Abstract. Vegetable crops can be significant sources of nutritionally important dietary carotenoids, and Brassica are sources that also exhibit antioxidant and anticarcinogenic activity. The family Brassicaceae contains a diverse group of plant species commercially important in many parts of the world. The six economically important Brassica species are closely related genetically. Three diploid species (B. nigra, B. rapa, B. oleracea) are the natural progenitors of the amphidiploid species (B. juncea, B. napus, B. carinata). The objective of this study was to characterize the accumulation of important dietary carotenoid pigments among the genetically related Brassica species. High-performance liquid chromatographic quantification revealed significant differences in carotenoid and chlorophyll pigment concentrations among the Brassica species. Brassica rapa accumulated the highest concentrations of antheraxanthin [0.79 mg/100 g fresh weight (FW)], lutein (8.89 mg/100 g FW), and zeaxanthin (0.75 mg/100 g FW). The highest concentrations of β-carotene (4.41 mg/100 g FW) and total chlorophyll (125.9 mg/100 g FW) were found in B. juncea. Brassica nigra accumulated the highest concentrations of 5,6-epoxy-lutein (0.41 mg/100 g FW) and violaxanthin (2.28 mg/100 g FW), whereas B. oleracea accumulated the highest concentrations of neoxanthin (2.10 mg/100 g FW). For many of the pigments analyzed, the amphidiploids B. carinata and B. napus accumulated significantly less carotenoid concentrations than the diploid species and B. juncea. Brassica convey unique health attributes when consumed in the diet. Identification of genetic relationships among the Brassica species would be beneficial information for improvement programs designed to increase carotenoid values.

The family Brassicaceae (Cruciferae) represents a diverse group of plant species commercially important in many parts of the world. The plants produce condiment mustard; leafy, stored, processed, and picked vegetables; seed oils for margarine, salad oils, cooking oils, and industrial uses; animal fodders; and green manure crops (Williams and Hill, 1986). The six economically important Brassica species in world production are genetically related. Three diploid species—B. nigra, B. rapa, B. oleracea—are the natural progenitors of the amphidiploids species: B. juncea, B. napus, and B. carinata. The “cole crops,” such as broccoli, brussels sprouts, cabbage, cauliflower, curly kale, and kohlrabi, make up B. oleracea. Oil seed types, as well as Chinese cabbage, turnip, and pak-choi are found within B. rapa. Brassica nigra, popularly known as black mustard, is used for its seed oil content. The cross between B. nigra and B. oleracea produces B. carinata. It is a tall, leafy plant mainly restricted to Ethiopia. The cross between B. oleracea and B. rapa produces B. napus. Rutabagas are a variety of B. napus, as well as the economically important oilseed rape. The cross between B. rapa and B. nigra produces B. juncea, the group known as mustard. The mustards are consumed in the southern United States as mustard greens, or are used as a condiment or spice (Williams and Hill, 1986).

The three common diploid species in the genus Brassica have haploid numbers of 8 (B. nigra), 9 (B. oleracea), and 10 (B. rapa) (Nwankiti, 1970). It is believed that these numbers represent a phylogenetically ascending series (Manton, 1932). The higher chromosome number species in the genus are believed to be amphidiploids and have haploid numbers of 17 (B. carinata), 18 (B. juncea), and 19 (B. napus). The genetic arrangement of the diploid and amphidiploid Brassica species is credited to the U (1935). The diagram is referred to as the triangle of U (Nwankiti, 1970) (Fig. 1). Study of interspecific hybridization and subsequent pairing within the genus led to the designation of two distinct groups: 1) monogenic diploids of B. nigra (2n = 16; BB), B. oleracea (2n = 18; CC), and B. rapa (2n = 20; AA); and 2) amphidiploids of B. juncea (2n = 36; AABB), B. carinata (2n = 34; BBCC), and B. napus (2n = 38; AAACC) (Prakash and Hinata, 1980).

