JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 6503–6509 6503
DOI:10.1021/jf1006962

Effects of Exogenous Abscisic Acid on Yield, Antioxidant Capacities, and Phytochemical Contents of Greenhouse Grown Lettuces

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Antioxidants and phytochemicals in vegetables are known to provide health benefits. Strategies that enhance these properties are expected to increase the nutritional values of vegetables. The objective of this research is to assess the effects of exogenous abscisic acid (ABA) on yield, antioxidant capacities, and phytochemical content of lettuces grown in a greenhouse. Red loose leaf lettuce (cv. Galactic) and green loose leaf lettuce (cv. Simpson Elite) were cultivated using a randomized complete block design. Three concentrations of ABA in water [0 (control), 150, 300 ppm] were sprayed on the 30th and 39th days after sowing, and lettuces were harvested on the 46th day. Exogenous ABA significantly decreased yield of green and red lettuces. Total phenolic and total anthocyanin contents in red lettuce treated with ABA were significantly higher than in controls, whereas no significant differences were observed in green lettuce. ABA significantly induced the accumulation of chlorophyll b and total carotenoids in lettuces. The phenolic compounds identified and quantified in red and green lettuces included caffeoyltartaric acid, 5-*O*-caffeoylquinic acid, dicaffeoyltartaric acid, 3,5-dicaffeoylquinic acid, and quercetin 3-(6″-malonyl)-glucoside. Additionally, cyanidin 3-glucoside, cyanidin 3-(3″-malonoyl)-glucoside, and cyanidin 3-(6″-malonoyl)-glucoside in red lettuces were quantified. No significant effects of ABA on these individual phytochemicals were observed in green lettuces, whereas ABA significantly elevated the content of individual phytochemicals in red lettuces except for 5-*O*-caffeoylquinic acid. Differences among red lettuces with or without exogenous ABA were visualized on the score plots of principal component analyses. Loading plot indicated that multiple phenolic compounds contributed to the observed differences in red lettuces.

KEYWORDS: Lettuce; abscisic acid; anthocyanin; quercetin; phytochemicals; antioxidants

INTRODUCTION

Lettuce is one of the most consumed vegetables in many parts of the world including the United States. It is of particular interest in nutrition due to its content of antioxidants and phytochemicals (*1*). Phytochemicals contribute to both the sensory and health-promoting properties of vegetables. Many studies suggested that phytochemicals have positive effects in preventing cancer and cardiovascular diseases, scavenging oxidative free radicals, and reducing the incidence of many other chronic diseases (*2*).

The phytochemicals in lettuce include caffeic acid and its derivatives, flavonols, anthocyanins, chlorophyll and carotenoids (*3*). They are mostly secondary metabolites synthesized during the normal growth of plant or in response to the environmental and other stresses, such as UV radiation, wounding, and infections (*3*, *4*). Phytochemicals act as natural phytoalexins to protect plants against these stresses. Environmental conditions and different processing methods are known to impact the accumulation of phytochemicals. For example, the exposure of lettuce to light caused a decrease of flavonol content but a significant increase of anthocyanin content (*5*). Various antibrowning agents had been evaluated for their influence on phenolic compounds in lettuce, and the results showed that oxalic acid and ascorbic acid were more effective in preserving the phenolic compounds compared to cysteine and citric acid (*6*).

Endogenous ABA is synthesized from xanthophylls, and its accumulation in plants occurs when they are subjected to drought, salt, desiccation, cold, and infection stresses (*7*). ABA exerts prominent roles in plants in response to these environmental stresses (*7*). ABA induced the synthesis of hydrogen peroxide and superoxide under stressful conditions and caused oxidative stress (*8*). On the other hand, ABA also stimulates the antioxidant defense system in plants to yield more antioxidants that can resist the oxidative damage (*9*). Indeed, an upregulation of antioxidant gene expression and increase on activities of antioxidant enzymes were observed in previous studies in plants after ABA application (*8*, *10*).

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Published on Web 04/26/2010

A few studies have suggested that exogenous ABA positively influences phytochemical content in selected crops. ABA applications on grapevines were found to stimulate the accumulation of the anthocyanins in red grapes (*11*). ABA significantly increased the content of chlorophyll and carotenoid in leaves of maize seedlings (*9*). The effects of ABA on leafy vegetables, such as lettuce, have not been investigated. We hypothesized that exogenous application of ABA may positively impact the antioxidant capacity and phytochemical content in lettuce. We also postulated that ABA may have different effects on phytochemicals of diverse structures or different genotypes of lettuces. The present study was designed to test these hypotheses by evaluating the effects of the exogenous ABA on the yield, antioxidant capacities, and phytochemical contents of lettuces.

MATERIALS AND METHODS

Chemicals. AAPH (2,2′-azotis(2-amidinopropane)) was a product of Wako Chemicals Inc. (Bellwood, RI). 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and cyanidin 3-rutinoside were purchased from Sigma-Aldrich (St. Louis, MO). Folin−Ciocalteu reagent, Tween 20, quercetin, chlorogenic acid, and other chemicals were products of Fisher Scientific (Pittsburgh, PA). The (*S*)-abscisic acid was received as a gift from Valent BioSciences Corporation (Libertyville, IL).

