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Characterization of Nutritionally Important Carotenoids in Bunching Onion

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***Abstract*. Members of the *Allium* genus are consumed for their culinary flavor attributes, but also contain antioxidant and anticarcinogenic phytochemicals. Bunching onions (*Allium fistulosum* L.) are commonly used in Asian cuisine, in which both leaves and pseudostems are consumed. Carotenoids and chlorophylls are important classes of phytochemicals gaining attention for their health attributes. The goal of our study was to characterize carotenoids and chlorophylls and identify possible genetic and environmental influences on carotenoid concentrations among *A. fistulosum* accessions. Twelve USDA-ARS accessions were field grown in Knoxville, TN, and Geneva, NY, during the summer of 2007. After harvest, carotenoid and chlorophyll pigments were evaluated in leaf and pseudostem tissues using high-performance liquid chromatography. We were able to identify the presence of antheraxanthin, β-carotene, chlorophyll *a* and *b*, lutein, neoxanthin, and violaxanthin in leaf tissues; however, pigments were not found in pseudostem tissues. Carotenoid and chlorophyll concentrations did not differ among accessions or between locations. It is possible that accessions evaluated in this study were a narrow genetic base or were selected based on flavor attributes and not leaf tissue pigmentation.**

Allium species have been valued for culinary and medicinal attributes for more than 4000 years. Members of the genus posses therapeutic *S*-alk(en)yl-L-cysteine sulfoxides (Eady et al., 2008), antibacterial compounds (Kim, 1997), high concentrations of flavonoids, and various antioxidants (Štajner et al., 2006). There has been extensive research into the health properties of bulb onion (*Allium cepa* L.) and garlic (*A. sativum* L.); however, little is known about the composition and concentrations of therapeutic compounds in other *Allium* species. Bunching onion (*A. fistulosum* L.) is commonly cultivated in China, Korea, and Japan, where it is grown for its foliar tissues and consumed either cooked or as a fresh vegetable (Umehara et al., 2006). With the popularity of Asian cuisine, bunching onions are being consumed more in Western diets.

Carotenoids are lipid soluble pigments integrated into light-harvesting complexes (Croce et al., 1999a, 1999b). Carotenoids function as light-harvesting pigments by channeling photons to the photosynthetic reaction center and functioning as photoprotectants by quenching damaging free radicals or through active nonphotochemical quenching of excess light energy (Croce et al., 1999b; Niyogi, 1999). Carotenoid formation is highly conserved throughout all plant species with six primary functioning carotenoids (antheraxanthin, β-carotene, lutein, neoxanthin, violaxanthin, and zeaxanthin) normally present in leafy tissues (Sandmann, 2001). Carotenoid accumulation in plant tissue is influenced by physiological, biochemical, and genetic factors interacting simultaneously with the growing environment (Chenard et al., 2005; Kopsell et al., 2003, 2004, 2005). Noticeable differences in leaf and fruit tissue colorations among different vegetable species are consistent with differences in carotenoid accumulations and types of carotenoids present (Gross, 1991; Kimura and Rodriguez-Amara, 2003; Sommerburg et al., 1998). Moreover, carotenoid variation among genotypes within species can also be significant (Kopsell et al., 2005; Kurilich et al., 1999).

Associations of increased fruit and vegetable consumption with the prevention of chronic diseases are shedding new light on the roles of chlorophylls as valuable phytochemicals (Ferruzzi and Blakeslee, 2007). Recent evidence is also suggesting that dietary chlorophylls may possess biological activities associated with cancer prevention, antimutagenic activity, and induction of apoptosis in tumor cells (Balder et al., 2006; Egner et al., 2003). Alternatively, dietary chlorophylls may reduce intestinal absorption of potentially harmful chemical mutagens and carcinogens (Hartman and Shankel, 1990; Natsume et al., 2004). Dietary chlorophylls from green vegetables such as bunching onions may soon be recognized for their health properties.

Genetic variability for total carotenoid and chlorophyll pigments among different *Allium* species has previously been reported (Štajner et al., 2006); however, characterization of individual carotenoids present in the leaf and pseudostem tissues and genetic variations among bunching onion germplasm is lacking. Therefore, the goal of this study was to characterize carotenoid and chlorophyll pigment concentrations among different plant accessions available as germplasm from the USDA-ARS Plant Genetic Resources Unit. This study will identify and quantify carotenoid pigments present in the tissues of bunching onions. Data may be useful for dieticians assessing the nutritional contributions of bunching onions in regard to carotenoids. Data may also be valuable to plant improvement programs designed to increase the nutritional value of onions and allied crops.

