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Anthocyanin Accumulation during Potato Tuber Development

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Abstract. The color of red potato tubers is due to an accumulation of anthocyanins in periderm and peripheral cortex tissues. The objective of this study was to characterize changes in anthocyanin content and tuber surface color during tuber development. Using the red tuber-producing potato (*Solanum tuberosum* L.) cultivar Norland, we observed that chroma (intensity of redness) and anthocyanin content per unit of surface area of greenhouse-grown tubers decreased as tuber weight increased. There was no increase in hue (tint) during the same developmental periods. Using high-performance liquid chromatography (HPLC), we determined that pelargonidin and peonidin are the major anthocyanidins (aglycones of anthocyanins) in the tuber periderm. Northern blot analyses indicated that steady-state mRNA levels of dihydroflavonol reductase (DFR), an anthocyanin biosynthetic enzyme, continued throughout tuber development. These results suggest that anthocyanins are synthesized throughout tuber development, and that cell division and/or enlargement contribute to a decline in chroma and anthocyanin concentration.

'Dark Red Norland', a fresh-market red potato cultivar and color sport of 'Red Norland' and 'Norland', has been one of the top 10 potato cultivars in seed acreage in the United States since 1993 (Johnson, 1995). Successful marketing of these potatoes depends primarily on their appearance, so growers want cultivars that develop and maintain good appearance. The skin color of red potatoes is due to the presence of anthocyanins in the periderm and peripheral cortex (Burton, 1989). Anthocyanins are thought to act as attractants of pollinators and animals to fruit for seed dispersal, as well as protectants of leaves against ultraviolet irradiation (Mazza and Miniati, 1993). However, the function of anthocyanins in potato periderm is not well understood.

Anthocyanins are glycosylated flavonoids, with chemical structures based on an aromatic flavylium cation. The aglycone forms are called anthocyanidins, and the six most frequently occurring anthocyanidins in plants are cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin (Harborne, 1967). Using paper chromatography, Harborne (1960) determined that red tubers of *Solanum phureja* contained rhamnosylglucosides of cyanidin and pelargonidin. He found other anthocyanins in *S. tuberosum* ssp. *tuberosum*, with R_f values that were later reported (Howard et al., 1970) to be those of acylated forms of the rhamnosylglucosides of peonidin and pelargonidin. Howard et al. (1970) showed that tetraploid *S. tuberosum* ssp. *tuberosum* tubers contained single anthocyanins (different cultivars have different anthocyanins) while diploids contained mixtures. Sasche (1973) determined that 'Urgenta' and 'Desirée' potatoes contained pelargonidin-3(p-coumaroyl-rhamnosylglucoside)-5-glucoside and peonidin-feruloyl-3-rhamnosyl-glucoside-5-glucoside. Using two-dimensional nuclear magnetic resonance techniques, Andersen et al. (1991) identified the anthocyanin in a blue-pigmented *S. tuberosum* tuber (variety unknown) as petunidin 3-*O*-[6-*O*-4-*O*-E-p-coumaroyl-α-L-rhamnopyranosyl)-β-D-glucopyranoside]-5-*O*-β-D-glucopyranoside.

Anthocyanin biosynthesis in flowers and leaves has been well studied, and much of the biosynthetic pathway and regulation of genes coding for biosynthetic enzymes have been characterized (Mol et al., 1989). Phenylalanine, from the shikimic acid pathway, is converted to cinnamic acid by phenylalanine ammonia lyase. Cinnamic acid is converted into coumaric acid, which is activated to the CoA form. Coumaroyl-CoA unites with three acetate moieties donated by malonyl-CoA to form chalcone through the action of chalcone synthase. Chalcone isomerase converts chalcone to flavanone, which is transformed into dihydroflavonol by flavanone 3-hydroxylase. Dihydroflavonol is reduced by dihydroflavonol reductase (DFR) to form leucoanthocyanin, which is converted to anthocyanin by leucoanthocyanidin hydroxylase and UDP-glucose: flavonoid 3-*O*-glucosyltransferase. Different dihydroflavonol substrates of DFR result in different leucoanthocyanidins. For example, dihydromyricetin is converted to leucodelphinidin, dihydrokaempferol to leucopelargonidin, and dihydroquercitin to leucocyanidin. The petunia (*Petunia ×hybrida* Hort. Vilm.-Andr.) DFR exhibits substrate specificity and reduces dihydromyricetin, but not dihydrokaempferol (Forkmann and Ruhnau, 1987), whereas the maize (*Zea mays* L.) enzyme can reduce dihydrokaempferol (Meyer et al., 1987). DFR is active late in the anthocyanin biosynthetic pathway and is therefore useful as an indicator of anthocyanin biosynthesis in biological studies, although it is also involved in the production of leucocyanidins and proanthocyanidins (Stafford, 1990).