Lettuce and ABA Applications. Red loose leaf lettuce (cv. Galactic) and green loose leaf lettuce (cv. Simpson Elite) (Johnny's Selected Seeds, Winslow, ME) were cultivated in a greenhouse in Gainesville, FL. Lettuce seeds were sowed into 200-cell Speedling flats (Speedling Incorporated, Sun City, FL) in the greenhouse on July 6, 2009, and transplanted into 3.8 L plastic pots filled with Metro-Mix 200 (SunGro Horticulture, Bellevue, WA) three weeks after sowing. Plants were fertilized weekly with a 20N−8.8P−16.6K soluble fertilizer solution. ABA was dissolved in water in 150 ppm and 300 ppm. Tween 20 was added as a leaf wetting agent using a 315 μL/L concentration. Control solution contained Tween 20 without ABA. The experiment was arranged in a randomized complete block design with four replications. In each block (replication), each cultivar received foliar spray of ABA in three concentrations [0 (control), 150, and 300 ppm]. There were five pots with two lettuce plants per pot in each treatment. The first foliar spray was carried out on the 30th day after sowing using 3.5 mL per pot on the upper surface of the leaves using a calibrated hand-held garden sprayer. The second spray was done on the 39th day after sowing using 7.0 mL per pot. Lettuces were harvested on the 46th day by cutting the plants from roots. One lettuce plant from each pot was weighed for yield measurement. The second and third outer leaves were removed from the other lettuce plant in each pot. For each treatment per replication, a composite sample of ten leaves from five lettuce plants was freeze-dried and moisture losses were recorded. Freeze-dried lettuce samples were blended into powder for solvent extraction.

Polyphenol Extraction. Freeze-dried lettuces (0.25 g) were weighed into 30 mL screw-capped glass tubes, and 20 mL of methanol:water:acetic acid (85:15:0.5, v/v) was added as extraction solvent. The extraction tubes were vortexed for 30 s and sonicated for 5 min, kept at room temperature for 20 min and vortexed again for another 30 s. The tubes were centrifuged at 3000 rpm for 10 min. Supernatants were decanted out and kept at −20 °C until further analysis.

Folin−Ciocalteu Assay. Total phenolic content was determined by the Folin−Ciocalteu assay. Lettuce extracts were mixed with diluted Folin−Ciocalteu reagent and 15% sodium carbonate. Absorbance at 765 nm was measured on a SPECTRAmax 190 microplate reader (Molecular Devices, Sunnyvale, CA) after 30 min of incubation at room temperature. Gallic acid was used to generate a standard curve. Results of total phenolic content for lettuces were expressed as milligram gallic acid equivalent per gram of freeze-dried leaf samples (mg GAE/g).

Oxygen Radical Absorbance Capacity ($ORAC_{FL}$) Assay. Lettuce extracts were incubated with fluorescein as a free radical probe and AAPH as a free radical generator (*12*). The kinetics of fluorescein degradation was read on a Spectra XMS Gemini microplate reader (Molecular Devices, Sunnyvale, CA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was used to generate a standard curve. The results of the ORAC antioxidant capacity of lettuces were expressed as μmol Trolox equivalent per gram of freeze-dried leaf samples (μmol TE/g).

DPPH Assay. The DPPH scavenging activities of freeze-dried lettuces were measured using a published method (*13*). Twenty grams of DPPH was dissolved into 100 mL of methanol to make a DPPH stock solution. DPPH working solution was freshly prepared by mixing 3.5 mL of DPPH stock solution and 6.5 mL of methanol. Absorbance at 515 nm was measured on a SPECTRAmax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The initial absorbance of DPPH working solution was between 0.9 and 1.0. The lettuce extracts (50 μL) were added to 950 μL of DPPH working solution and incubated in darkness for 60 min. Trolox solutions from 100 to 1000 μM were added to DPPH working solution as standards. The results of the DPPH scavenging activity of lettuces were expressed as μmol Trolox equivalent per gram of freeze-dried leaf samples (μmol TE/g).

Total Anthocyanin Assay. Total anthocyanin content was measured by the pH differential assay (*14*). Lettuce extracts were adjusted to pH 1 and pH 4.5 using 0.025 M potassium chloride buffer and 0.4 M sodium acetate buffer, respectively. Absorbance at 520 and 700 nm was measured on a DU 730 Life Science UV/vis spectrophotometer (Beckman Coulter, Fullerton, CA) after 30 min of incubation at room temperature. Absorbance (A) was calculated using $(A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$. Total anthocyanin content (mg of cyanidin 3-rutinoside/g) was calculated using $(A \times 595 \times 25 \times 1000)/(26900 \times 1)$. Results of total anthocyanin content for lettuces were expressed as milligram cyanidin 3-rutinoside equivalent per gram of freeze-dried samples (mg cyanidin 3-rutinoside/g).

