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Comparison of Flavonoid Profiles in Sprouts of Radiation Breeding Wheat Lines (*Triticum aestivum* L.)

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Abstract: Wheat (*Triticum aestivum* Linn.; Poaceae), one of the most popular food crops worldwide, contains basic and essential nutrients and various health benefiting phytochemicals. Among them, flavonoids have attracted significant interest owing to their various health-promoting properties. In this study, 35 wheat mutant lines were developed via gamma-irradiated mutation breeding from the original cultivar. The effects of radiation breeding on the endogenous phytochemical production in the sprouts of these mutant lines were investigated using high performance liquid chromatography-diode array detector-electrospray ionization mass spectrometry (HPLC-DAD-ESIMS) with multivariate analysis for the first time in this study. Fourteen characteristic peaks, including eleven flavone *C*-glycosides, two flavone *O*-glycosides, and one flavone, were identified. In addition, the contents of three flavone *C*-glycosides, namely, isoschaftoside, isoorientin, and isoscoparin, in 37 wheat sprout samples from the original cultivar, certificated cultivar, and the mutant lines were determined. A heat map combined with hierarchical clustering showed variation in the relative content for the flavonoids between the 37 wheat sprout samples, clustering into three groups. On principal component analysis scores scatter and loading plots, significant differences in the levels of flavonoids were found between the samples and several markers responsible for group separation were detected. These results provide a scientific reference for the phytochemical variation in wheat mutant lines, thereby aiding in further mutation mechanism studies and for the quality control of the improved wheat cultivars.

Keywords: *Triticum aestivum* Linn.; sprout; wheatgrass; radiation-induced mutation breeding; flavone *C*-glycoside

1. Introduction

Wheat (*Triticum aestivum* L.; Poaceae) is one of the major cereal crops worldwide; its grain is energy-rich owing to the high carbohydrate content and is a rich source of nutrients, including proteins, minerals, and dietary fiber [1]. Recently, the use of wheat parts, such as straw, bran, germ, root, or sprout, has increased; therefore, the breeding of wheat parts has been focused on increasing the accumulation of bioactive compounds that are beneficial to human health [2]. The sprout of *T. aestivum* is generally known as wheatgrass. Since the 1980's, its consumption has been popular in Western countries in the form of health functional foods and detox juices [3,4]. Wheatgrass is preferred by farmers because sprouting is a simple and cost-effective process; furthermore, it has a short production cycle, small space production requirement in the greenhouse, and a significantly

high yield [4,5]. Wheatgrass contains high amounts of chlorophyll, vitamins, minerals, amino acids, dietary fiber, and biologically active compounds, and is mostly consumed as fresh juice or tablets [6]. Preclinical and clinical studies have reported that wheatgrass juice and tablets are effective in treating myelodysplastic syndrome [7], thalassemia major [8,9], and hematological disease related to breast cancer [10]. Furthermore, wheatgrass has diverse biological activities, such as anti-oxidative [11], anti-hyperglycemia [11], anti-adipogenesis [12], anti-allergic [13], and hepatoprotective activities [14]. These medicinal utilities of wheatgrass can be attributed to the presence of biologically active compounds, such as flavonoids.

Flavonoids are one of the major groups of plant secondary metabolites found in vegetables, fruits, and cereals. Flavonoids have been reported to have a broad spectrum of health-promoting effects, involving in various biological and pharmaceutical functions, such as anti-obesity, anti-cholesteric, anti-inflammatory, antiviral, anti-cardiovascular, anticancer, antitumor, and antioxidant activities [15,16]. For this reason, flavonoids are significant constituents of plants that play a key role in nutraceutical, pharmaceutical, medicinal, and cosmetic applications [17], as well as serve as chemical markers for the quality control of medicinal plants [18]. Flavonoids also play an important role in plant resistance in general, and are beneficial to the plants themselves by possessing the function to produce physiological active compounds, protect from environment stress, be attractants, and deter feeding [19].