Materials and Methods

Plant culture and harvest. Assessment of tissue carotenoid variability among bunching onion genotypes was performed using 12 USDA-ARS accessions (Table 1). Seeds from each accession were sown and cultured under protected culture using a Cornell Mix described by Boodley and Sheldrake (1977) at the Plant Genetic Resource Unit in Geneva, NY, on 19 Mar. 2007. Seedlings of each accession were shipped to Knoxville, TN, on 23 Apr. 2007.

At the Geneva, NY, location, seedlings were transplanted to the field on 15 May 2007. Transplants were placed 16 cm apart in double rows set 92 cm apart on black plastic covering a Honeoye silt loam soil (fine-loamy, mixed, active, mesic Glossic Hapludalf). Preplant fertilizer applications were 112.1N–43.6P–83.1K (in kg·ha^{-1} from potassium nitrate, monoammonium phosphate, and muriate of potash). The plot also received two fertigation applications of 14 kg N/ha (as

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ammonium nitrate) through drip irrigation 14 and 28 d after transplanting. Plots consisted of three individual plants for each accession set in single rows of the plastic and were arranged in a randomized complete block replicated three times.

At the Knoxville, TN, location, seedlings were grown under protected culture and were transplanted to the field on 11 May 2007. Transplants were placed 12 cm apart in double rows set 50 cm apart on black plastic (86.4 cm in width) covering a Sequatchie silt loam soil (fine-loamy, siliceous, thermic Humic Hapudult). Preplant fertilizer and fertigation applications were the same as those described for the Geneva, NY, location. The experimental design was also similar to the Geneva, NY, location. Onions were harvested and all three plants were bulked as one sample on 27 to 28 June in Knoxville, TN, and on 4 to 6 July in Geneva, NY. Plants were stored on ice, transported to the laboratory, and cleaned of any residual soil. The root plate was removed and the plants were divided into pseudostem and leaf tissues at the separation layer between the tissues. Geneva, NY, samples were packed in dry ice and shipped to Knoxville for analysis. All samples were frozen at –80 °C before lyophilization (Model 6L FreeZone; LabConCo, Kansas City, MO).

Tissue extraction. Tissues were freeze-dried at a constant temperature of –25 °C before extraction. Pigments were extracted from freeze-dried tissues according to Kopsell et al. (2004) and analyzed according to Kopsell et al. (2007a). A 0.1-g tissue subsample was rehydrated with 0.8 mL of ultrapure H_2O and placed in a water bath set at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl-β-8′-apo-carotenoate (Sigma Chemical Co., St. Louis, MO) was added to determine extraction efficiency. The addition of 2.5 mL of tetrahydrofuran (THF) stabilized with 25 mg·L^{-1} of 2,6-Di-*tert*-butyl-4-methoxyphenol was performed after sample hydration. The sample was then homogenized in a Potter-Elvehjem (Kontes, Vineland, NJ) tissue grinding tube using ≈25 insertions with a pestle attached to a drill press set at 540 rpm. During homogenization, the tube was immersed in ice to dissipate heat. The tube was then placed into a clinical centrifuge for 3 min at 500 g_n. The supernatant was removed and the sample pellet was resuspended in 2 mL THF and homogenized again with the same extraction technique. The procedure was repeated for a total of four extractions to obtain a colorless supernatant. The combined supernatants were reduced to 0.5 mL under a stream of nitrogen gas (N-EVAP 111; Organomation Inc., Berlin, MA) and brought up to a final volume of 5 mL with methanol (MeOH). A 2-mL aliquot was filtered through a 0.2-µm polytetrafluoroethylene filter (Model Econofilter PTFE 25/20; Agilent Technologies, Wilmington, DE) using a 5-mL syringe (Becton, Dickinson and Company, Franklin Lakes, NJ) before high-performance liquid chromatography (HPLC) analysis.

High-performance liquid chromatography pigment analysis. An Agilent 1200 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, CA) was used for pigment separation. Chromatographic separations were achieved using an analytical scale (4.6 mm i.d. × 250 mm) 5 µm, 200 Å polymeric RP-C_{30} column (ProntoSIL, MAC-MOD Analytical Inc., Chadds Ford, PA), which allowed for effective separation of chemically similar pigment compounds. The column was equipped with a 5-µm guard cartridge (4.0 mm i.d. × 10 mm) and holder (ProntoSIL) and was maintained at 30 °C using a thermostatted column compartment. All separations were achieved isocratically using a binary mobile phase of 11% methyl *tert*-butyl ether, 88.99% MeOH, and 0.01% triethylamine (v/v/v). The flow rate was 1.0 mL·min^{-1} with a run time of 53 min followed by a 2-min equilibration before the next injection. Eluted compounds from a 10-µL injection were detected at 453 (carotenoids and internal standard), 652 [chlorophyll *a* (Chl *a*)], and 665 Chl *b*) nm and data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies). Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection using external standards (β-carotene, Chl *a*, Chl *b*, lutein, neoxanthin, violaxanthin, zeaxanthin from ChromaDex Inc., Irvine, CA).

