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Genetic analysis of pigmented tuber flesh in potato

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Abstract Interest in anthocyanin-pigmented potato tuber flesh is increasing. To genetically map and characterize loci that influence this trait, diploid potato clone 10618-01, which has partially pigmented flesh, was crossed with diploid 320-02, which has white flesh. Almost all progeny exhibited purple coloration in the flesh, with some clones having only a small percentage of tissue pigmented, other clones having most tissue pigmented, and the majority of clones showing intermediate color phenotypes. The two parents and 228 progeny were genotyped with 493 AFLP, 8 CAPS, and 13 SSR markers. QTLs influencing extent of flesh pigmentation were detected on chromosomes 5, 8, and 9. The potato homolog of *Petunia an1*, a basic helix-loop-helix (bHLH) transcriptional regulator of anthocyanin biosynthesis, was found to co-localize with the QTL on chromosome 9. A CAPS marker based on this gene was used to evaluate a collection of 21 tetraploid potato clones with highly or fully pigmented red or purple flesh, as well as 53 cultivars with white or yellow flesh. All 21 pigmented-flesh clones shared a marker allele that was present in only 21 of the 53 white and yellow clones, suggesting that a common bHLH allele contributes toward, although it is clearly not sufficient for, highly or fully pigmented tuber flesh in cultivated potato.

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Introduction

Consumer interest in potatoes with red or purple flesh has been increasing over the past decade, in part because of novel appearance, and in part because of the perceived benefits of higher antioxidant content (Tsuda et al. 2000; Ross and Kasum 2002; Brown et al. 2003, 2005, 2007; Scalbert et al. 2005). Red and purple tuber flesh color results from the accumulation of anthocyanin pigments (Lewis et al. 1998; Rodriguez-Saona et al. 1998; Naito et al. 1998; Eichhorn and Winterhalter 2005).

Pigmented tuber flesh is conferred by the *Pf* locus (De Jong 1987). *Pf* is tightly linked with *I*, which is required for pigmentation of tuber skin and maps to chromosome 10 (De Jong 1987; Dodds and Long 1955; van Eck et al. 1994). The *I* locus is also known as *D* in tetraploid potatoes (Salaman 1910). *Pf* alone is not sufficient for tuber flesh to be completely pigmented; potatoes with this gene may exhibit a small, intermediate, or large degree of flesh coloration. In many plants, tissue-specific accumulation of anthocyanins is mediated by R2R3MYB genes and/or bHLH regulators (Cone et al. 1986; Ludwig et al. 1989; Ludwig and Wessler 1990; Quattrocchio et al. 1998, 1999; Selinger et al. 1998). The potato ortholog of *Petunia an*2, an R2R3MYB regulator of anthocyanin production, maps to the same region of the genome as *Pf* and *I* (De Jong et al. 2004).

Several other potato genes are also known to influence potato color. The *R* locus, which co-segregates with dihydroflavonol 4-reductase (De Jong et al. 2003), is required for the production of red anthocyanins, while the *P* locus, which codes for flavonoid 3′,5′-hydroxylase (Jung et al. 2005), is required for production of purple pigments.

Several recent studies have reported on potential health benefits of consuming potatoes with anthocyanin-pigmented flesh. For example, rats fed with purple potato flakes have