Diverse cultivars of *T. aestivum* have been obtained by hybridization, genetic modification, or mutation so as to improve crop productivity and quality. In mutation breeding, mutagens, such as chemical (i.e., ethyl methanesulphonate and methylnitrosourea), physical (i.e., ultraviolet lights, gamma-rays, and proton beams), or combined mutagens, have been used for developing cultivars with novel mutational characteristics, while preserving the unique and excellent properties of the plant [20–22]. More than 2500 mutant varieties have been developed via physical mutagen treatment and have been registered with the Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA) [23]. Our research group developed novel wheat lines generated by gamma-radiation mutagenesis, exhibiting improved agronomic traits and various phenotypes. Several previous studies have reported the secondary metabolite profiling of *T. aestivum*, including flavonoids and phenolic acids [24–28], however there have been no studies on the sprouts of gamma-irradiated wheat mutant lines. Thus, the metabolomics for the flavonoid composition of the sprouts of gamma-irradiated mutant lines as well as original and certified cultivars of *T. aestivum* were investigated by the differences in their chemical composition according to the influence of radiation breeding.

In this study, the composition and content of the flavonoids of the wheat sprout samples were determined by high performance liquid chromatography-diode array detector-electrospray ionization mass spectrometry (HPLC-DAD-ESIMS) in order to evaluate for the selection of potential improved varieties. In addition, the marker flavonoids responsible for their differentiation were identified by multivariate analysis.

2. Materials and Methods

2.1. General Procedures

HPLC-DAD-ESIMS was performed using an Agilent 1200 series system and Agilent 6120 single quadruple MS system (Agilent Technologies Co., Santa Clara, CA, USA) equipped with a YMC-Triart C18 column (5 μm, 250 × 4.6 mm; YMC Co., Kyoto, Japan). All of the data were processed using ChemStation software (Agilent Technologies Co., Santa Clara, CA, USA). A [^{60}Co] γ-irradiator (150 TBq capacity; AECL, Ottawa, Ontario, Canada) was used for the gamma irradiation. The standard compounds, i.e., isoschaftoside (Extrasynthese, Genay, France), isoorientin (Chengdu Biopurify Phytochemicals Ltd., Chengdu, China), and isoscoparin (Wuhan ChemFaces Biochemical Co., Ltd., Hubei, China), were obtained from Sigma-Aldrich (Sigma Chemical Co., St Louis, MO, USA). All of the other chemicals and solvents used in this study were of analytical grade.

2.2. Plant Materials

The wheat mutant lines were generated by treating the seeds of the original cultivar with 200 Gy of gamma (^{60}Co) irradiation [29]. These mutants were selected according to their diverse phenotypic variants and exhibited a stable inheritance of these phenotypes for 4 years. The plants were authentic and grown by Dr. Jin-Beak Kim and Dr. Min-Jeong Hong (Korea Atomic Energy Research Institute). The voucher specimens (PL 01–61) were deposited at the Radiation Breeding Research Center, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute. Herein, 100 g of wheat seeds were sown and germinated in square pots (54 × 27 × 3 cm^3) filled with soil. The germinated wheat was grown in a well-controlled growth room at 22–23 °C, with a relative humidity of 60% under long-day conditions (16-h daylight). The wheat sprouts were sampled after 7 days of sowing. The sprouts were air-dried, chopped, and stored at −20 °C in polyethylene plastic bags until further analysis.

2.3. Preparation of Standard Solutions and Sample Preparation

Dried sprouts of the wheat mutant lines and the original and certified cultivars were individually chopped, and a sample (1 g for each) was extracted with 95% ethanol (30 mL) using sonication for 1 h. The extracted solutions were evaporated in vacuo in order to produce dryness. Each of the 95% ethanol extracts were accurately weighed and were dissolved in methanol at 10 mg/mL; then, they were filtered through a polyvinylidene fluoride syringe filter (0.45 μm) for the HPLC analysis. Each of the standards, isoschaftoside, isoorientin, and isoscoparin, were accurately weighed and were dissolved in methanol at 1.0 mg/mL. The stock solutions were diluted to afford a series of standard solutions at five different concentrations (10, 20, 40, 100, and 200 μg/mL) for the quantitative analysis.

2.4. Qualitative and Quantitative Analysis

Qualitative and quantitative analyses were performed using the Agilent 1200 series LC system coupled with an Agilent 6120 quadrupole single mass spectrometer detector. All of the data were processed using ChemStation software. This study was conducted on a YMC-Triart C18 column (5 μm, 250 × 4.6 mm, YMC Co.). The mobile phase comprised 0.1% formic acid in water (*v*/*v*; solvent A) and 0.1% formic acid in acetonitrile (*v*/*v*; solvent B), and was performed as follows: 0–25 min, 15%–35% B; 25–30 min, 35%–100% B; 30–35 min, 100% B; 35–36 min, 100%–15% B; and 36–40 min, 15% B. The total flow rate was maintained at 0.8 mL/min and the injection volume was 10 μL. The chromatograms were acquired at 280 nm using a DAD detector. The mass spectrometry conditions were set as follows: negative ionization mode (ESI$^-$); scan range = *m*/*z* 100–1000; scan rate = 1.06 s/cycle; capillary voltage = 4000 V; drying gas flow = 10 L/min (N_2); nebulizer pressure = 30 psi; and drying gas temperature = 350 °C. Each experiment was conducted in triplicate, and all of the data are presented as the mean ± standard deviation (SD).