HPLC−ESI-MS″ Analyses of Phytochemicals. An Agilent 1200 HPLC system consisting of an autosampler, a binary pump, a column compartment, a diode array detector and a fluorescent detector (Agilent Technologies, Palo Alto, CA) was interfaced to a HCT ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). Lettuce extracts were filtered through 0.45 μm filter units, and 20 μL was injected without further purification. An Agilent Zorbax Stablebond SB-C18 column (250 mm × 4.6 mm, 5 μm particle size, Agilent Technologies, Palo Alto, CA) was used for separation of phytochemicals. The binary mobile phase consisted of (A) formic acid:water (5:95 v/v) and (B) methanol. For the analysis of flavonols and caffeic acid derivatives, a 40 min gradient was used. The gradient is described as follows: 0−30 min, 5−40% B linear; 30−40 min, 40% B isocratic; followed by 5 min of re-equilibration of the column before the next run. The detection wavelength on diode array detector was 330 nm for caffeic acid derivatives and flavonols. Electrospray ionization in negative mode was performed using nebulizer 65 psi, drying gas 11 L/min, drying temperature 350 °C, and capillary 4000 V. The full scan mass spectra of the flavonoids were measured from m/z 100 to m/z 2000. For anthocyanin analysis, a 68 min gradient was used. The gradient is described as follows: 0−2 min, 0−5% B linear; 2−10 min, 5−20% B linear; 10−15 min, 20% B isocratic; 15−30 min, 20−30% B linear; 30−35 min, 30% B isocratic; 35−50 min, 30−45% B linear; 50−55 min, 45% B isocratic; 55−65 min, 45−5% B linear; 65−68 min, 5% B isocratic; followed by 5 min of re-equilibration of the column before the next run. The detection wavelength was 520 nm. Electrospray ionization in positive mode was performed using nebulizer 45 psi, drying gas 11 L/min, drying temperature 350 °C, and capillary 4000 V. The full scan mass spectra of the anthocyanins were recorded from m/z 350 to m/z 1500. Auto MS^2 was conducted with 50% compound stability and 60% trap drive level. Quercetin, chlorogenic acid and cyanidin 3-rutinoside were used as external standards to quantify flavonol, caffeic acid derivatives, and anthocyanins, respectively. Data were collected and calculated using Chemstation software (Version B. 01.03, Agilent Technologies, Palo Alto, CA).

Chlorophyll, Carotenoid Extraction and Estimation. A method reported by Lichtenthaler et al. (*15*) was adopted with minor modifications. Freeze-dried samples (0.25 g) were extracted with 5 mL of chloroform:methanol solvent (2:1 v/v) in 15 mL screw-capped glass tubes. The extraction tubes were vortexed for 30 s and sonicated for 2 min, and kept on ice for 20 min. After filtration, 1 mL of extract was dried under vacuum in an ISS110 Speedvac evaporator (Fisher Scientific, Pittsburgh, PA). The dried extracts were dissolved in 2 mL of 80% of acetone and sonicated for 30 s. The extracts were centrifuged at 13300 rpm for 5 min. Absorbance of supernatants was measured at 663, 646, and 470 nm on a DU 730 Life Science UV/vis spectrophotometer (Beckman Coulter, Fullerton, CA) using 80% of acetone as a blank. Chlorophyll a and b content (mg/g) was

Table 1. Yields, Total Phenolic Content, Total Anthocyanin Content, And Antioxidant Capacity of Lettuces Treated with Exogenous ABA of Different Concentrations[a]

cultivar	treatment	yield (g fresh wt/plant)	total phenol (GAE mg/g)	ORAC (TE μmol/g)	DPPH (TE μmol/g)	total anthocyanins (mg cyanidin 3-rutinoside/g)
green leaf lettuce (cv. Simpson Elite)	water control	79.0 ± 17.8 a	13.07 ± 3.12 a	57.80 ± 2.06 a	47.15 ± 13.74 a	nd[b]
	150 ppm ABA	52.2 ± 4.4 b	12.89 ± 1.45 a	54.07 ± 4.75 a	51.08 ± 9.93 a	nd
	300 ppm ABA	48.2 ± 10.9 b	13.42 ± 1.09 a	54.40 ± 1.83 a	57.24 ± 5.14 a	nd
red leaf lettuce (cv. Galactic)	water control	64.0 ± 7.8 a	28.22 ± 3.23 b	74.43 ± 3.49 b	59.14 ± 2.59 a	1.74 ± 0.51 b
	150 ppm ABA	42.7 ± 11.3 b	31.76 ± 4.43 ab	79.89 ± 2.83 ab	62.89 ± 3.42 a	3.52 ± 1.12 a
	300 ppm ABA	29.8 ± 9.0 b	33.10 ± 2.13 a	80.77 ± 2.29 a	64.35 ± 2.73 a	3.91 ± 1.03 a

[a] Total phenolic content, total anthocyanin content, and antioxidant capacity are mean ± standard deviation of lettuce from four blocks on basis of freeze-dried leaf samples. For each cultivar, means within a column followed by the same letter are not significantly different at $p \leq 0.05$. [b] Not detected.

calculated using $12.21A_{663} - 2.81A_{646}$ and $20.13A_{646} - 5.03A_{663}$, respectively. Total chlorophyll concentration (mg/g) was the sum of chlorophyll a and b. Total carotenoid content (mg/g) was calculated using $A_{1cm}^{1\%} = 2290$ at 470 nm as reported by Lichtenthaler et al. (*15*).

Statistical and Multivariate Analyses. Samples were analyzed in duplicate, and the average values were used. Data were expressed as mean ± standard deviation of lettuce samples from four blocks. One-way analyses of variance with Tukey−Kramer HSD all-pairs comparison of the means were performed using JMP software (Version 8.0, SAS Institute Inc., Cary, NC). Data (except the yield measurement) were $\log_{10}$ transformed before ANOVA analyses. A difference of $p \leq 0.05$ was considered as significant. Principal component analyses were done with the same software using the contents of total carotenoid, chlorophyll a and b, caffeoyltartaric acid, 5-*O*-caffeoylquinic acid, dicaffeoyltartaric acid, 3,5-dicaffeoylquinic acid, quercetin 3-(6″-malonyl)-glucoside, cyanidin 3-glucoside, cyanidin 3-(3″-malonoyl)-glucoside, and cyanidin 3-(6″-malonoyl)-glucoside as variables without data transformation or normalization.