Table 1 CAPS markers developed in this study

Marker	Approx. product size (bp)	Restriction enzyme	Chromosome	Primer sequences
21BA	470/400	None	10	F: GTGATTATGTCATCCAAAAGTTTATAG R: GAATTTCTGAGGTTGAGGTCTTA
ans	700	*Hae*III	8	F: TATTGCTTGTACTTCTATTTTTCGAGATAG R: CTTGGCATATTCACTTGTTGCT
bch6	400	*Bcr*I	6	F: AACAACCTCACATGTTTCTCCAA R: CAAATGTACCCAACATTTCGGTTA
chi	800	*Mse*I	5	F: ATAGAGGTTTGGAGATTGAAGG R: ACTACACTTTGCTGCAGGGGA
chs	1600	*Alu*I	5	F: GCGACTCCTTCGAACTGTG R: TGAAGTTTTTCGGGCTTTAGGC
CT203	760	*Alu*I	10	F: AGTGACGATGATGACAGAGGAGAA R: AAATGGACTAAAGCATATAGCCGG
GP24	900	*Alu*I	6	F: CTGCAGTCAAGGGATACATTT R: GCGTCTCTGCAATCTATTTCT
jaf13	1480	*Rsa*I	8	F: GAAGATCCTAACCTCATTCAGCAAATAAAA R: GTTGCTTAAAATTATGGAGGCACTGA
Stan1	1600	*Taq*I	9	F: CGGCCCTAGTTATGATGAATTATCACA R: ACCTCCACTTTAAGTTCCCTTAGC
UGPase	600	*Rsa*I	11	F: CACCTTGACTGATGAGGGCTAT R: TGGCACCAGCAGCTACTCTA
zep	1000	*Bfu*CI	2	F: AGAGGGATTTAAGTGCTATCAGAG R: CCAGTATAACAAGTGTAGCCAGAG

significantly higher serum antioxidant potential and hepatic Cu/Zn-superoxide dismutase in the liver (Han et al. 2006). Potato anthocyanin may also help combat both prostate cancer (Reddivari et al. 2007) and breast cancer (Thompson et al. 2009). To more efficiently manipulate tuber flesh color in our applied breeding program, we would like to better understand the genetic basis for flesh pigmentation. Toward this end, we constructed a diploid population that segregates for degree of tuber flesh coloration, and report here on a QTL analysis of the pigmented flesh trait, as well as a follow-up marker analysis of tetraploid potato cultivars with and without pigmented tuber flesh.

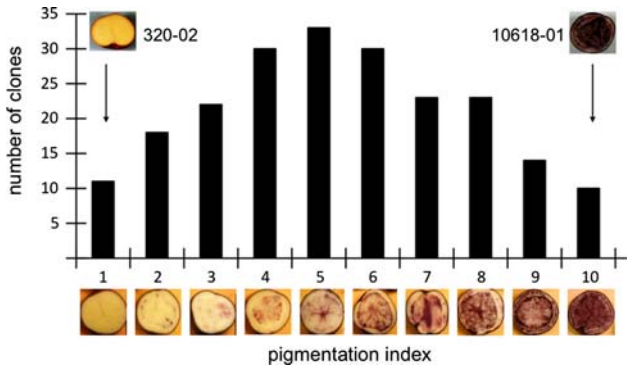

Fig. 1 Distribution of flesh color phenotypes observed in 2006 in the F_1 progeny of a cross between diploid clones 10618-01 and 320-02

Materials and methods

Plant materials

Diploid potato clone 10618-01, which has purple, partially pigmented tuber flesh, was crossed as a female with white-fleshed diploid 320-02 to form an F_1 mapping population consisting of 228 clones. Both diploid parents were kindly provided by H. De Jong (AAFC, Fredericton, NB). WIS clones with pigmented tuber flesh were kindly provided by S. Janksy (USDA-ARS, Madison, WI). POR04PG01-2 was kindly provided by C. Brown (USDA-ARS, Prosser, WA). NY clones were provided by the Cornell University potato breeding program.

Phenotyping

The extent of tuber flesh coloration was evaluated with tubers produced in the greenhouse. Each clone was grown in two pots, and the largest tuber from each pot was scored. The average score of the two tubers was used for QTL analysis. All 228 progeny, as well as both parents, were genotyped. After genotyping, two daughter clones appeared to be identical; one of the two was excluded from subsequent analysis. Thirteen of the remaining 227 clones did not tuberize well or died in 2006, and were not included in any QTL analyses. A further 15 clones did not tuberize well or

Table 2 QTLs detected by Kruskal–Wallis (KW) and Interval Mapping (IM) using phenotype data from 2006