2.5. Chemometrics

The peak area data set (280 nm) obtained for the sprouts of the 37 wheat samples was normalized, and was submitted to hierarchical clustering analysis (HCA) with a heatmap using "heatmaply" package in R software (version 4.0.2) [30–33]. The scores scatter and loading plots were generated after principal component analysis (PCA) in SIMCA 15.0.2 (Umetrics, Umeå, Sweden).

3. Results and Discussion

3.1. Identification of the Compounds

The flavonoids in the 37 wheat sprout samples, including the original cultivar (PL01), certified cultivar ("Geumgang"; PL26), and mutant lines (PL05, PL06, PL08–PL11, PL13–PL20, PL23–PL25, PL41, PL42, PL44, PL46–PL48, and PL50–PL61), were analyzed using HPLC-DAD-ESIMS. Then, 14 peaks in their chromatograms were speculated and identified by comparing their retention time,

ultraviolet (UV) spectra, and negative molecular ions with those of the compounds purchased in this study or with those reported previously [24–28]. Peaks 3, 5, and 11 were assigned to isoschaftoside, isoorientin, and isoscoparin, respectively, which were unambiguously identified by comparing the retention times and molecular weights with those of the reference standards. On the basis of the retention times of peaks 3, 5, and 11, the remaining peaks were tentatively identified by comparing their retention times and the molecular weights of the precursor ion with those reported previously [24–28]. The identification results of the compounds are listed in Table 1. Peaks 1 and 2 show the molecular ions of *m*/*z* 579 $[M\text{-}H]^-$ and *m*/*z* 563 $[M\text{-}H]^-$, respectively, and have retention times higher than that of peak 3 (isoschaftoside), and were reported previously as luteolin 6-*C*-pentoside 8-*C*-hexoside and apigenin 6-*C*-hexoside 8-*C*-pentoside [25–27], respectively; thus, in this study, peaks 1 and 2 were identified as luteolin 6-*C*-hexoside 8-*C*-pentoside and apigenin 6-*C*-hexoside 8-*C*-pentoside, respectively. Peak 4, appearing between the retention times of peaks 3 (isoschaftoside) and 5 (isoorientin), with a molecular ion of *m*/*z* 593 $[M\text{-}H]^-$, was identified as apigenin-6,8-di-*C*-hexoside [27]. The peaks appearing between peaks 5 (isoorientin) and 11 (isoscoparin) were identified by comparing their relative retention times and molecular ions with previously reported values [26,27]. Peaks 6, 7, 8, 9, and 10 were previously identified as luteolin 6-*C*-hexoside 8-*O*-deoxyhexoside, apigenin 6-*C*-hexoside 8-*O*-deoxyhexoside, crysoeriol 6-*C*-hexoside 8-*O*-deoxyhexoside, apigenin 8-*C*-glucoside, and apigenin 6-*C*-glucoside, respectively. Peaks 12 and 14 were tentatively identified as tricin 7-*O*-malonylhexoside and tricetin trimethyl ether, respectively, as reported previously [26]. Peak 13 was assumed to be crysoeriol 7-*O*-malonylhexoside by comparing its molecular ions with those reported previously [34], although this compound was not reported in *T. aestivum*.

Table 1. UV–VIS absorption maxima and main electrospray ionization mass spectrometry (ESIMS) peaks of the flavonoids tentatively identified in the wheat sprout samples.