RESULTS

Yields, Total Phenolic Content, Total Anthocyanin Content, and Antioxidant Capacities. Exogenous ABA significantly decreased the yields of both green and red leaf lettuce cultivars compared with the control (**Table 1**). ABA at 300 ppm appeared to be more effective in reducing the yield than ABA at 150 ppm, however, the difference was not statistically significant.

ABA did not affect total phenolic content and antioxidant capacities in green lettuces (**Table 1**). Total phenolic content and $ORAC_{FL}$ in red lettuces was elevated by 300 ppm ABA application, but not the 150 ppm ABA. No difference was observed in DPPH scavenging capacities. The exogenous ABA application significantly increased the content of total anthocyanins in red leaf lettuces. However, no significant differences were observed between lettuces treated with 150 ppm and 300 ppm ABA.

Phytochemical Identification and Quantitation. Five phenolic compounds were identified in both green and red lettuces while three anthocyanins were identified only in red lettuces (**Figure 1**). Identification was based on mass spectra (**Figure 2**) and confirmed by comparing to previous reports (*3*, *4*, *16*). Peak **1** was identified as caffeoyltartaric acid in accordance to $[M - H]^-$ m/z 311 and a product ion at m/z 149 formed by tartaric acid. Peak **2** had deprontonated ion at m/z 353 and a product ion at m/z 191, suggesting the existence of a caffeic acid and a quinic acid moiety. The peak was identified as 5-*O*-caffeoylquinic acid. Peak **3** was identified as dicaffeoyltartaric acid due to the loss of two caffeic acid moieties suggested by m/z 311 and m/z 149. Similarly, peak **4** was identified as 3,5-dicaffeoylquinic acid. Peak **5** had $[M - H]^-$ m/z 549 and product ion at m/z 300 $[M - H - 249]^-$, suggesting the loss of malonylglucoside and the existence of quercetin. This peak was identified as quercetin 3-(6″-malonyl)-glucoside. Peak **6** (m/z 449 $[M]^+$, product ion at m/z 287) was cyanidin 3-glucoside. Peaks **7** and **8** had the same molecular weight of 535 ($[M]^+$, m/z) and product ion at m/z 287 formed by cyanidin. They were identified as cyanidin 3-(3″-malonoyl)-glucoside and cyanidin 3-(6″-malonoyl)-glucoside, respectively, by comparing to previous reports (*3*, *4*, *16*). In green lettuce, dicaffeoyltartaric acid was the major caffeic acid derivative, whereas both 5-*O*-caffeoylquinic acid and dicaffeoyltartaric acid were major caffeic acid derivatives in red lettuce. In red leaf lettuces, cyanidin 3-(6″-malonoyl)-glucoside accounted for 93% of the total anthocyanins.

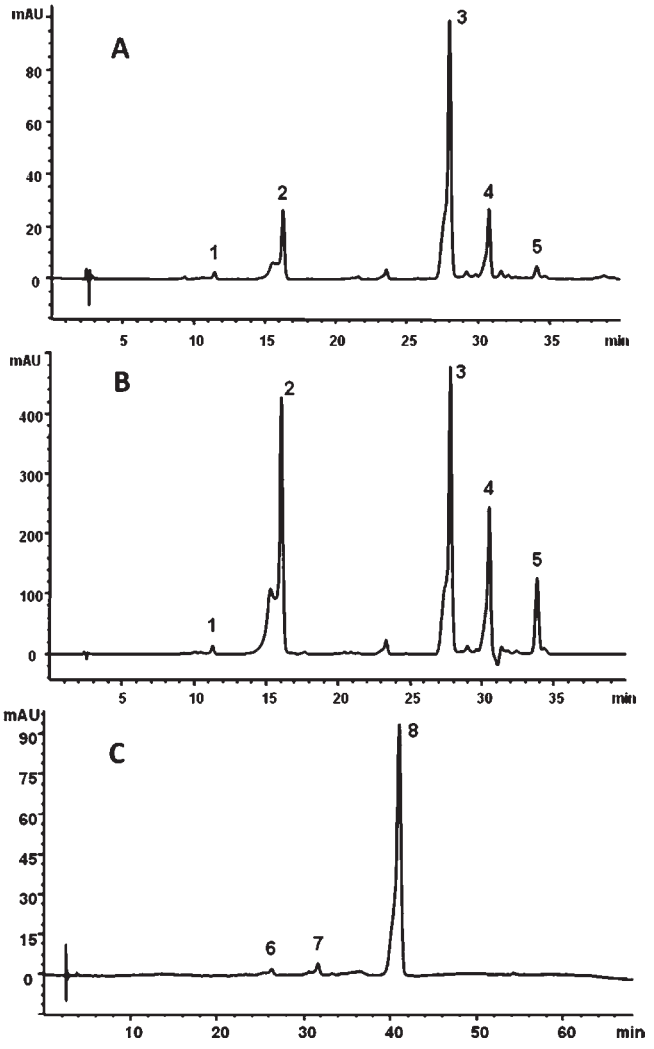

Figure 1. HPLC chromatograms of lettuces: **A**, green leaf lettuce (cv. Simpson Elite) at 330 nm; **B**, red leaf lettuce (cv. Galactic) at 330 nm; and **C**, red leaf lettuce (cv. Galactic) at 520 nm. Peaks **1**, **2**, **3**, **4**, and **5** in both green and red leaf lettuces are caffeoyltartaric acid, 5-*O*-caffeoylquinic acid, dicaffoeyltartaric acid, 3,5-dicaffeoylquinic acid, and quercetin 3-(6″-malonyl)-glucoside, respectively. Peaks **6**, **7**, and **8** in red leaf lettuce are cyanidin 3-glucoside, cyanidin 3-(3″-malonoyl)-glucoside, and cyanidin 3-(6″-malonoyl)-glucoside, respectively.