QTL model	Parent	QTL parameters			
		Chromosome	Marker[a]	Significance value	Percent variation (r^2)
KW	10618-01	9	E32M48-233	0.0001	N/A
		5	E32M49-442	0.001	N/A
	320-02	8	P14M37-134	0.0005	N/A
		5	E35M54-162	0.001	N/A
IM	10618-01	9	E32M48-233	LOD 3.6	8.1
		5	E32M49-442	LOD 3.6	8.1
	320-02	8	P14M37-134	LOD 2.9	6.5
		5	E35M54-162	LOD 3.7	8.1

[a] Marker with highest significance score

died in 2007, and were not used for QTL analyses that year. Flesh coloration was scored on an arbitrary 1–10 scale (1 = no flesh pigmentation, 10 = almost completely pigmented), where the distribution of pigment was assessed in equatorial cross sections of mature tubers. Flesh coloration was scored in both 2006 and 2007.

Marker analysis

Genomic DNA was extracted from plants grown in the greenhouse using a Qiagen DNA kit, following the manufacturer's instructions. AFLP markers were generated with 14 Pst+2/Mse+3 and 10 Eco+2/Mse+3 AFLP primer combinations according to Vos et al. (1995), using ^{33}P-labeled *Pst*I or *Eco*R1 primers. AFLP amplification products were separated on a 5% denaturing polyacrylamide gel. Sizes of amplification products were estimated by comparison to a Sequamark 10 base ladder (Research Genetics, Huntsville, AL). Images were visualized by exposing film against dried acrylamide sequencing gels. Eight CAPS markers (Konieczny and Ausubel 1993) (SbeII—Chen et al. 2001; F35H-4F/4R—Jung et al. 2005; 21BA, *bch*6, CT203, GP24, *UGPase*, and *zep* are described in Table 1) and 13 polymorphic SSR markers of known chromosomal location (STM0003, STM1104, STM1106, STM1053, STM2020, STM2022, STM3009, STM3010, STM3016, StI011, StI014, StI041, and StI049) (Milbourne et al. 1998; Feingold et al. 2005) were used to identify chromosomes.

Mapping

Marker data were analyzed with JoinMap 3.0 (Van Ooijen and Voorrips 2001). Linkage groups were assembled using the Kosambi function (Kosambi 1943). Twenty-two linkage groups were assembled at LOD thresholds of eight or greater. Chromosomes 2 and 7 of female parent 10618-01 were assembled at LOD 6 and LOD 5, respectively. Each linkage group was labeled with at least one anchor marker of known location.

QTL analysis

QTL analysis was performed with the program MapQTL 5 (Van Ooijen 2004). Two analysis models (Kruskal–Wallis and Interval Mapping) were used. A LOD threshold of 2.85 for declaring significance ($P < 0.05$) for interval mapping was established by empirically permuting the data 1000 times. Linkage map and QTL locations were visualized using MapChart 2.1 (Voorrips 2002). QTL analysis was repeated with phenotypic data from 2006 and 2007.

Association of QTLs with anthocyanin pathway genes

CAPS markers were designed against five potato anthocyanin pathway genes (*ans*, *Stan1*, *chi*, *chs*, *jaf13*; Table 1). For each CAPS marker, genomic DNA was amplified using the following thermal profile: 94°C for 2 min, then 35 cycles of [94°C, 20 s; 72°C, 60 s; 56°C, 30 s]. PCR products were digested with the corresponding restriction enzyme for 3 h, then visualized on a 2% agarose gel.

Results

The progeny of a cross between diploid 10618-01, which has purple skin and partially colored (purple and white) tuber flesh, and diploid 320-02, which has red skin and white flesh, segregated extensively for extent of purple color in tuber flesh. After harvest in 2006, the flesh of 11 progeny did not appear to be pigmented at all, the flesh of 10 progeny were heavily pigmented, while the remaining 193 progeny displayed intermediate degrees of purple flesh coloration (Fig. 1). All progeny had purple tuber skin. The extent of tuber flesh coloration was scored on a 1–10 scale (Fig. 1).