Peak No.	R_t (min) [1]	UV λ_{max} (nm)	ESI-NI (m/z) [2]	Identification	Reference
1	10.9	270, 350	579	Luteolin 6-*C*-pentoside 8-*C*-hexoside	[16–18]
2	11.9	270, 335	563	Apigenin 6-*C*-hexoside 8-*C*-pentoside	[16–18]
3	12.9	270, 335	563	Apigenin 6-*C*-arabinoside 8-*C*-glucoside (Isoschaftoside)	Standard
4	13.2	270, 335	593	Apigenin-6,8-di-*C*-hexoside	[18]
5	13.8	270, 350	447	Luteolin 6-*C*-glucoside (Isoorientin)	Standard
6	14.8	270, 350	593	Luteolin 6-*C*-hexoside 8-*O*-deoxyhexoside	[17,18]
7	16.0	270, 335	577	Apigenin 6-*C*-hexoside 8-*O*-deoxyhexoside	[17,18]
8	16.6	270, 345	607	Crysoeriol 6-*C*-hexoside 8-*O*-deoxyhexoside	[17,18]
9	17.1	270, 335	431	Apigenin 8-*C*- hexoside	[17,18]
10	17.7	270, 335	431	Apigenin 6-*C*- hexoside	[17,18]
11	18.6	270, 345	461	Crysoeriol 6-*C*-glucoside (Isoscoparin)	Standard
12	19.7	270, 335	577	Tricin 7-*O*-malonylhexoside	[17]
13	21.2	270, 345	547	Crysoeriol 7-*O*-malonylhexoside	[25]
14	22.7	270, 330	343	Tricetin trimethyl ether	[17]

[1] Retention time on LC analysis. [2] Molecular ion $[M–H]^-$ in negative ion mode.

Typically, flavonoids are found as *O*- or *C*-glycosylic forms. Flavonoids generally accumulate as *O*-glycosylated derivatives in most plant, while flavone *C*-glycosides is predominantly found in cereal crops [35]. *O*-glycosides have been reported mainly as 3- and 7-*O*-glycosides, however *C*-glycosides have been found as mostly 6- and 8-*C*-glycosides. In this study on the flavonoid profiling of the sprouts of wheat mutant lines, 11 flavone *C*-glycosides and two flavone *O*-glycosides were identified. In previous reports, the characteristic fragment patterns of flavonoid *C*-glycosides in the negative ion mode of the MS or MS/MS have been reported as follows [36–39]: The product ion $[M-H-H_2O]^-$ was found from 6-*C*-glycosyl flavones among mono *C*-glycosyl flavonoid isomers [36]; in the di-*C*-glycosyl flavonoids, the characteristic fragment ions, $[aglycone+113]^-$ and $[aglycone+83]^-$, were detected; the relative intensities of ions $[(M-H)-90]^-$ for 6-*C*-pentosyl-8-*C*-hexosyl-flavone and $[(M-H)-120]^-$ for 6-*C*-hexosyl-8-*C*-pentosyl-flavone were high when compared with each other [37,38]; from the viewpoint of *C*-glycosyl flavones *O*-glycosylated in the phenolic hydroxyl, a loss of a sugar moiety from *O*-glycosylation on the phenolic hydroxyl was observed, and typical fragmentation ions of *C*-glycosyl flavones (explained foregoing) were shown [39]. These series of fragment ions arising from the MS^2 and MS^3 spectra could be obtained using LC/MS systems with a triple quadruple or quadrupole time-of-flight analyzer. In this study, it was difficult to determine these characteristic fragment ions in the total ion current chromatograms of the sprouts of 37 wheat sprout samples because of the use of a single quadruple mass spectroscopy.

3.2. Quantitative Analysis of Flavonoids in the Wheat Mutants

The contents of the three commercially available compounds, i.e., isoschaftoside, isoorientin, and isoscoparin, were quantified in 95% aqueous ethanol extracts of the wheat sprout samples using the method described in Section 2.4. As shown in Table 2, the linearity, regression equation, and linear ranges of the three compounds were determined. Calibration curves showed a high degree of linearity with high-correlation coefficient values ($R^2 > 0.9995$). The limits of detection (LOD) and limits of quantification (LOQ) for the three compounds were in the range of 0.354–1.036 and 1.071–3.138 μg/mL, respectively, indicating that the analytical method was effective and suitably sensitive. The intra- and inter-day precisions were determined by analyzing the replicates of the three compounds on the same day and on three consecutive days, measured by calculating the relative standard deviation (RSDs) of the contents of all of the compounds. The RSDs of the precision of the intra- and inter-day analyses were in the range of 0.15%–2.79% and 0.23%–1.73%, respectively (Table 3). These optimized analysis conditions could be useful for the standardization and quality assessment of the wheat sprout materials or the products derived from them.