Carotenoids are C40 isoprenoid polynye plant secondary compounds that form lipid-soluble yellow, orange, and red pigments (Zaripheh and Erdman, Jr., 2002). The carotenoids can be divided into two groups: 1) hydrocarbon carotenes (C40H40) and 2) their oxygenated derivative, referred to as xanthophylls. Carotenoids span the thylakoid membranes of chlorophyll (Chl) complexes and function in accessory roles for light harvesting, photoprotection, and structural stabilization (Demmig-Adams et al., 1996; Tracewell et al., 2001). Carotenoid pigments protect photosynthetic structures by quenching excited triplet Chl (‘‘Chl’’ to dissipate excess energy (Frank and Cogdell, 1996) and by binding singlet oxygen (O2) to inhibit potential oxidative damage (Demmig-Adams et al., 1996; Tracewell et al., 2001). Carotenoids are produced in the plastids and are derived from isopentenyl diphosphate (IPP). In the first step in biosynthesis, IPP is isomerized to dimethylallyl diphosphate, which becomes the substrate for the C20 geranylgeranyl diphosphate (GGPP) (Bramley, 2002). The first step unique to carotenoid biosynthesis is the condensation of two molecules of GGPP to form the first C40 carotenoid, phytoene, via phytoene synthase (Gross, 1991). The carotenoid pathway branches at the cyclization reactions of lycopene to produce carotenoids with either two β-rings (e.g., β-carotene, zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin) or carotenoids with one β-ring and one ε-ring (e.g., α-carotene and lutein) (Cunningham, 2002) (Fig. 2).
There has been increased interest in the nutritional and medicinal importance of dietary carotenoids (Faulks and Southon, 2005; Frazer and Bramley, 2004; Yeum and Russell, 2002). Dietary intake of carotenoids has been associated with reduced risk of lung cancer and chronic eye diseases, including cataract and age-related macular degeneration (Johnson et al., 2000; Le Marchand et al., 1993). Studies have indicated that consumption of a variety of vegetables providing a mixture of carotenoids was more strongly associated with disease reduction than individual carotenoid supplements (Johnson et al., 2000; Le Marchand et al., 1993). Plants can be significant sources of carotenoids in the diet, and Brassica vegetables are relatively abundant sources that exhibit antioxidant and anticarcinogenic activity (Kurilich et al., 1999). Therefore, the objective of this study was to characterize the variation in accumulation of important dietary carotenoids among the six genetically related Brassica species.

Materials and Methods

Seeds of rapid-cycling populations of B. nigra, B. rapa, B. oleracea, B. juncea, B. napus, and B. carinata (Crucifer Genetics Cooperative, Department of Plant Pathology, The University of Wisconsin, Madison, Wis.) were sown into 2.5 × 2.5-cm growing cubes (Grodan A/S, Dk-2640, Hedehusene, Denmark) and germinated in a growth chamber room (Environmental Growth Chambers, Chagrin Falls, Ohio) at 23°C/17°C day/night under a 16-h photoperiod on 2 Dec. 2004 and again on 11 Jan. 2005. Photosynthetically active radiation at the canopy height was 1000 μmol m⁻² s⁻¹ (LI-188B; LI-COR, Inc., Lincoln, Neb.). The first true leaves appeared 4 to 7 d after planting (DAP). Nutrients were applied as needed with 200 mg L⁻¹ Peter’s 20N–6.9P–16.6K water-soluble fertilizer (The Scotts Company, Marysville, Ohio). At 14 DAP, the plantlets were transferred to 11-L containers (Rubbermaid Inc., Wooster, Ohio) filled with 9 L of a nutrient solution (Hoagland and Arnon, 1950). Elemental concentrations of the nutrient solutions were N (105 mg L⁻¹), P (15.3 mg L⁻¹), K (117.3 mg L⁻¹), Ca (80.2 mg L⁻¹), Mg (24.6 mg L⁻¹), S (32.0 mg L⁻¹), Fe (0.5 mg L⁻¹), B (0.25 mg L⁻¹), Mo (0.005 mg L⁻¹), Cu (0.01 mg L⁻¹), Mn (0.25 mg L⁻¹), and Zn (0.025 mg L⁻¹). Solutions were aerated with an air blower (model 25E133W222; Spencer, Winsor, Conn.) connected to air stones. Water was added daily to maintain initial solution volumes in each container.