Light is an important environmental factor involved in the induction and regulation of expression of the chalcone synthase, chalcone isomerase, and DFR genes (Kubasek et al., 1992; Murray et al., 1994; van Tunen et al., 1988). Potato tubers, growing underground, would not normally be exposed to light, or otherwise would turn green (Dean, 1994). Hence, potato tubers provide an alternative system for studying anthocyanin biosynthesis. Although much information is available regarding the inheritance of red coloration, not much is known about the accumulation of anthocyanins during tuber development. The aim of this study was to characterize changes in surface color and anthocyanin content of red potato tubers during tuber development, as a first step toward developing methods to enhance or maintain their color.

Materials and Methods

Plant material. 'Norland' potato plants were grown in a greenhouse maintained at about 21 °C at the Univ. of Minnesota.

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Only one potato cultivar was used for these experiments because we wanted to characterize one system well for future experiments. Seed tubers were obtained from R. Korsman (Gilbert, Minn.), a commercial seed producer. Experiments were conducted continually throughout the year. Due to space limitations, only 10 to 20 plants were grown at any one time and were replaced about 13 weeks after planting. Plants were grown in Strong Lite Universal soil mix (Strong Lite Horticultural Products, Pine Bluff, Ark.) and fertilized 5 d/week with a 0.2 $g \cdot L^{-1}$ solution of 20N–20P–20K. To provide about the same daylength throughout the year, plants were grown under a 12-h photoperiod supplemented by Sylvania 400 and 1000 metal halide lights giving an average photon flux density of 672 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 15 Sept. through 15 Mar. Tubers were harvested 7 weeks after planting from some of the plants, then at 2-week intervals from other plants until 13 weeks after planting and all plants had been harvested. This was done to obtain tubers of different developmental stages. Mass of tubers was used as an indication of developmental stage (Ross et al., 1994). All harvested tubers were used unless diseased or severely damaged or malformed. As this was a descriptive study, we took all usable tubers, measured color, anthocyanin and anthocyanidin concentrations, and periderm cell sizes, and examined these characteristics as related to tuber mass.

Surface color measurement. Tubers of different masses were harvested from several plants and at several times of year, washed with distilled water, surface-dried to remove any free moisture, and individually weighed. The surface color of an individual tuber was measured at three locations with a Minolta CR-200 chromameter. This measurement is based on the Commission Internationale de L'Eclairage (CIE) scale (McGuire, 1992). Linearized nonlinear regression analyses were done using CoStat software (CoHort Software, Minneapolis).

Anthocyanin extraction and quantification. Immediately after harvest, tubers were washed with distilled water and dried. Potato periderm was broken carefully with a 7-mm-diameter cork borer, and discs were peeled from the cored areas. Five discs were used per assay and extracted with 1 mL of methanol-HCl (0.1% HCl, v/v) solution and held at 4 °C for 24 h in the dark. A 1-mL aliquot was measured spectrophotometrically at 513 nm using a Beckman DU-50 spectrophotometer.

High-performance liquid chromatography (HPLC) analyses. Two grams of periderm tissue was extracted with 40 mL of the methanol-HCl solution at 4 °C for 24 h in the dark. Anthocyanidins were prepared from these extracts according to the method of Murray and Hackett (1991). Forty milliliters of each extract was taken to near dryness under a stream of nitrogen, dissolved in 600 µL of 2 N HCl, hydrolyzed at 100 °C for 1 h, then quickly cooled in ice, and mixed with 600 µL of methanol. The solution was filtered through a 0.450-µm PTFE centrifuge filter and used immediately for HPLC analysis. The procedure described above was carried out in the dark or in subdued light because of the instability of anthocyanidin pigments (Hong and Wrolstad, 1986).