To identify loci influencing extent of flesh coloration, the progeny and both parents were evaluated with 514 molecular markers including 493 AFLP, 13 SSR, and 8 CAPS markers. Analysis with JoinMap 3.0 readily separated markers into 12 maternal and 12 paternal linkage

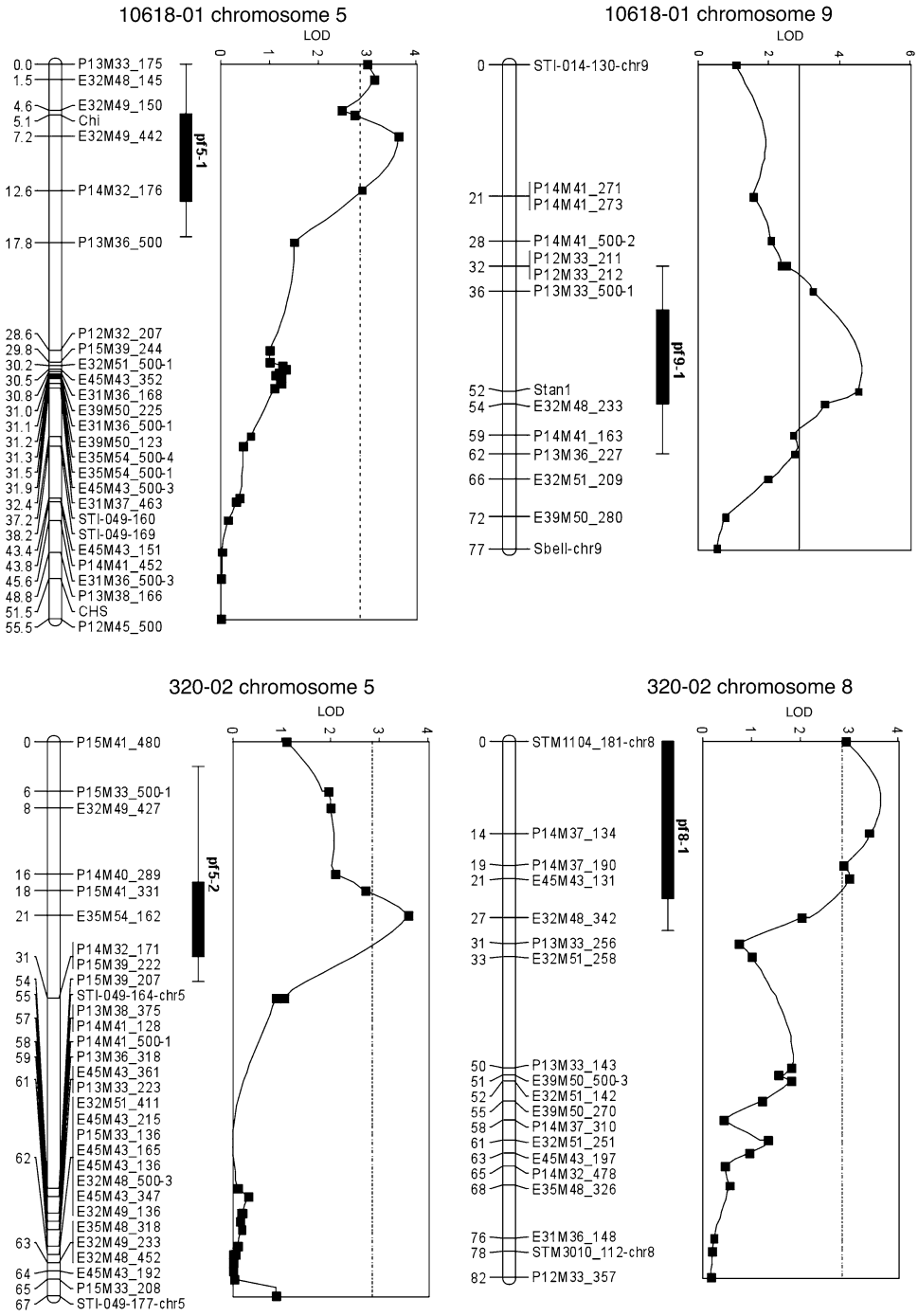

Fig. 2 Location of QTLs that influenced extent of tuber flesh coloration in 2006. Map locations for anthocyanin-related genes *chi*, *chs*, and *Stan1* are also shown