Each experimental replication consisted of three randomized blocks, with plots...
consisting of individual containers holding six plants of one Brassica species, each placed into 2-cm round holds set at 10.6 × 9.5-cm spacing on the lid. Plants were harvested just before anthesis 27 DAP (B. rapa, B. juncea, B. nigra) and 34 DAP (B. carinata, B. napus, B. oleracea). Leaf tissue was removed from six plants for each Brassica species and was immediately stored at −80 °C before lyophilization. The frozen plant samples were lyophilized for a minimum of 72 h (model 6L FreeZone; LabConCo, Kansas City, Mo.).

Carotenoid/chlorophyll analysis. Plant pigments were extracted from freeze-dried tissues according to Kopsell et al. (2004) and analyzed according to Emenhiser et al. (1996). A 0.10-g subsample was rehydrated with 0.8 mL ultra pure H2O at 40 °C for 20 min. After incubation, 0.8 mL of the internal standard ethyl-β-8’-apo-carothenoate (Sigma Chemical Co., St. Louis) was added to determine extraction efficiency. A total of 2.5 mL tetrahydrofuran (THF) stabilized with 25 ppm 2,6-Di-tert-butyl-4-methoxyphenol was also added. The sample was homogenized in a Potter–Elvehjem (Kontes, Vineland, N.J.) tissue grinding tube using ≈25 insertions with a pestle attached to a drill press (Sears, Roebuck and Co., Hoffman Estates, Ill.) set at 540 rpm. During homogenation, the tube was immersed in ice to dissipate heat. The tube was then placed into a clinical centrifuge for 3 min at 500 g. 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 1.5 mL under a stream of nitrogen gas (N2-EVAP 111; Organomation, Berlin, Mass.) in a water bath set at 40 °C, and was brought up to a final volume of 5 mL with methanol (MeOH). A 2-mL aliquot was filtered through a 0.2-μm polytetrafluoroethylene (PTFE) filter (model Econofilter PTFE 25/20; Agilent Technologies, Wilmington, Del.) using a 5-mL syringe (Becton, Dickinson and Company, Franklin Lakes, N.J.) before analysis using high-performance liquid chromatography (HPLC).

An Agilent 1100 series HPLC unit with a photodiode array detector (Agilent Technologies, Palo Alto, Calif.) was used for pigment separation. Chromatographic separations were achieved using an analytical scale (4.6 mm i.d. × 250 mm) of 5 μm and a 200-A polymeric C18 reverse-phase column (ProntoSIL, MAC-MOD Analytical Inc., Chadds Ford, Pa.), which allowed for effective separation of chemically similar carotenoid compounds (Fig. 3). The column was equipped with a guard cartridge (4.0 mm i.d. × 10 mm) and holder (ProntoSIL), and was maintained at 30 °C using a thermal column compartment. All separations were achieved isocratically using a binary mobile phase of 11% methyl tert-butyl ethylanol, 88.9% MeOH, and 0.1% triethylamine (v/v/v). The flow rate was 1.0 mL·min⁻¹, with a run time of 53 min, followed by a 10-min equilibration before the next injection. Eluted compounds from a 20-μL injection loop were detected at 453 nm (carotenoids and internal standard), 652 nm (Chl a), and 665 nm (Chl b), and data were collected, recorded, and integrated using ChemStation Software (Agilent Technologies). Internal standard percent recovery ranged from 62% to 100%, with a mean for all samples at 80%. Peak assignment for individual pigments was performed by comparing retention times and line spectra obtained from photodiode array detection using external standards (antheraxanthin, neoxanthin, 5,6-epoxylutein, lutein, violaxanthin, zeaxanthin from Carotenature, Lupsingen, Switzerland; β-carotene, Chl a, Chl b from Sigma Chemical Co.). The concentration of the external pigment standards were determined spectrophotometrically using the following λ<sub>max</sub> values: antheraxanthin, 2349 in ethanol (ETOH), λ<sub>max</sub> = 446 nm; neoxanthin, 2270 in ETOH, λ<sub>max</sub> = 438 nm; 5,6-epoxylutein, 2463 in ETOH, λ<sub>max</sub> = 440 nm; lutein, 2468 in ETOH, λ<sub>max</sub> = 440 nm; violaxanthin, 2499 in ETOH, λ<sub>max</sub> = 441 nm; zeaxanthin, 2540 in ETOH, λ<sub>max</sub> = 452 nm; β-carotene, 2592 in hexane, λ<sub>max</sub> = 452 nm; Chl a, 819 in ETOH, λ<sub>max</sub> = 665 nm; and Chl b, 441 in ETOH, λ<sub>max</sub> = 649 nm (Davies and Köst, 1988). Spinach standard reference material (Sturried Spinacht 2385, National Institute of Science and Technology, Gaithersburg, Md.) was used for method validation.