Data sets were analyzed by the GLM procedures of SAS (Cary, NC) with cultivar means separated by least significant difference of $\alpha = 0.05$. Leaf tissue pigment data are presented on a fresh weight (FW) basis.

Results and Discussion

Leaf tissue carotenoid and chlorophyll pigments were not influenced by growing location or by accession (Tables 1 and 2). The USDA Nutrient Database lists *A. fistulosum* as averaging 0.60 mg/100 g FW of β-carotene and 1.14 mg/100 g FW of lutein + zeaxanthin (USDA Nutrient Database, 2008). Previously, total carotenoids in the leaf tissues of *A. fistulosum* (cultivar not reported) were reported to be 2.87 mg·g^{-1} FW (Štajner et al., 2006). Umehara et al. (2006) reported β-carotene values in the leaves of *A. fistulosum* L. cv. Kujyoasagikei to be 4.63 mg/100 g FW. Carotenoid values for accessions in the current study differ from values reported previously (Tables 1). These previous reports used different extraction and analytical methods to determine carotenoid concentrations. Our current analytical protocol includes methods proven to be more efficient at tissue pigment

Table 1. Mean concentrations[z] (mg/100 g fresh weight) for carotenoid pigments in leaf tissues of bunching onion (*Allium fistulosum* L.) USDA-ARS accessions field-grown in Knoxville, TN, and Geneva, NY.

	Leaf tissue carotenoids (mg/100 g fresh wt)					
Accession lot	β-carotene	Antheraxanthin	Lutein	Neoxanthin	Violaxanthin	Total
G 32145	1.74 ± 0.53	0.11 ± 0.06	2.92 ± 0.86	0.72 ± 0.37	0.48 ± 0.24	5.97 ± 1.76
PI 462345	1.58 ± 0.39	0.13 ± 0.06	2.96 ± 1.02	0.69 ± 0.18	0.69 ± 0.36	6.03 ± 1.57
PI 280562	1.55 ± 0.62	0.10 ± 0.09	2.52 ± 0.41	0.47 ± 0.36	0.46 ± 0.25	5.10 ± 1.31
PI 546228	1.52 ± 0.42	0.10 ± 0.08	2.72 ± 0.97	0.69 ± 0.30	0.58 ± 0.24	5.61 ± 1.61
PI 508401	1.51 ± 0.32	0.12 ± 0.07	2.82 ± 0.40	0.72 ± 0.17	0.40 ± 0.18	5.57 ± 0.60
PI 436539	1.47 ± 0.44	0.15 ± 0.10	2.85 ± 1.07	0.78 ± 0.30	0.61 ± 0.21	5.86 ± 1.16
PI 656924	1.45 ± 0.41	0.13 ± 0.07	2.31 ± 0.34	0.60 ± 0.27	0.36 ± 0.20	4.84 ± 0.94
G 32088	1.43 ± 0.42	0.11 ± 0.05	2.95 ± 0.68	0.68 ± 0.23	0.53 ± 0.23	5.70 ± 1.11
PI 462357	1.41 ± 0.35	0.12 ± 0.07	2.30 ± 0.35	0.64 ± 0.18	0.45 ± 0.26	4.92 ± 0.67
PI 546343	1.37 ± 0.24	0.10 ± 0.07	2.48 ± 0.95	0.77 ± 0.32	0.62 ± 0.39	5.33 ± 1.74
PI 274254	1.27 ± 0.29	0.09 ± 0.04	2.50 ± 0.53	0.64 ± 0.12	0.56 ± 0.28	5.06 ± 0.81
PI 546171	1.23 ± 0.43	0.13 ± 0.07	2.63 ± 0.97	0.61 ± 0.17	0.45 ± 0.24	5.05 ± 1.46
$LSD_{0.05}$[y]	0.48	0.09	0.92	0.28	0.31	1.49

[z]Composition of leaf samples from three replications of three plants grown at two locations ± SD.
[y]Least significant difference among genotypes at $\alpha = 0.05$.

Table 2. Mean concentrations[z] (mg/100 g fresh weight) for chlorophyll pigments in leaf tissues of bunching onion (*Allium fistulosum* L.) USDA-ARS accessions field-grown in Knoxville, TN, and Geneva, NY.

