuged at 4,000 rpm for 10 min, and the supernatant was filtered with a 0.45 μm PVDF syringe filter. The filtrate was analyzed with HPLC (HPLC-DAD, Agilent Technologies, Santa Clara, CA, USA).

Saponarin and Its Metabolite Analysis with HPLC. The linearity of the standard curve for apigenin, luteolin, lutonarin, and saponarin was in the ranges of 1.0 to 50 mg L^{-1} ($R^2 > 0.999$). The limit of quantitation (LOQ) was 0.5 mg kg^{-1} for saponin, and 1.0 mg kg^{-1} for apigenin, luteolin, and lutonarin, respectively. C18 column (3.0 × 100 mm, 3.5 um, Agilent Technologies, Santa Clara, CA, USA) was used for the quantitative analysis of saponarin and its precursors apigenin, luteolin, and lutonarin. The mobile phase was 0.1% aqueous formic acid and acetonitrile. The detailed instrumental condition is provided in Table S2. The analyses of saponarin precursors, 4-coumaric acid, phenylalanine, isovitexin, naringenin, and naringenin-chalcone were performed with LC-MS/MS (Agilent 6420 TripleQuad, Agilent Technologies Inc., Santa Clara, CA, USA). The detailed instrumental conditions are presented in Table S3.

Measuring Antioxidant Enzyme Activities. The antioxidant enzyme activities for APX and CAT were conducted following the method by Kantharaj et al.[41] The fresh YBL sample (100 mg, fresh weight) was rapidly frozen in liquid nitrogen and then homogenized with 200 μL of extraction buffer consisting of 50 mM HEPES and 0.1 mM EDTA for APX and potassium phosphate buffer for CAT. The extract was centrifuged at 12 000 rpm for 15 min at 4 °C, and the supernatant was measured for the activity. APX and CAT activities were determined by evaluating the ascorbate and H$_2$O$_2$ decreases, respectively. The reaction mixture for APX contained 800 μL of extraction buffer, 50 μL of 0.6 mM ascorbic acid, 100 μL of 0.06 mM H$_2$O$_2$, and 50 μL of the enzyme solution. APX activity was then measured at a wavelength of 290 nm. The activity for CAT was measured with 620 μL of 50 mM PBS (pH 7.0), 330 μL of 0.1% H$_2$O$_2$, and 50 μL of the enzyme solution at 240 nm. The activities of APX and CAT were monitored at 10 s intervals for a total of 1 min.

Measuring MDA in YBL. For the quantitative analysis of MDA, the MDA standard was prepared by hydrolysis of TMP.[42] TMP standard solutions ranging from 0.36 to 10.8 μM were prepared with 0.1 M HCl. The solutions were hydrolyzed at 90 °C for 30 min. After hydrolysis, the reaction mixture was cooled with ice, and absorbance was obtained at 535 nm. For the MDA in fresh YBL, 2 g of fresh YBL was homogenized with 10 mL of 0.4% TCA solution in an ice bath. The extract was centrifuged for 40 min. One mL of the supernatant was added to 2 mL of 1% TBA with 20% TCA solution, then stored at 90 °C for 30 min. After cooling the samples on ice, they were centrifuged at 10 000 rpm for 5 min. The supernatant was filtered using a 0.45 μm PVDF syringe filter, and the absorbance was measured at 535 nm.

RNA Expression Analysis. Total RNA was extracted from young barley leaves using the IQeasy Plus Plant RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea). The RNA concentration was quantified with the BioPhotometer D30 (Eppendorf, Hamburg, Germany), and only high-quality RNA samples were used for subsequent experiments. RNA expression level was determined by quantitative real-time polymerase chain reaction (qRT-PCR, QuantStudio 1, Applied Biosystems, MA, USA) with a SYBR green qPCR premix (Prime Q-Mastermix, Daejeon, Korea). GAPDH1 was used as a reference gene for quantitative gene expression analysis in YBL. Prior to performing the qRT-PCR analysis, complementary DNA (cDNA) was prepared by reverse transcription using AccuPower RT PreMix & Master Mix (Bioneer, Daejeon, Korea) following the protocol. The primers for qRT-PCR analysis are listed in Table S4.

Statistical Analysis. Statistical analysis of the data was conducted using the R statistical program (version 4.2.3, R Foundation). Response surface methodology (RSM) was used for optimizing the salt concentration and light quantity with Minitab software (version 21, Minitab Inc., State College, PA, USA).

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c09809.

> Biosynthetic gene expression comparison to the light quantity of 6500 K and K$^+$ treatment (Figure S1); White LED light spectrum of 3000 K (a) and 6500 K (b) for the barley leaf cultivation (Figure S2); Regression coefficients (β), determination coefficients (R^2), and F-test value of predicted second-order polynomial models for saponarin content and dry weight by PPFD and Na$^+$/K$^+$ salt (Table S1); Instrumental condition of HPLC for quantitative analysis of saponarin, apigenin, luteolin, and lutonarin (Table S2); Instrumental condition of LC-MS/MS for quantitative analysis of the saponarin precursors (Table S3); Primers sequences for qRT-PCR analysis (Table S4) (PDF)

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Author Contributions

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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