Data sets were analyzed by the GLM procedures of SAS (Cary, N.C.) with species means separated by Duncan’s multiple range test (α = 0.05). Data were recorded on both a fresh weight (FW); in milligrams per 100 g and dry weight (DW; in milligrams per gram) basis.

Results and Discussion

Carotenoid pigment accumulation in leaf tissues differed among the Brassica species (Table 1). Lutein accumulation ranged from 8.89 mg/100 g FW (B. rapa) to 6.55 mg/100 g FW (B. carinata). Leaf tissue lutein averaged 8.64 mg/100 g FW among the diploid Brassica species (B. rapa, B. nigra, B. oleracea) and 7.35 mg/100 g FW among the amphidiploid species (B. juncea, B. carinata, B. napus). Carotenoid 5,6-epoxides are widespread in nature. Their conversion is mediated by a mixed-function oxygenase, which requires nicotinamide adenine dinucleotide phosphate (NADPH) and O2 as cofactors (Goodwin, 1980). The accumulation of 5,6-epoxylutein ranged from 0.41 mg/100 g FW (B. nigra) to 0.25 mg/100 g FW (B. carinata). The accumulation of 5,6-epoxyxanthin averaged 0.40 mg/100 g FW for the diploid Brassica species and 0.33 mg/100 g FW for the amphidiploid species.

The accumulation of β-carotene was similar for B. rapa, B. juncea, B. nigra, and B. oleracea, and was significantly less for B. carinata and B. napus (Table 1). The accumulation of β-carotene averaged 3.93 mg/100 g FW for the diploid Brassica species and 3.14 mg/100 g FW for the amphidiploid species. Insertion of the oxygen function in the carotenoid biosynthetic pathway occurs after the cyclization of lycopene (Cunningham and Grant, 1998). Zeaxanthin forms from the hydroxylation of β-carotene. Only one Brassica species (B. rapa) accumulated significantly higher zeaxanthin concentrations than all the other species. The accumulation of zeaxanthin averaged 0.46 mg/100 g FW for the diploid Brassica species and 0.28 mg/100 g FW for the amphidiploid species. Epoxidation of zeaxanthin, via antheraxanthin, results in the formation of violaxanthin. The rearrangement of the epoxide group is the proposed mechanism for the conversion of violaxanthin to neoxanthin (Gross, 1991). Antheraxanthin accumulation ranged from 0.79 mg/100 g FW (B. rapa) to 0.41 mg/100 g FW (B. napus). The accumulation of antheraxanthin averaged 0.62 mg/100 g FW for the diploid Brassica species and 0.55 mg/100 g FW for the amphidiploid species. The built-in ability for photoprotective energy dissipation within the photosynthetic apparatus of higher plants occurs through reversible changes in the violaxanthin content in the leaves. The xanthophyll, or violaxanthin, cycle is involved in the deoxidation of violaxanthin in high light conditions, and
the reoxidation of zeaxanthin in dark conditions via antheraxanthin (Demmig-Adams and Adams, 1996; Gross, 1991). The accumulation of violaxanthin in *B. juncea* and *B. nigra* was significantly higher than the other *Brassica* species. The accumulation of violaxanthin averaged 1.71 mg/100 g FW for the diploid *Brassica* species and 1.47 mg/100 g FW for the amphidiploid species. The accumulation of neoxanthin also differed among the *Brassica* species. The accumulation of neoxanthin averaged 10.08 mg/100 g FW for the amphidiploid species and 1.68 mg/100 g FW for the amphidiploid species. The *Brassica* species accumulated much higher concentrations of Chl pigments. Chlorophyll pigments differed among the *Brassica* species (Table 2). Chlorophyll *a* accumulation ranged from 100.1 mg/100 g FW (*B. juncea*) to 52.4 mg/100 g FW (*B. carinata*). Leaf tissue Chl *a* averaged 93.4 mg/100 g FW among the diploid *Brassica* species and 73.6 mg/100 g FW among the amphidiploid species. Chlorophyll *b* accumulation ranged from 28.8 mg/100 g FW (*B. oleracea*) to 18.3 mg/100 g FW (*B. carinata*). The accumulation of Chl *b* averaged 26.2 mg/100 g FW for the diploid *Brassica* species and 22.5 mg/100 g FW for the amphidiploid species. Total Chl concentrations ranged from 125.9 mg/100 g FW (*B. juncea*) to 71.7 mg/100 g FW (*B. carinata*). Total leaf tissue Chl averaged 119.5 mg/100 g FW among the amphidiploid species and 96.1 mg/100 g FW among the amphidiploid species.