Anthocyanidins were separated using a Millipore model 510 HPLC equipped with a Millipore 680 automated gradient controller, Hewlett-Packard 1040A photodiode array detector, and Vydac C_{18} guard and analytical (4.6 mm ID × 25 cm, 5-µm particle size) columns. The mobile phases used were A) 0.1% trifluoroacetic acid and B) 0.1% trifluoroacetic acid in 90% acetonitrile. Samples were eluted using the following program: isocratic elution with 10% B and 90% A from 0 to 5 min, linear gradient to 25% B and 75% A from 5 to 35 min, isocratic elution at 25% B and 75% A from 35 to 40 min, linear gradient to 27% B and 73% A from 40 to 80 min, isocratic elution at 27% B and 73% A from 80 to 85 min, linear gradient to 30% B and 70% A from 85 to 95 min, isocratic elution at 30% B and 70% A from 95 to 100, linear gradient to 100% B from 100 to 105 min, and linear gradient to 10% B and 90% A from 105 to 110 min. The flow rate used was 0.8 $mL \cdot min^{-1}$ at room temperature. Anthocyanidin levels were quantified at 520 nm. Standard anthocyanidins (Indofine Chemical Co., Inc., Somerville, N.J.) were used to determine retention times of the different anthocyanidins. Identification of each compound was based on the elution order of the anthocyanidins, which is related to their polarity (Casteele et al., 1983) and their on-line spectra obtained by photodiode array detection. Determinations were done twice per tuber developmental stage with similar results.

Cell size determinations. Tubers of different masses were collected, washed with distilled water, and dried. Tubers were cut into pieces small enough to mount on a Vibratome 1000 (Lancer, St. Louis) and sectioned tangentially across the periderm. The sectioned periderm tissues were placed on slides to which a small amount of distilled water was added, covered with coverslips, and observed immediately to avoid compression of cells due to drying. Sections were examined by light microscopy, and three photographs were taken of randomly chosen areas per section. The number of cells in a given area was counted in each photograph, and the mean cell number per area for each section was obtained. Total cell area was divided by cell number to obtain the mean cell cross-sectional area per section. Linearized nonlinear regression analyses were done using CoStat software.

Northern blot analyses. Total RNA was extracted from young tomato (*Lycopersicon esculentum* L.) leaves, whole, unpeeled swollen potato stolon tips, and peeled potato tuber periderm using lithium chloride purification as described by Rochester et al. (1986). A minimal amount of cortex tissue was included with the periderm tissues. For northern analysis, 30 µg of total