Table 2. Linear regression and LOD and LOQ values for the three representative flavonoids identified from the wheat sprout samples.

Peak No.	Compounds	Regression Equation [1]	R^2 [2]	Linear Range (μg/mL)	LOD [3] (μg/mL)	LOQ [4] (μg/mL)
3	Isoschaftoside	y = 23.268x + 7.4461	0.9995	10.00–200.00	1.036	3.138
5	Isoorientin	y = 18.126x + 24.042	0.9999	10.00–200.00	0.474	1.437
11	Isoscoparin	y = 19.823x + 8.613	0.9999	10.00–200.00	0.354	1.071

[1] y = peak area; x = concentration of standard solution. [2] R^2 is coefficient values. [3] LOD is the limit of detection (signal to noise = 3). [4] LOQ is the limit of quantification (signal to noise = 10). LOD: limits of detection; LOQ: limits of quantification.

Table 3. Intra- and inter-day precisions of the three representative flavonoids identified from the wheat sprout samples.

Peak No.	Compounds	Concentration (μg/mL)	Inter-Day		Intra-Day	
			Mean ± SD [1] (μg/mL)	RSD [2] (%)	Mean ± SD [1] (μg/mL)	RSD [2] (%)
3	Isoschaftoside	20	21.59 ± 0.15	0.69	20.09 ± 0.70	0.35
		40	41.15 ± 0.36	0.15	41.13 ± 0.02	0.38
		100	102.12 ± 0.20	0.35	102.74 ± 1.06	1.03
5	Isoorientin	20	20.53 ± 0.57	2.79	19.90 ± 0.04	0.23
		40	40.53 ± 0.06	0.87	40.06 ± 0.69	1.73
		100	98.97 ± 0.35	0.20	97.86 ± 1.06	1.08
11	Isoscoparin	20	20.33 ± 0.34	1.68	20.01 ± 0.11	0.57
		40	40.30 ± 0.07	0.17	40.09 ± 0.20	0.49
		100	99.82 ± 1.18	1.18	98.58 ± 0.57	0.57

[1] Values are mean ± standard deviation in tinplate. [2] Relative standard deviation. SD: standard deviation; RSD: relative standard deviation.

This established method was successfully applied for the determination of the three compounds in the extracts of the wheat sprout samples, and the results are listed in Table 4. The original cultivar (PL01) had a higher content of isoschaftoside and isoscoparin than the certificated cultivar (PL26, "Guemgang"), while PL26 showed that the content of isoorientin was twice as high as that of PL01. Among the sprout samples of the wheat mutant lines bed by gamma-irradiation on the original cultivar, 10 mutant lines, namely, PL06, PL08, PL11, PL16, PL19, PL20, PL25, PL44, PL50, and PL55, showed that the contents of all three components increased compared with the original cultivar; in particular, PL19 and PL50 exhibited a significant increase in the content of the three ingredients. On the contrary, the mutant lines with a reduced or almost similar content for the three components were PL05, PL17, PL24, PL48, PL52, PL53, PL54, PL57, PL60, and PL61. In nine mutant lines (PL10, PL15, PL23, PL41, PL42, PL46, PL47, PL56, and PL59), out of the three components, only isoorientin was accumulated at higher levels than the original cultivar. A mutant line, PL09, had an increased level of only isoscoparin compared with the original cultivar. In addition, the mutant lines with an increased content of isoschaftoside and isoorientin and decreased content of isoscoparin were PL18, PL51, and PL58; the mutant line of PL13 had a slightly increased content of isoorientin and isoscoparin and decreased content of isoschaftoside; the mutant line PL14 accumulated a higher content of isoschaftoside and isoscoparin and lower content of isoorientin, compared with those of the original cultivar.

Flavonoids are generally found in glycoside form, particularly for cereal crops which predominantly synthesize flavone *C*-glycosides, e.g., vitexin, isoorientin, orientin, and isovitexin and their multiglycosides [40,41]. A review on flavonoid *C*-glycosides and their biological benefits can be summarized as follows [42]: flavonoid *C*-glycosides are more stable and show a higher antioxidant potential than the aglycones or flavonoid *O*-glycosides; flavonoid *C*-glycosides have been reported to have positive effects on human health, such as anticancer, hepatoprotective, antidiabetic, and anti-inflammatory properties, and other biological potentials; and flavonoid *C*-multiglycosides are better absorbed when unchanged in the intestine and distributed to other tissues, compared with flavonoid *C*-monoglycosides. Although this study is limited to the quantification of three components in the sprout samples, the mutant lines with an increased content of all three components compared with the original wheat cultivar could have the potential to be developed as improved wheat cultivars that can be used in the development of dietary supplements using the wheat sprouts.