groups. A total of 496 markers could be placed in linkage groups with a LOD score of 5 or higher. The map of 10618-01 totaled 753 cM in length and comprised 212 markers, while the map of 320-02 totaled 907 cM in length and was made up of 284 markers. All 24 linkage groups included at least one anchor marker of known chromosomal location.

Marker and year 2006 trait data were then analyzed using both nonparametric (Kruskal–Wallis) and parametric (interval mapping) approaches. Kruskal–Wallis analysis revealed significant ($P < 0.001$) loci on chromosome 5 of both parents: for 10618–01, at AFLP marker E32M49-442, and for 320–02, at marker E53M54-162 (Table 2). In addition, highly significant loci were detected on chromosome 8 of 320-02 at marker P14M37-134 ($P < 0.0005$) and on chromosome 9 of 10618-01 at marker E32M48-233 ($P < 0.0001$) (Table 2). QTLs at comparable locations were identified by interval mapping (Fig. 2, Table 2). The same loci were detected when phenotypic data for year 2007 was analyzed separately. Tuber pigmentation scores were not

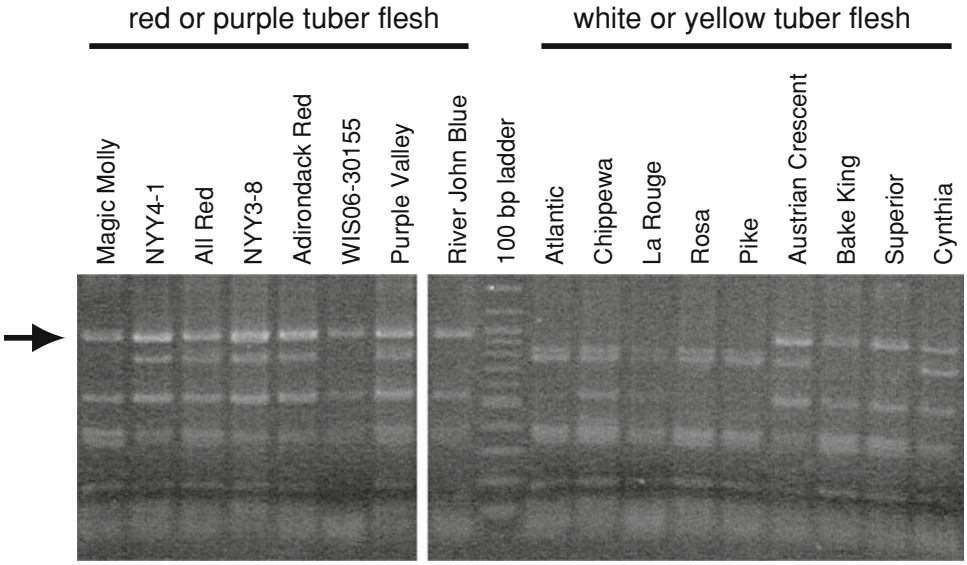

Fig. 3 Association between colored tuber flesh and a CAPS marker allele based on the potato homolog of *Petunia an1*. Genomic DNA was amplified with *Stan1* primers (Table 1), restricted with *Taq* I, and electrophoresed through a 2% agarose gel. An *arrow* denotes the approximately 980 bp band present in all clones tested with red or purple tuber flesh

identical in 2006 and 2007, but were highly correlated ($r^2 = 0.61$). Two additional loci were detected only in 2007, on chromosome 8 of 10618-01 at marker E32M48-318 (LOD 3.1) and chromosome 3 of 320-02 at marker E39M50-249 (also with LOD 3.1) (data not shown).