Vegetable tissues can be dried and encapsulated for use as lutein and β-carotene dietary antioxidant supplements. Pool-Zobel et al. (1997) and Müller et al. (1999) conducted experiments to determine whether carotenoids could protect against DNA damage and oxidative stress. The researchers used dried spinach powder (obtained from Völkel GmbH, Königmoos, Germany) to assess antioxidant potential. The lutein concentration for the dried spinach was 11.3 mg/g DW and neuronal lutein and lutein was discovered a high degree of pairing and chiasmata formation for the amphiploid *B. napus*. Brassica carinata had very low meiotic pairing and chiasmata compared with *B. napus*, with slightly higher pairing observed for *B. juncea*. Morphological (Prakash and Hinata, 1980) and chromosomal (Armstrong and Keller, 1982) differentiation identified *B. napus* to be the most distantly related of the genomic species. Cunha et al. (2004) used chloroplast DNA (cpDNA) to identify polymorphisms between the six genetically distinct *Brassica* species. The study concluded that cpDNA polymorphisms could discriminate among the three diploid *Brassica* species, but were unable to identify polymorphisms among the three amphidiploid species. Research shows that *B. rapa* and *B. oleracea* are very closely related species (Harberd and McArthur, 1980). In most cases for the current study, carotenoid pigment accumulation among these two *Brassica* species did not differ (Table 1).

Earlier studies revealed structural divergence among the monogenic *Brassica* species was based upon reductions in chro-

### Table 1. Mean values for carotenoid pigments (measured in milligrams per 100 g fresh weight) for the six genetically related *Brassica* species grown in a controlled environment.

| *Brassica* Species  | Lutein | β-carotene | Zeaxanthin | Antheraxanthin | Violaxanthin | Neoxanthin |
|---------------------|--------|------------|------------|---------------|--------------|------------|
| *B. rapa* 0.89 a    | 0.40 a | 0.75 a     | 0.79 a     | 1.35 b        | 1.93 ab      |            |
| *B. juncea* 0.75 a  | 0.39 a | 0.36 b     | 0.67 ab    | 2.17 a        | 2.03 ab      |            |
| *B. nigra* 0.85 a   | 0.41 a | 0.36 b     | 0.56 bc    | 2.28 a        | 1.97 ab      |            |
| *B. carinata* 0.65  | 0.25 b | 0.25 b     | 0.57 bc    | 1.11 b        | 1.32 c       |            |
| *B. oleracea* 0.48  | 0.39 a | 0.28 b     | 0.51 bc    | 1.51 b        | 2.10 a       |            |
| *B. napus* 0.76 a   | 0.36 a | 0.25 b     | 0.41 c     | 1.12 b        | 1.69 b       |            |
| Mean 0.80 0.37      | 0.35   | 0.37       | 0.59       | 1.59          | 1.84         |            |