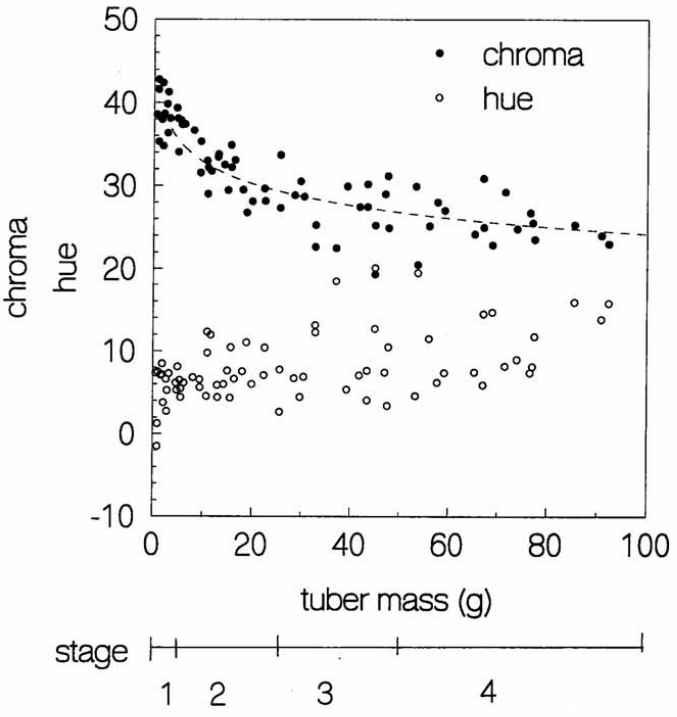


Fig. 1. Changes in the periderm color of 'Norland' potatoes during tuber development. Hue indicates tint and chroma indicates intensity of color. Each point represents a measurement from one tuber. The equation for the chroma values, $y = 41.43 - 3.75 \ln(x)$, was derived using linearized nonlinear regression, and has an $r^2 = 0.76$. No equation with an $r^2 > 0.23$ was found to fit the hue data. Stage 1 = tubers ≤5 g, stage 2 = tubers 5 g < 24.9 g, stage 3 = tubers 25 g < 49.9 g, and stage 4 = tubers >50 g.

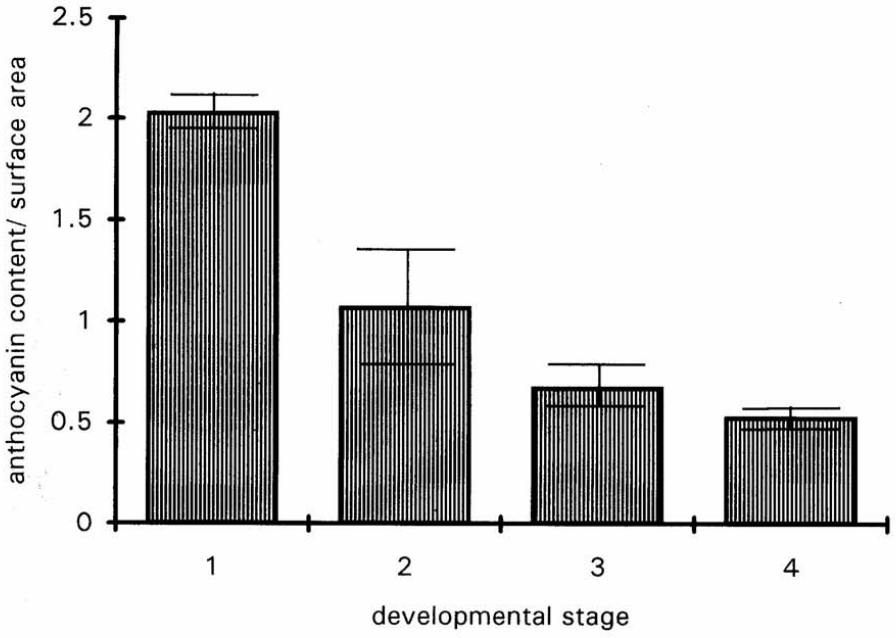
Table 1. Chroma, hue, and mean cell area of tubers during development. Stage 1 = tubers ≤5 g, stage 2 = tubers 5 g < 24.9 g, stage 3 = tubers 25 g < 49.9 g, and stage 4 = tubers > 50 g. SD = standard deviation. N = number of samples; N for chroma and hue are the same.

Stage	Chroma ± SD	N	Hue ± SD	Mean cell area ± SD (μm^2)	N
1	38.6 ± 2.7	15	5.5 ± 2.8	2181 ± 558	4
2	32.6 ± 3.4	23	7.3 ± 2.5	3951 ± 542	5
3	27.3 ± 3.6	17	5.8 ± 4.9	5638 ± 847	3
4	25.6 ± 2.7	17	10.7 ± 4.3	5119 ± 693	3

RNA (as determined by measuring the A_{260} of the final extract) was fractionated on a formaldehyde-agarose gel and blotted onto Genescreen Plus membrane (NEN Research Products, Boston). The membrane was prehybridized overnight in a 50% formamide solution containing 500 $\mu g \cdot mL^{-1}$ salmon sperm DNA at 42 °C (Murray et al., 1994). A radiolabeled probe was made using a tomato DFR cDNA (Bongue-Bartelsman et al., 1994) and the Megaprime (Amersham Corp., Arlington Heights, Ill.) random oligonucleotide labeling kit. Membranes were hybridized with the probe overnight at 42 °C, washed twice at room temperature for 15 min and once at 60 °C for 30 min in a 0.3× SSPE (1× = 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA, pH 7.5) solution with 0.1% SDS. Membranes were exposed to XAR film (Eastman Kodak Co., Rochester, N.Y.) with Cronex Lightning Plus intensifying screens (Du Pont, Wilmington, Del.) at –80 °C. Experiments were performed three times with similar results.