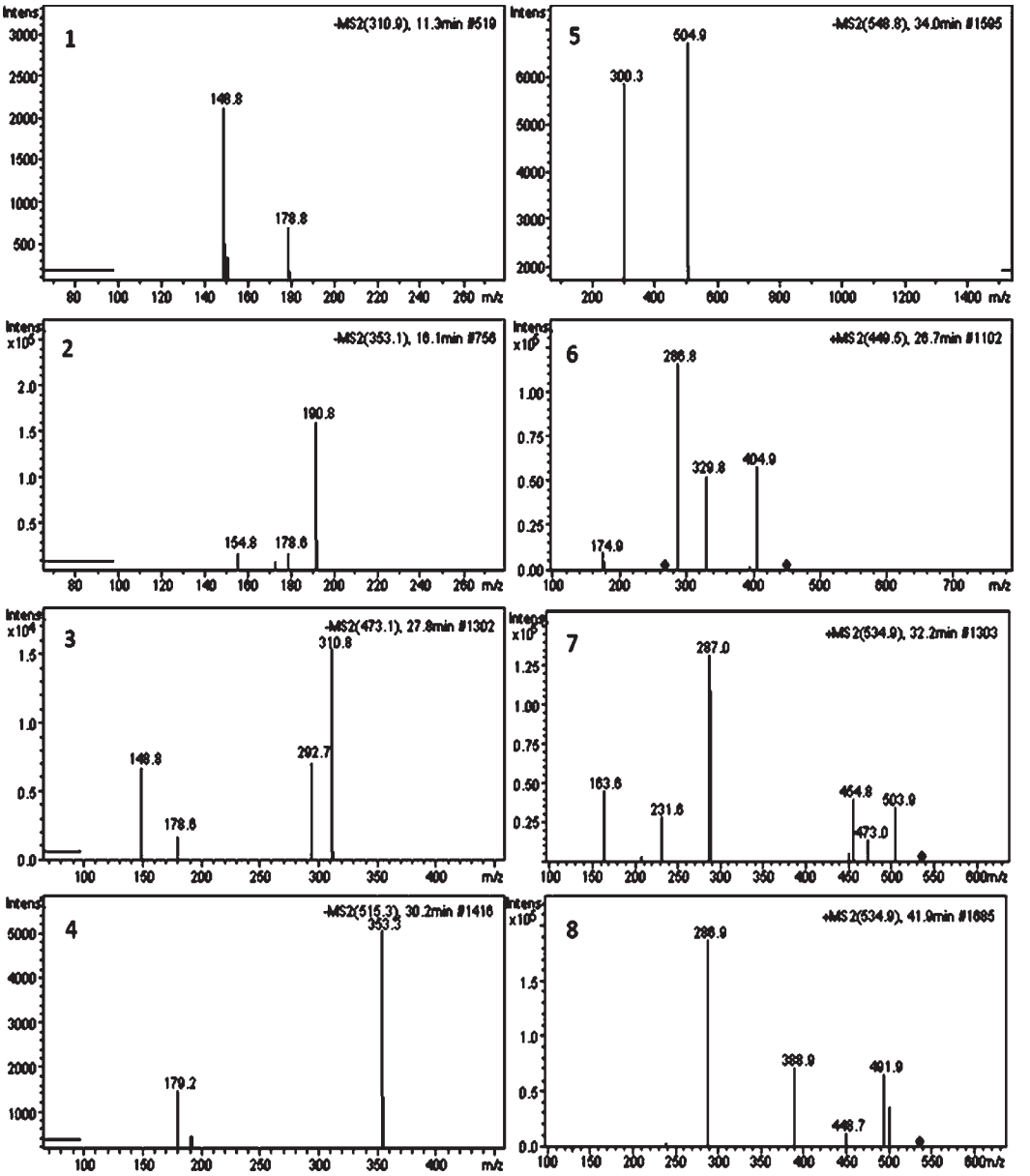

Figure 2. Product ion spectra (MS^2) of phytochemicals in lettuces. **1**, **2**, **3**, **4**, and **5**, at negative mode, are product ion spectra of caffeoyltartaric acid, 5-*O*-caffeoylquinic acid, dicaffoeyltartaric acid, 3,5-dicaffeoylquinic acid, and quercetin 3-(6″-malonyl)-glucoside, respectively. **6**, **7**, and **8**, at positive mode, are product ion spectra of cyanidin 3-glucoside, cyanidin 3-(3″-malonoyl)-glucoside, and cyanidin 3-(6″-malonoyl)-glucoside, respectively.