We subsequently tested whether any known anthocyanin biosynthetic or regulatory genes co-localize with these QTLs. Potato chromosome 5 is known to code for at least two anthocyanin biosynthesis genes, chalcone isomerase (*chi*) and chalcone synthase (*chs*); chromosome 8 is known to harbor a basic helix-loop-helix (bHLH) anthocyanin regulatory gene (homolog of *Petunia jaf13*), as well as anthocyanidin synthase (*ans*); and chromosome 9 is known to code for a bHLH gene homologous to petunia *anthocyanin 1* (*an1*) (Spelt et al. 2000; De Jong et al. 2004). CAPS markers were developed for all these genes (Table 1). Two of the five genes mapped under QTLs detected in 10618-01: *chi* on chromosome 5 and *Stan1*, the potato homolog of *an1*, on chromosome 9 (Fig. 2). *Stan*1 explained more phenotypic variation—11%—than AFLP marker E32M48-233, which explained 8.1% (Table 2). The relationship between the potato homologs of *jaf13* and *ans* with the QTL on chromosome 8 could not be evaluated, as neither CAPS marker was polymorphic in 320-02. The fifth gene, *chs*, mapped far from the QTL on chromosome 5 of 10618-01 (Fig. 2).

CAPS markers based on *ans*, *chi*, *Stan1*, and *jaf13* were tested for possible relationship with pigmented flesh in a panel of diverse potato germplasm consisting of 21 tetraploid potato clones with red or purple flesh and 53 clones with white or yellow tuber flesh. The *Stan1* CAPS marker revealed a common digestion product, about 980 bp in size, in all 21 of the clones with pigmented flesh (Fig. 3 and Table 3). This same digestion product was present in only 21 of 53 white- and yellow-fleshed clones (Fig. 3 and Table 3), suggesting that a common bHLH allele contributes toward, but is not sufficient, for the ability to accumulate anthocyanin in potato tuber flesh. No association with flesh color was observed with CAPS markers based on *jaf13*, *ans* or *chi* in the same panel.

Discussion

This study detected loci on three chromosomes—5, 8, and 9—that mediate degree of tuber flesh pigmentation. Alleles influencing this trait descended from both the white- and purple-fleshed parents, with the white-fleshed parent contributing alleles from chromosome 5 and 8, and the purple-fleshed parent contributing alleles from chromosomes 5 and 9.

The only locus that has previously been implicated in pigmentation of tuber flesh, *Pf*, is presumably located on chromosome 10, as *Pf* is tightly linked to *I* (De Jong 1987), and *I* has been mapped to chromosome 10 (Van Eck et al. 1994). If *Pf* segregated in this cross, we would have expected half the progeny to exhibit white flesh. Instead, only 11 of 214 progeny had unpigmented flesh, suggesting that 10618-01 is homozygous for *Pf*, and that *Pf* is necessary, but not sufficient, for anthocyanin-pigmented tuber flesh. No polymorphic markers from chromosome 10 segregated aberrantly, so *Pf* must have been transmitted to either half or all progeny. As tuber skin color did not segregate in this cross—all progeny had purple-skinned tubers—the genes required for anthocyanin production per se were present in all progeny. Thus, the relatively few white-fleshed progeny must have been white for a reason other than lacking a necessary biosynthetic gene.

Table 3 Presence/absence of ≈980 bp *Stan1* marker allele in a panel of potato clones with and without anthocyanin-pigmented tuber flesh