Table 4. Contents of three flavonoids in the wheat sprout samples.

Cultivar No.	Contents (*w*/*w*, mg/g of Extract) [1]			
	Isoschaftoside	**Isoorientin**	**Isoscoparin**	**Sum**
PL01 (original)	23.12 ± 2.14	3.89 ± 1.26	1.81 ± 0.04	28.81 ± 3.43
PL05	16.71 ± 1.34	1.34 ± 0.11	1.78 ± 0.02	19.82 ± 1.48
PL06	24.19 ± 1.76	7.77 ± 1.18	2.79 ± 0.22	34.76 ± 3.16
PL08	26.88 ± 0.68	4.90 ± 0.10	2.34 ± 0.36	34.12 ± 1.14
PL09	18.16 ± 0.30	3.28 ± 0.15	2.77 ± 0.02	24.20 ± 0.47
PL10	15.94 ± 0.29	4.56 ± 0.05	1.60 ± 0.04	22.10 ± 0.38
PL11	29.49 ± 0.29	8.06 ± 0.36	1.91 ± 0.32	39.46 ± 0.96
PL13	15.96 ± 0.17	3.96 ± 0.07	2.02 ± 0.06	21.68 ± 0.30
PL14	30.32 ± 1.17	3.14 ± 2.71	2.82 ± 0.06	36.28 ± 3.95
PL15	23.58 ± 0.89	6.12 ± 0.28	1.88 ± 0.03	31.58 ± 1.19
PL16	25.57 ± 0.97	5.84 ± 1.44	1.96 ± 0.03	33.36 ± 2.44
PL17	21.62 ± 0.31	3.00 ± 0.80	1.77 ± 0.13	26.39 ± 1.24
PL18	34.53 ± 0.73	4.56 ± 0.22	1.56 ± 0.19	40.65 ± 1.14
PL19	37.56 ± 1.19	9.20 ± 0.12	2.34 ± 0.06	49.10 ± 1.37
PL20	29.59 ± 1.70	11.15 ± 0.33	2.02 ± 0.02	42.76 ± 2.04
PL23	19.41 ± 0.05	9.88 ± 0.24	1.89 ± 0.06	31.17 ± 0.35
PL24	17.99 ± 1.00	2.66 ± 0.02	1.46 ± 0.01	22.11 ± 1.03
PL25	31.53 ± 0.58	4.61 ± 0.06	2.34 ± 0.08	38.49 ± 0.72
PL26 (Geumgang)	18.31 ± 0.74	9.41 ± 0.52	1.16 ± 0.05	28.88 ± 1.32
PL41	22.00 ± 0.57	4.89 ± 0.74	1.53 ± 0.07	28.41 ± 1.38
PL42	21.40 ± 0.20	8.81 ± 0.22	1.44 ± 0.11	31.65 ± 0.53
PL44	24.20 ± 0.71	10.63 ± 0.26	1.84 ± 0.25	36.68 ± 1.23
PL46	22.41 ± 0.42	6.75 ± 0.09	1.21 ± 0.34	30.37 ± 0.86
PL47	12.35 ± 0.98	5.66 ± 1.18	1.12 ± 0.28	19.13 ± 2.44
PL48	18.79 ± 0.24	2.58 ± 0.70	1.56 ± 0.09	22.93 ± 1.04
PL50	30.64 ± 0.87	10.61 ± 0.70	2.36 ± 0.45	43.61 ± 2.02
PL51	24.25 ± 0.23	4.22 ± 0.63	1.71 ± 0.07	30.18 ± 0.93
PL52	23.38 ± 1.50	2.96 ± 0.15	1.97 ± 0.12	28.30 ± 1.76
PL53	22.37 ± 0.32	3.34 ± 0.11	1.15 ± 0.04	26.86 ± 0.47
PL54	19.49 ± 0.78	2.80 ± 0.45	1.16 ± 0.14	23.45 ± 1.36
PL55	39.27 ± 0.31	10.29 ± 0.22	1.86 ± 0.02	51.42 ± 0.54
PL56	18.56 ± 0.75	3.95 ± 0.52	1.30 ± 0.06	23.80 ± 1.33
PL57	22.78 ± 0.08	3.25 ± 0.01	1.90 ± 0.02	27.93 ± 0.11
PL58	24.20 ± 0.74	7.46 ± 0.02	1.39 ± 0.06	33.05 ± 0.82
PL59	22.05 ± 0.78	5.13 ± 0.74	1.24 ± 0.46	28.42 ± 1.99
PL60	21.90 ± 0.40	3.29 ± 0.33	1.26 ± 0.05	26.44 ± 0.77
PL61	23.19 ± 1.24	2.82 ± 0.01	1.38 ± 0.03	26.01 ± 0.24