Table 2. Content of Individual Caffeic Acid Derivative and Flavonol in Lettuces Treated with Exogenous ABA of Different Concentrations[a]

cultivar	treatment	caffeoyltartaric acid (mg/g)	5-*O*-caffeoylquinic acid (mg/g)	dicaffeoyltartaric acid (mg/g)	3,5-dicaffeoylquinic acid (mg/g)	quercetin 3-(6″-malonyl)-glucoside (mg/g)
green leaf lettuce (cv. Simpson Elite)	water control	0.06 ± 0.03 a	1.24 ± 0.51 a	1.92 ± 0.63 a	0.59 ± 0.25 a	0.16 ± 0.07 a
	150 ppm ABA	0.06 ± 0.02 a	1.25 ± 0.28 a	1.84 ± 0.35 a	0.49 ± 0.09 a	0.18 ± 0.06 a
	300 ppm ABA	0.06 ± 0.02 a	1.45 ± 0.17 a	2.02 ± 0.25 a	0.59 ± 0.09 a	0.19 ± 0.03 a
red leaf lettuce (cv. Galactic)	water control	0.04 ± 0.01 b	6.60 ± 1.46 a	2.55 ± 0.32 b	1.87 ± 0.31 b	1.70 ± 0.18 b
	150 ppm ABA	0.06 ± 0.01 a	7.49 ± 1.25 a	3.38 ± 1.12 ab	2.41 ± 0.70 ab	2.27 ± 0.37 a
	300 ppm ABA	0.08 ± 0.03 a	7.72 ± 0.88 a	3.77 ± 0.48 a	2.55 ± 0.34 a	2.30 ± 0.16 a

[a] Results are mean ± standard deviation of lettuce from four blocks on basis of freeze-dried leaf samples. For each cultivar, means within a column followed by the same letter are not significantly different at $p \leq 0.05$.

The exogenous ABA application did not change composition or pattern of phytochemicals in lettuces.

As shown in **Tables 2** and **3**, no significant differences were observed in the contents of the individual flavonoid among control and ABA treated green lettuces. However, the exogenous ABA application showed a significantly positive influence on accumulation of individual flavonoid in red lettuces except for 5-*O*-caffeoylquinic acid. The ABA treated red lettuces had higher levels of caffeoyltartaric acid, quercetin 3-(6″-malonyl)-glucoside, cyanidin 3-glucoside, cyanidin 3-(3″-malonoyl)-glucoside,

and cyanidin 3-(6″-malonoyl)-glucoside compared to controls. However, no significant differences were observed with two different levels of ABA treatment (150 ppm and 300 ppm). Contents of dicaffeoyltartaric acid and 3,5-dicaffeoylquinic acid were significantly higher in red lettuces with 300 ppm ABA compared to controls whereas no differences were observed between 150 ppm ABA treated lettuces and controls. Total anthocyanin content in red lettuces quantified with HPLC was comparable to those measured using pH differentiation (**Table 1**). Higher contents of total phenolics and total anthocyanins in ABA treated red lettuces were in accordance with the increase of individual flavonoid.

Chlorophyll and Carotenoid Content. Although no significant differences were observed in total chlorophyll and chlorophyll a in green lettuces with or without ABA treatment, green lettuces treated with ABA showed significantly higher levels of chlorophyll b and total carotenoid content compared to controls (**Table 4**). However, no significant differences were observed in results obtained from two different concentrations of ABA (150 ppm and 300 ppm). Similar results were observed in red lettuces. The exogenous application of ABA significantly enhanced chlorophyll b and total carotenoid content whereas chlorophyll a and total chlorophyll were not affected by the exogenous ABA application.

Principal Component Analyses. Samples with similarities cluster on score plot of principal component analysis and segregate from samples of different properties. The first three principal components accounted for 52%, 23%, and 12% of the total variances for green lettuces. No obvious separation was observed for green lettuces treated with water, 150 ppm and 300 ppm (**Figure 3A**). For principal component analysis of red leaf lettuce, the first three principal components accounted for 69%, 18%, and 5% of the total variances, respectively. Contrary to the green lettuces, red lettuces treated with 300 ppm ABA segregated from water-treated control on the score plot (**Figure 3 B**). The red leaf lettuce treated with 150 ppm were scattered in between. The impact of individual phytochemicals to the overall variance in red lettuces was visualized on the loading plot of principal component analysis (**Figure 4**). Compounds **1**, **3**, **4**, **5**, **6**, **7**, **8** [caffeoyltartaric acid, dicaffeoyltartaric acid, 3,5-dicaffeoylquinic acid, quercetin 3-(6″-malonyl)-glucoside, cyanidin 3-glucoside, cyanidin 3-(3″-malonoyl)-glucoside, cyanidin 3-(6″-malonoyl)-glucoside] formed a cluster on the loading plot, suggesting that the concentrations of these compounds are positively correlated and they had similar impacts on overall variance. These compounds primarily impacted the variances on component 1, but showed no or little influence on component 2. The loading plot also suggested compounds **10** and **2** (chlorophyll a and 5-*O*-caffeoylquinic acid) were inversely correlated.

Table 3. Content of Individual Anthocyanin in Red Lettuce (Cv. Galactic) Treated with Exogenous ABA of Different Concentrations[a]

treatment	cyanidin 3-glucoside (mg/g)	cyanidin 3-(3″-malonoyl)-glucoside (mg/g)	cyanidin 3-(6″-malonoyl)-glucoside (mg/g)
water control	0.03 ± 0.01 b	0.05 ± 0.02 b	1.23 ± 0.46 b
150 ppm ABA	0.07 ± 0.03 a	0.11 ± 0.04 a	2.51 ± 0.92 a
300 ppm ABA	0.08 ± 0.02 a	0.12 ± 0.03 a	2.93 ± 0.64 a

[a] Results are mean ± standard deviation of lettuce from four blocks on basis of freeze-dried leaf samples. For each cultivar, means within a column followed by the same letter are not significantly different at $p \leq 0.05$.