Potato clone	Flesh color	*Stan1* 980 bp fragment present (1 = yes, 0 = no)
Adirondack Blue	Purple	1
Adirondack Red	Red	1
All Red	Red	1
Huckleberry	Red	1
Magic Molly	Purple	1
NYH52-1	Purple	1
NYS48-6	Purple	1
NYY3-8	Red	1
NYY4-1	Red	1
POR04PG01-2	Purple	1
Purple Peruvian	Purple	1
Purple Valley	Purple	1
River John Blue	Purple	1
WIS00-4252-1	Purple	1
WIS01-1131-1	Purple	1
WIS01-1131-5	Red	1
WIS06-3124	Purple	1
WIS06-30155	Purple	1
WIS06-30244	Purple	1
WIS06-30340	Purple	1
WIS99-2743	Purple	1
Allegany	White	0
Amandine	Yellow	1
Andover	White	0
Atlantic	White	0
Austrian Crescent	Yellow	1
Bake King	White	1
Bintje	Yellow	1
Carola	Yellow	0
Chieftain	White	0
Chippewa	White	0
Cynthia	Yellow	0
Desiree	Yellow	1
Eva	White	0
German Butterball	Yellow	1
Idarose	White	1
Katahdin	White	0
Kennebec	White	0
Keuka Gold	Yellow	1
La Rouge	White	0
Lehigh	Yellow	0
Lenape	White	0
Monona	White	0
Nordonna	White	1
Norland	White	1
NY97	White	1
NY99	White	0
NY115	White	0
NY118	White	1
NY120	White	1
NY121	White	0
NY123	White	0
NY127	White	0
NY128	White	0
NY129	White	0
NY130	White	0
NY132	White	0
NYT15-1	White	0
Pike	White	0
Prince Hairy	White	1
Reba	White	0
Red La Soda	White	0
Redsen	White	1
Rideau	White	1
Rosa	White	0
Salem	White	0
Sandy	Yellow	0
Serrana Inta	Yellow	1
Snowden	White	0
Stirling	White	1
Superior	White	1
Sylvia	Yellow	0
Yagana	Yellow	1
Yukon Gold	Yellow	1

Although the genes underpinning flesh coloration QTLs were not conclusively established in this study, two promising candidates—*chi* (for a QTL on chromosome 5) and a bHLH transcription factor similar to *Petunia an1* (for a QTL on chromosome 9)—were identified. Both of these genes mapped close to, or under, the peak of the respective QTLs. It is not obvious how *chi*, an anthocyanin biosynthetic gene, might influence degree of flesh pigmentation; perhaps this gene exhibits functional variation in its promoter region, leading to differences in the range of tissues in which it can be expressed. That the potato homolog of *Petunia an1* may play a role in tissue-specific expression was not surprising, as bHLH regulators of anthocyanin biosynthesis, such as *delila* in *Antirrhinum majus* (Goodrich et al. 1992), *B* in *Zea mays* (Selinger et al. 1998), *ivs* in *Ipomoea tricolor* (Park et al. 2004), *tt8* of *Arabidopsis thaliana* (Nesi et al. 2000; Baudry et al. 2006), the rice *Purple leaf* (*Pl*) locus (Sakamoto et al. 2001), and the rice

red grain locus *Rc* (Sweeney et al. 2006) are all known to mediate tissue-specific expression of anthocyanins.

Further evidence that the potato homolog of *Petunia an1* (or a gene tightly linked to it) is associated with pigmented tuber flesh came from a comparison of varieties with and without pigmented flesh. All 21 pigmented flesh clones tested to date share an approximately 980 bp *Stan1* CAPS marker allele. Eight of the pigmented flesh clones evaluated were developed in Wisconsin (WIS clones), six were developed in New York (NY and Adirondack clones), one was developed in Alaska (Magic Molly), one was developed in Washington (POR clone), one was developed in Korea (Purple Valley), and the remaining four are of unknown origin. Though potato clones that accumulate anthocyanin in tuber flesh are not uncommon in Andean landraces, this trait has generally been selected against in modern potato breeding, just as pigmented kernels were selected against in maize (Johannessen et al. 1970) and pigmented grains were selected against in rice (Sweeney et al. 2007). Nevertheless, as understanding of the potential health benefits conferred by anthocyanins has increased over the past decade, interest in consuming anthocyanin-rich plant tissues has also increased dramatically. Markers based on *Stan1* may thus prove useful for those seeking to more efficiently manipulate the nutritionally important trait of pigmented tuber flesh in applied potato breeding programs.

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