Results

PERIDERM COLOR AND ANTHOCYANIN CONCENTRATION. Tuber surface chroma (intensity of redness) decreased as tuber mass increased (Fig. 1). Chroma values initially decreased rapidly (Table 1), but the rate of change lessened after tubers reached a mass of 5 g. Based on these data, tubers were separated into four groups, such that stage 1 = tuber mass ≤5 g; stage 2 = tuber mass 5 g < 24.9 g; stage 3 = tuber mass 25 g < 49.9 g; stage 4 = tuber mass > 50 g. There was no increase in hue (tint) during tuber development (Table 1). The mean hue value calculated from all stages was 8.02, which represents a purplish-red to red color.



Periderm anthocyanin content per unit of surface area (absorbance at 513 nm of extractions from five 7-mm-diameter disks) decreased as tuber mass increased (Fig. 2). Anthocyanin content was reported on a surface area basis, as that would represent the color viewed by the human eye and measured by the chromameter. Indeed, changes in anthocyanin concentration matched positively with changes in chroma during tuber development. The periderm anthocyanin concentration of stage 2 tubers was about half that of stage 1 tubers, and continued to decrease slightly after stage 2.

PERIDERM ANTHOCYANIDINS. HPLC analyses of anthocyanidins extracted from stages 1 to 4 tuber periderm showed that pelargonidin and peonidin accounted for >90% of the total anthocyanidins. The ratio of pelargonidin to peonidin was usually 4.5–6:1 for all stages (data not shown).

CHANGES IN PERIDERM CELL SIZE. Mean peridermal cell cross-sectional areas during tuber development are illustrated in Fig. 3. The mean periderm cell cross-sectional area of stage 2 tubers was 1.8 times that of stage 1 tubers, coincident with the 2-fold decrease in anthocyanin concentration (Table 1).

During tuber growth, the number of layers of periderm increased, in agreement with previous observations (Rastovski et al., 1987). In transverse section, peridermal cells appeared flattened, so cell growth was not equal in three dimensions. Stage 1 tuber periderm consisted of about three cell layers. Stage 4 tuber periderm contained about six cell layers, with the two layers closest to the cortex having a pink color, while the other layers were more red (data not shown).

EXPRESSION OF DFR DURING TUBER DEVELOPMENT. DFR mRNA was detectable at all four stages of tuber development (Fig. 4). The apparent molecular weight of the putative potato DFR mRNA transcript was the same as the tomato leaf DFR band, reported as 1.5 kb by Bongue-Bartelsman et al. (1994).

Discussion

The tuber surface color intensity (chroma) changed during tuber development, declining during stages 1 and 2. This change in chroma coincided with a decline in periderm anthocyanin content per unit surface area. The decline in anthocyanin content per unit of surface area could result from degradation of anthocyanins, which is difficult to measure, and dilution due to increases in cell size. The approximately 2-fold increase in periderm

Fig. 2. Mean anthocyanin content per given surface area of the periderm of different tuber stages. Anthocyanin content of methanol-HCl extracts was measured spectrophotometrically at 513 nm. Error bars = SD. Stage 1 = tubers ≤5 g, stage 2 = tubers 5 g < 24.9 g, stage 3 = tubers 25 g < 49.9 g, and stage 4 = tubers >50 g.