	Leaf tissue chlorophylls (mg/100 g fresh wt)		
Accession lot	Chlorophyll *b*	Chlorophyll *a*	Total
G 32145	11.04 ± 3.38	16.25 ± 8.22	27.29 ± 10.98
PI 462345	12.02 ± 4.12	18.11 ± 7.41	30.13 ± 5.64
PI 280562	8.46 ± 2.88	13.41 ± 9.27	21.87 ± 11.98
PI 546228	9.76 ± 3.76	17.17 ± 7.60	26.93 ± 11.02
PI 508401	9.13 ± 1.54	12.49 ± 8.91	21.62 ± 9.06
PI 436539	11.32 ± 3.32	21.30 ± 9.27	32.62 ± 10.88
PI 656924	8.46 ± 2.42	11.67 ± 9.69	20.13 ± 11.47
G 32088	9.91 ± 2.97	17.98 ± 8.12	27.89 ± 9.78
PI 462357	9.03 ± 2.31	15.52 ± 8.04	24.55 ± 9.60
PI 546343	9.91 ± 2.95	18.30 ± 7.81	28.21 ± 9.09
PI 274254	9.19 ± 1.66	17.04 ± 4.28	26.24 ± 5.08
PI 546171	8.07 ± 2.92	12.92 ± 6.35	20.99 ± 8.32
$LSD_{0.05}$[y]	3.60	9.13	10.83

[z]Composition of leaf samples from three replications of three plants grown at two locations ± SD.
[y]Least significant difference among genotypes at $\alpha = 0.05$.

separation and detection, which may account for the differences for carotenoid concentrations among the studies (Emenhiser et al., 1995). Leaf tissue pigments were well separated (see Kopsell et al., 2007b for a representative chromatogram); however, pseudostem tissue pigments were below HPLC detection limits. To the best of our knowledge, this is the first study to characterize the major carotenoid pigments present in the leaf tissues of *A. fistulosum* germplasm.

Intraspecific genetic variation for leaf tissues carotenoids has been reported for dicot vegetables. Lutein ranged from 4.8 to 13.4 mg/100 g fresh weight and β-carotene ranged from 3.5 to 10.0 mg/100 g fresh weight in *Brassica oleracea* L. var. *acephala* (Kopsell et al., 2004). Lutein ranged from 4.2 to 8.3 mg/100 g FW, zeaxanthin ranged from 0.2 to 0.6 mg/100 g FW, and β-carotene ranged from 3.4 to 7.7 mg/100 g FW in *Ocimum basilicum* L. (Kopsell et al., 2005). Lutein ranged from 6.5 to 13.0 mg/100 g FW and β-carotene ranged from 4.1 to 10.9 mg/100 g FW in *Spinacia oleracea* L. (Kopsell et al., 2006). Lutein ranged from 0.1 to 5.8 mg/100 g FW and β-carotene ranged from 0.1 to 9.1 mg/100 g FW in *Lactuca* species (Kimura and Rodriguez-Amaya, 2003; Mou, 2005). Lack of genetic variation for leaf tissue carotenoid among the *A. fistulosum* accessions in the current study may reflect a narrow genetic pool possibly present in the germplasm held in the repository. The sample size of only 12 accessions may not have been enough to capture variation present within the species. It may also be possible that the germplasm parental material was selected based on flavor attributes and not carotenoid concentrations. Unique metabolism of secondary sulfur metabolites within the *Alliums* contributes to their intense flavors. *Brassica* species metabolize sulfur into glucosinolates responsible for flavor intensity. In a previous study, Kopsell et al. (2003) demonstrated that sulfur fertility increases glucosinolate compounds, but did not influence leaf tissue carotenoid accumulations among different *B. oleracea* L. var. *acephala* cultivars. Therefore, selections for flavor intensity would not be expected to impact leaf tissue carotenoid accumulations in *B. oleracea* and possibly *A. fistulosum*.

Bulb onions are rich sources of flavonoids and *S*-alk(en)yl-L-cysteine sulfoxides shown to convey beneficial health properties when consumed in the diet. Leaf tissues of *Allium* species will contain nutritionally important carotenoid and chlorophyll pigments. The current study demonstrates high concentrations of a variety of valuable pigments in leaf tissues of *A. fistulosum* accessions. These compounds were shown to be similar at two environments and among 12 accessions. Information from this study may be valuable for plant improvement programs, which use this germplasm to develop *A. fistulosum* or allied crops with higher concentrations of nutraceutical pigments.

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