DISCUSSION

ABA has been applied to increase accumulation of anthocyanins in the skin of red grapes (*17*). The concentrations used in this

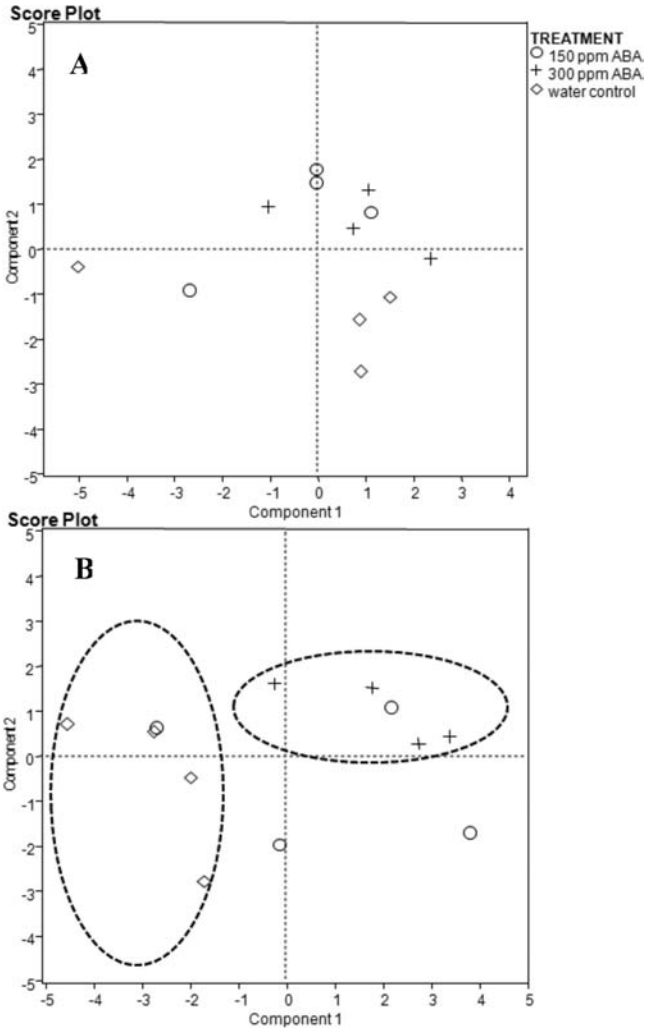

Figure 3. Score plots of principal component analysis of lettuces: **A**, green leaf lettuces (cv. Simpson Elite); **B**, red leaf lettuces (cv. Galactic).

Table 4. Content of Total Chlorophyll, Chlorophyll a, Chlorophyll b, and Total Carotenoid in Lettuces Treated with Exogenous ABA of Different Concentrations[a]

cultivar	treatment	Total chlorophyll (mg/g)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total carotenoid (mg/g)
green leaf lettuce (cv. Simpson Elite)	water control	7.15 ± 0.77 a	5.34 ± 0.63 a	1.81 ± 0.20 b	0.91 ± 0.13 b
	150 ppm ABA	7.94 ± 0.85 a	5.74 ± 0.68 a	2.20 ± 0.21 a	1.06 ± 0.13 a
	300 ppm ABA	7.64 ± 0.47 a	5.28 ± 0.38 a	2.37 ± 0.12 a	1.08 ± 0.08 a
red leaf lettuce (cv. Galactic)	water control	7.63 ± 0.63 a	5.58 ± 0.41 a	2.04 ± 0.28 b	0.97 ± 0.11 b
	150 ppm ABA	7.29 ± 0.67 a	5.12 ± 0.51 a	2.17 ± 0.17 b	1.09 ± 0.13 ab
	300 ppm ABA	7.85 ± 0.36 a	5.38 ± 0.29 a	2.46 ± 0.09 a	1.19 ± 0.04 a

[a] Results are mean ± standard deviation of lettuce from four blocks on basis of freeze-dried leaf samples. For each cultivar, means within a column followed by the same letter are not significantly different at $p \leq 0.05$.

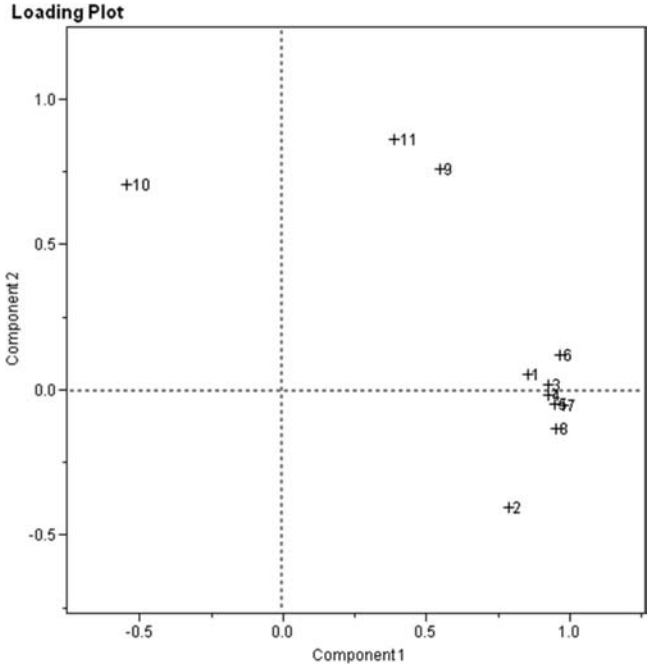

Figure 4. Loading plot of principal component analysis of red lettuces based on principal components 1 and 2: **1**, caffeoyltartaric acid; **2**, 5-*O*-caffeoylquinic acid; **3**, dicaffeoyltartaric acid; **4**, 3,5-dicaffeoylquinic acid; **5**, quercetin 3-(6″-malonyl)-glucoside; **6**, cyanidin 3-glucoside; **7**, cyanidin 3-(3″-malonoyl)-glucoside; **8**, cyanidin 3-(6″-malonoyl)-glucoside; **9**, total carotenoid; **10**, chlorophyll a; **11**, chlorophyll b.