[1] Values calculated as an average of three replications and are expressed as mg/g of extract ± standard deviation.

3.3. Multivariate Analysis

The flavonoid profiling of wheat spouts has been studied [24–28], however the variation of flavonoids in the sprouts of the wheat mutant lines according to gamma-irradiated mutation breeding using metabolomics combined with multivariate analysis has not been studied so far. The differences among the samples were difficult to see in the chromatograms; however, the HCA with a heatmap and PCA scores scatter and loading plots showed a clear separation of clusters with the application of a multivariate analysis to the normalized data set.

The peak area values acquired by HPLC-DAD (280 nm) were exported for sample classification and multivariate analysis. HCA with a heatmap was performed to determine the similarities in the chemical composition of the wheat mutant lines induced from the original cultivar, and to highlight variations in content for flavonoids between the mutant lines (Figure 1). In the results of HCA, the 37 wheat sprout samples were clustered into three groups. Group I contained the certificated cultivar (PL26, "Geumgang") and the 10 mutant lines (PL11, PL19, PL20, PL23, PL42, PL44, PL46, PL55,

PL58, and PL59), and formed a cluster with high peak area values for peaks 4, 5, 8, and 10. Group II contained 11 mutant lines (PL09, PL13, PL16, PL47, PL56, PL54, PL60, PL61, PL53, PL10, and PL48). Among them, PL09, PL13, and PL16, with a relatively high content of peaks 1 and 11 clustered into a subgroup, as well as other subgroups, generally showed a low content of all of the peaks. In group III, 14 mutants (PL25, PL52, PL41, PL05, PL24, PL17, PL51, PL57, PL18, PL08, PL15, PL50, PL06, and PL14) were merged with the original cultivar (PL01), and this group showed the distribution of a high or low content of each peak. Two groups (II and III) were merged into one super group.

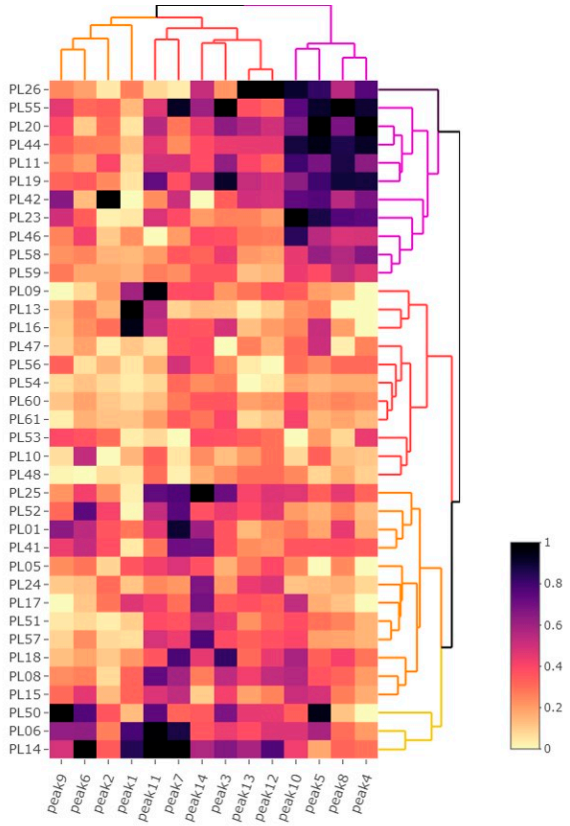

Figure 1. Hierarchical clustering analysis (HCA) with a heatmap for the sprouts of the wheat cultivars.