### Table 2. Mean values for chlorophyll pigments (measured in milligrams per 100 g fresh weight) for the six genetically related *Brassica* species grown in a controlled environment.

| *Brassica* Species | Chlorophyll *a* | Chlorophyll *b* | Total chlorophyll |
|--------------------|-----------------|-----------------|------------------|
| *B. rapa*          | 99.8 a          | 25.3 ab         | 125.1 a          |
| *B. juncea*        | 100.1 a         | 25.8 ab         | 125.9 a          |
| *B. nigra*         | 87.1 a          | 24.4 b          | 111.5 a          |
| *B. carinata*      | 52.4 c          | 18.3 c          | 70.7 c           |
| *B. oleracea*      | 93.2 a          | 28.8 a          | 121.9 a          |
| *B. napus*         | 68.4 b          | 23.3 b          | 91.7 b           |
| Mean               | 83.5            | 24.3            | 107.8            |

### Table 3. Mean values for carotenoid pigments (measured in milligrams per gram dry weight) for the six genetically related *Brassica* species grown in a controlled environment.

| *Brassica* Species  | Lutein       | β-carotene | Zeaxanthin | Antheraxanthin | Violaxanthin | Neoxanthin |
|---------------------|--------------|------------|------------|---------------|--------------|------------|
| *B. rapa* 0.87 a    | 0.04 a       | 0.40 a     | 0.07 a     | 0.08 a        | 0.13 b       | 0.19 ab    |
| *B. juncea* 0.86 a  | 0.04 a       | 0.43 a     | 0.04 b     | 0.07 ab       | 0.21 a       | 0.20 a     |
| *B. nigra* 0.83 ab  | 0.04 a       | 0.38 a     | 0.04 b     | 0.05 c        | 0.22 a       | 0.18 ab    |
| *B. carinata* 0.52  | 0.02 c       | 0.22 b     | 0.02 b     | 0.05 bc       | 0.10 b       | 0.12 c     |
| *B. oleracea* 0.80  | 0.04 ab      | 0.36 a     | 0.03 b     | 0.05 c        | 0.14 b       | 0.20 a     |
| *B. napus* 0.71 b   | 0.03 ab      | 0.24 b     | 0.02 b     | 0.04 c        | 0.10 b       | 0.16 bc    |
| Mean 0.77 0.03      | 0.34         | 0.34        | 0.04       | 0.06          | 0.12         | 0.18       |

*Composition of leaf samples from six replications, six plants each. Mean separation within columns by Duncan’s multiple range test, α = 0.05.*
to the maternal donor B. rapa than to the paternal donor B. nigra, and B. napus is almost equally related to the maternal and paternal donors of B. rapa and B. oleracea respectively. The results suggest that the maternally donated cytoplasmic genomes have some effect on nuclear genome evolution in the amphidiploid species (Cunha et al., 2004; Song et al., 1996). Large genomic diversity was found among and within the three cultivated amphidiploid species of *Brassica* (Song et al., 1996).

In the current study, genetic differences were identified for carotenoid and Chl pigment concentrations within and among the diploid and amphidiploid *Brassica* species. *Brassica* species also produce glucosinolate secondary compounds that have important anticarcinogenic properties in humans. Quantitative and qualitative differences in glucosinolate production have been linked to genome differences among rapid-cycling *Brassica* species (Hill et al., 1988). For many of the pigments analyzed in the current study, the amphidiploids *B. carinata* and *B. napus* accumulated significantly less carotenoid concentrations than the diploid species and *B. juncea*. The six genetically related *Brassica* species share common evolutionary ancestry, and breeding techniques are currently used for interspecific hybridizations and species improvement. *Brassica* species convey unique health attributes when consumed in the diet, and are shown to accumulate high levels of carotenoids (USDA, 2005). Therefore, identification of the genetic relationships that exist among the *Brassica* species for carotenoid concentrations would be beneficial information for improvement programs designed to increase nutritional values.

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