study were the same as those applied on grapes (150 and 300 ppm) (*17*). It was reported that application of ABA on grapevines after veraison increased the anthocyanin content in grape skin by 100% (*18*). However, it should be noted that anthocyanin accumulation caused by ABA does not appear to be a universal phenomenon. There are reports indicating anthocyanin or catechin level decreased in plants treated with ABA (*19*, *20*).

In this study, the exogenous application of ABA significantly induced the accumulation of anthocyanins, flavonols, and caffeic acid derivatives in red lettuces. A possible explanation is that these three classes of phytochemicals share a common phenylpropanoid synthetic pathway. Caffeic acid is the early product of this pathway. Anthocyanins and flavonols are the downstream products (*21*). The phenylalanine ammonia-lyase and cinnamate 4-hydroxylase are the first two steps of phenolic synthesis. ABA has been reported to increase the activity of phenylalanine ammonia-lyase (*22*) and transcription of cinnamate 4-hydroxylase (*23*).

ABA plays an important role in adaptive responses to the environmental stresses, such as heat stress, water stress, and salt stress (*7*). Plants accumulate endogenous ABA when subjected to environmental stresses. ABA, as a critical signal transduction compound, subsequently upregulates the transcription of stress-responsive genes to protect the plants (*24*). Although basal synthesis of anthocyanins is independent of ABA (*25*), ABA stimulates the synthesis of anthocyanins and other phenolic compounds (*25*). Several mechanisms are thought to be involved. Endogenous ABA is known to induce the synthesis of hydrogen peroxide in plants. The resultant hydrogen peroxide causes oxidative stress and damages the tissue and cells of the plants (*25*). Phytochemicals with antioxidant capacities are synthesized in response to this stress (*9*). Other plant growth regulators, such as salicylic acid and jasmonates, are also known to regulate flavonoid biosynthesis in vegetables (*26*). Therefore ABA effects on stress-responsive gene regulation and flavonoid level may also result from interacting with other plant growth hormones (*27*).

The total phenolic content and antioxidant capacity of lettuces measured by $ORAC_{FL}$ were consistent with a previous report (*28*) . The increase of phenolic content in red lettuce treated by 300 ppm of ABA was consistent with a higher antioxidant capacity measured by $ORAC_{FL}$. However, no significant differences were observed in DPPH scavenging activity. Worth noting were the differences in antioxidant capacities measured by DPPH and $ORAC_{FL}$ assays. This is likely due to the differences in reaction mechanisms because DPPH scavenging activity is based on electron transfer while ORAC antioxidant capacity uses hydrogen atom transfer (*29*). Increases of carotenoids in lettuces (both green and red cultivars) were observed. This is consistent with the observation on grape leaves (*30*). The increase of carotenoid content may share similar mechanisms to phenolic compounds.

In contrast to red lettuces, no increases of phytochemical contents were observed in green lettuces. It suggested that different cultivars of lettuce have differential mechanisms coping with exogenous ABA. This is consistent with a previous report on rice which showed that flavonoid accumulation in response to stress and ABA was genotype dependent (*31*). Such a phenomenon may be explained by mutation or absence of specific transcription factors associated with flavonoid biosynthesis in crops of different cultivars (*32*).

Growing environments are known to impact phytochemical synthesis in plants. It has been reported that lettuces grown in greenhouse accumulated less flavonoid compounds than those in open field (*5*, *33*). This is because UV light is a potent inducer of flavonoid synthesis and lettuce in greenhouse received less UV radiation (*5*). However, the increases of phytochemicals in grape leaves by UV-B radiation or drought stress were suggested to be mediated by ABA (*30*). This present study was conducted in a greenhouse and, therefore, did not address effects of ABA in open field.

ABA is a natural compound synthesized by all plants, and no toxic effects have been reported. It appears to be a promising agent to increase phytochemical contents in fruits and vegetables. However, exogenous ABA significantly decreased the yields of both lettuce cultivars indicating a trade-off between new growth of plant and phytochemical synthesis. Decreases in yield may be explained by the capacity of ABA to downregulate enzymes needed for photosynthesis (*34*). Such a decrease of crop yield may hinder practical uses of ABA. This drawback may be alleviated in part by applying ABA at different concentrations or growing stages. Decrease of lettuce size by ABA suggested that yield can be increased by applying a higher planting density in the field. Additional experiments have been planned to explore these possibilities.

In conclusion, exogenous ABA application significantly increased contents of total phenols, total carotenoids, anthocyanins, flavonols, and caffeic acid derivatives in the red leaf lettuce. No increase in phytochemical content was observed in the green leaf lettuce. Exogenous ABA significantly decreased the yield of both lettuce cultivars.

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Received for review February 19, 2010. Revised manuscript received April 18, 2010. Accepted April 19, 2010.