The 37 samples were clustered into three groups depending on the PCA scores, based on a peak area value identified from the wheat sprout samples. A total variance of 54.0% was explained by the first two principle components (34.4% and 29.0% by PC1 and PC2, respectively) in the presented scores scatter plot. As shown in Figure 2A, the wheat sprout samples between group I and group II were clearly distinguished by PC1, while the group I and group III samples were readily discriminated by PC2. The corresponding PCA loading plot showed the derivation of seven markers responsible for the separation of two groups (I and III; Figure 2B). Four markers (peaks 4, 5, 8, and 10) were far from the center and were shifted in the same direction as group I, suggesting that these compounds could be distinguishable markers for group I. One of the mutants, PL14, was distinguished from other samples, and the loading plot showed that peaks 6, 7, and 11 were attributable to the distribution. The PCA plot colored by the content of the variables was provided, indicating each of the most effective variables (Figure S1). The same quantitative clustering as the HCA heatmap was observed in the additional unbiased PCA; thus this multivariate analysis revealed that all of the results were reliable. This result could be valuable information for quality markers in the breeding studies of the wheat mutant lines.

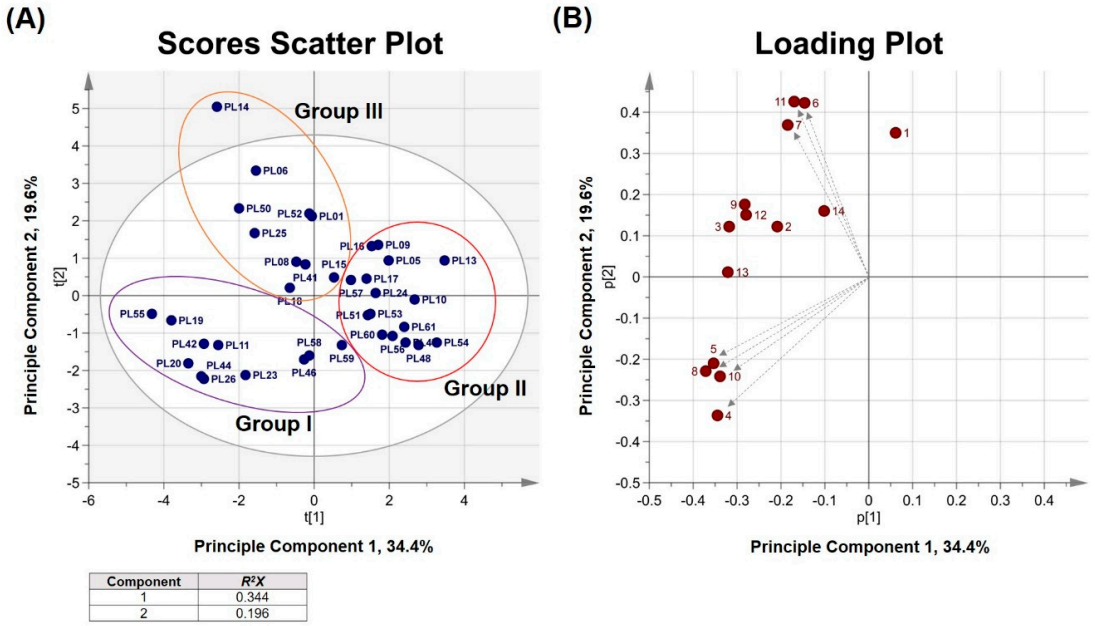

Figure 2. (**A**) Principal component analysis (PCA) scores scatter and (**B**) loading plots for the wheat sprout samples.

4. Conclusions

In this study, HPLC-DAD-ESIMS combined with a multivariate statistical analysis method was used to analyze the chemical compositions of the sprouts of 35 gamma-irradiated mutant lines, an original cultivar, and a certificated cultivar of wheat (*T. aestivum*). The results show that 14 components were tentatively identified, and a significant difference in the contents of the three compounds, i.e., isoschaftoside, isoorientin, and isoscoparin, was observed among the 37 wheat sprout samples. Multivariate analysis was used to speculate the distinguished compounds, such as apigenin-6,8-di-*C*-hexoside, isoorientin, crysoeriol 6-*C*-hexoside 8-*O*-deoxyhexoside, and apigenin 6-*C*- hexoside, comparing the relative content (the peak area at 280 nm) differences in the various components of the different samples. These results provide a scientific reference to further investigate the mutation mechanism of the radiation-induced wheat mutant cultivars or to evaluate the quality of the improved wheat cultivars.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/10/1489/s1: Figure S1: PCA scores scatter plots colored by the content of the variables (peaks 1–14).

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