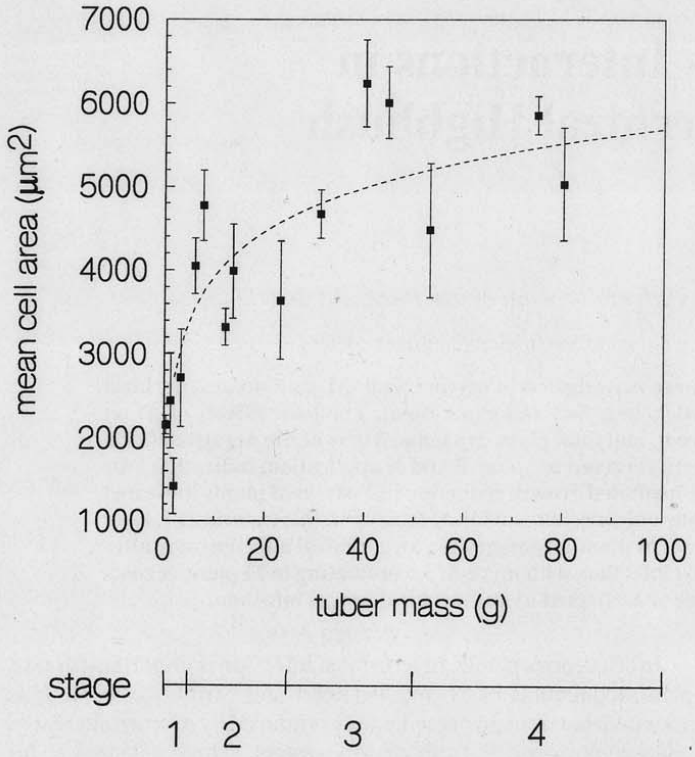


Fig. 3. Mean cell area of peridermal cells of different tuber developmental stages. Each point represents the mean of three determinations from one tuber. Error bars = SD. The equation for the mean cell areas, $y = 2191.6 + 758.6 \ln(x)$, was derived using linearized nonlinear regression, and has an $r^2 = 0.71$.

surface area seen in tangential sections between stages 1 and 2, coincident with the 2-fold decline in anthocyanin content per unit surface area, suggests that dilution may be the major factor leading to a decline in tuber color intensity.

The continued presence of DFR mRNA into stage 4 tubers suggests that biosynthesis of anthocyanins continues throughout tuber development. DFR enzyme activity was not determined, as we have not yet found a source of dihydrokaempferol, the substrate necessary to synthesize pelargonidin, the primary anthocyanidin in 'Norland' potato periderm. Also, products of DFR enzyme activity include proanthocyanins (Stafford, 1990), which would not contribute to the redness of potato tubers. Peridermal cell size did not change dramatically between tubers of 40 to 80 g fresh mass (Fig. 3). We believe that cell division occurs during this time, as the surface area of tubers and the number of peridermal cell layers increase with tuber growth.

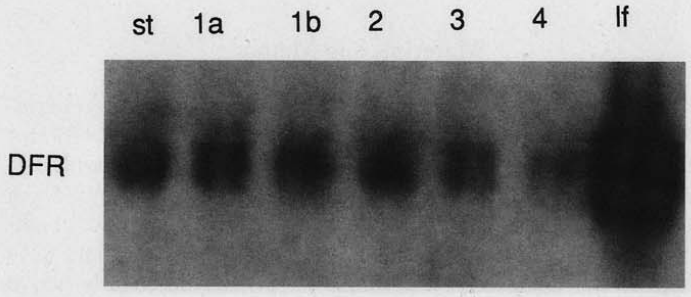


Fig. 4. Steady-state levels of DFR mRNA in potato periderm and tomato leaf tissue. st = Stolon tips; 1a = early stage 1 potato periderm; 1b = late stage 1 potato periderm; 2 = stage 2 potato periderm; 3 = stage 3 potato periderm; 4 = stage 4 potato periderm, and lf = pigmented, young tomato leaf. Thirty micrograms of total RNA was loaded per lane.

Although degradation of individual anthocyanins may be occurring throughout tuber development, we only measured levels of the anthocyanidins, or aglycone forms of anthocyanins, which are simpler to identify than the numerous anthocyanins. Although plants were grown and tubers harvested throughout the year, during which greenhouse conditions could be expected to vary, we showed that tuber color and anthocyanin concentration decreased during tuber development, suggesting that these decreases were not grossly affected by any variations in growing conditions the plants may have been exposed to. These data suggest that to maintain the stability or increase the color of red tubers, improvements probably should be made early in tuber development, perhaps by increasing synthesis of anthocyanin to overcome dilution due to cell division or losses due to degradation.

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