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A survey of cultivated heirloom tomato varieties identifies four new mutant alleles at the *green-flesh* locus

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Abstract The process of crop domestication occurs through the selection and subsequent propagation of novel alleles that improve traits of interest. Cultivated tomato (*Solanum lycopersicum*), particularly heirloom varieties, exhibit a wide range of variation in fruit size, shape and color. The *green-flesh* mutant of tomato possesses a stay-green phenotype resulting in fruits that ripen to a red-brown color, due to the retention of chlorophyll and the simultaneous accumulation of lycopene. The recent identification of the *GREEN-FLESH* gene provides a molecular tool with which to investigate the origin of a subset of cultivated tomato varieties that resemble the *green-flesh* mutant. Sequence analysis of the *GF* locus from 26 varieties revealed the existence of four previously unidentified null alleles. This study illustrates the potential of cultivated tomato varieties, including heritage cultivars, heirlooms, and land races, for uncovering new alleles in genes of interest.

Keywords Heirloom tomato · Fruit color · Chlorophyll degradation · Stay-green mutant · Genotyping · Fruit quality

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Introduction

Fruits of cultivated tomato exhibit tremendous phenotypic diversity, having varied sizes, shapes and colors. This diversity has arisen as a result of alleles at multiple loci, selection during domestication and subsequent improvement efforts. The phenotypic variation in cultivated tomato is particularly prevalent within heirloom varieties. Heirloom tomato varieties have been loosely classified in to four groups (Male 1999). Commercial heirlooms are typically varieties that were introduced by seed companies prior to 1940. Family heirlooms are varieties that have been selected by home gardeners or farmers and subsequently maintained and distributed. Created heirlooms are derived by deliberately crossing two heirloom varieties or an heirloom variety and a hybrid variety followed by selection of progeny with desired characteristics. Mystery heirlooms are varieties that arose through non-deliberate cross-pollination and have then been stabilized after several generations. The diversity found in heirloom tomato varieties is highly prized among horticulturalists and amateur gardeners and has proven to be a rich source of germplasm for studies investigating the genetic basis of fruit size, shape and color (Male 1999; Paran and van der Knaap 2007).

The color change that typically accompanies the ripening of fleshy fruits is one of the most conspicuous phenotypes associated with ripening and serves as an important signal to seed dispersing fauna that

the fruit is ripe and palatable. In many fruit, including tomato, this color change occurs through both the degradation of chlorophyll and the concomitant increase in the synthesis of carotenoids as chloroplasts are converted to chromoplasts. Fruit of the *green-flesh* (*gf*) mutant of tomato fail to degrade chlorophyll at the onset of ripening but accumulate carotenoids, leading to ripe fruit that are brown in color (Kerr 1956). Similarly, senescence-induced chlorophyll loss in leaves is also compromised in the *gf* mutant and thylakoid grana and light-harvesting chlorophyll-binding proteins (LHCP) persist during senescence (Cheung et al. 1993; Akhtar et al. 1999). The *gf* mutant maps to the long arm of tomato chromosome 8 and positional cloning revealed a single A → T nucleotide change in a tomato homolog of the *STAY-GREEN* gene of rice (Park et al. 2007; Barry et al. 2008). The mutation converts an invariant arginine residue at position 143 into a serine within the highly conserved central core of the protein (Barry et al. 2008). The biochemical function of SGR has yet to be determined, but mutations in *SGR* genes from several additional species, including Arabidopsis, pepper, pea, and *Festuca pratensis* inhibit chlorophyll degradation leading to stay-green phenotypes (Armstead et al. 2006, 2007; Ren et al. 2007; Sato et al. 2007; Barry et al. 2008; Borovsky and Paran 2008).

Many heirloom varieties of tomato have a ripe fruit phenotype reminiscent of the *gf* mutant and the identification of the *GF* gene has provided an opportunity to examine the molecular basis of fruit color in these varieties. In this study a set of 26 varieties with fruit described as brown, black and purple were evaluated for the presence of the *gf* mutant allele. Eight varieties carried the A → T nucleotide substitution, characteristic of the original *gf* mutant allele. Additional sequence analysis revealed that the other seventeen heirloom varieties each contained one of four new mutant alleles. Together, these data indicate that mutations at the *gf* locus have arisen on at least five separate occasions. Finally, four of the five alleles can be readily detected by PCR based markers allowing for rapid screening of this fruit quality trait in breeding programs. This study provides compelling evidence of the power of heirloom varieties of tomato for uncovering novel alleles at loci of interest.

Materials and methods

Plant material, growth conditions and evaluation of leaf de-greening

Tomato accessions homozygous for the *green-flesh* (*gf*) mutation, LA3534 were obtained from the Tomato Genetics Resource Center, UC Davis. The parental cultivar Ailsa Craig (AC) was originally obtained from the Glasshouse Crops Research Institute (Littlehampton, Sussex, UK). Heirloom tomato varieties were obtained from either TomatoFest (http://www.tomatofest.com/) or Tomato Bob's Heirloom Tomatoes (http://www.tomatobob.com/). Duplicate plants were grown in peat-based compost supplemented with fertilizer in greenhouses equipped with heating and supplemental lighting at Michigan State University, East Lansing, MI during spring 2008. A leaf de-greening assay was performed by harvesting expanding terminal leaflets from the fourth true leaf of young tomato plants prior to the onset of flowering. Leaves were excised and floated on water in sealed petri dishes. The dishes were wrapped in aluminum foil and placed in darkness for 2-weeks at room temperature.

Amplification and cloning of GF genomic clones

Genomic DNA was isolated from expanding tomato leaves as previously described (Barry et al. 2005). A 2.55 kb genomic fragment comprised of the exons and introns of *GF* was PCR amplified from genomic DNA, isolated from the Ailsa Craig tomato cultivar or stay-green heirloom tomato varieties. Amplification was performed using the *Pfu* Ultra™ DNA Polymerase (Stratagene, LaJolla, CA) and the primers U316068F: 5′-GGACTTTTATCAAACAGCTAACT TGCA-3′ and U316068R: 5′-GGCACAACCCAA CTTACAATAATTGTA-3′. PCR fragments were purified using the Charge Switch® PCR Purification Kit and cloned using the Zero Blunt® TOPO® PCR cloning kit (Invitrogen Corporation, Carlsbad, CA). Plasmid DNAs were purified from 3 ml cultures of *E. coli* grown overnight, using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega Corporation, Madison, WI). DNA sequence analysis was performed at the Research Technology Support Facility (http://www.genomics.msu.edu). DNA sequences were assembled using Sequencher™ version 4.7

(Genecodes Corporation, Ann Arbor, MI). A genomic sequence of *GF*, together with annotation of the allelic variants described in this report, has been deposited in Genbank with the accession number FJ647188.

Genotyping stay-green tomato varieties

Genotyping of the *gf* mutant allele was achieved through amplification a 304 bp fragment flanking the *gf* mutation using the primers gfallele-f: 5′-GTCC ATTGCCACATTAGTGGAGGCC-3′ and gfallele-r: 5′-GGATAATTCCTTAGTAAATTCTCATC-3′. The resulting fragments were sequenced using the nested primer gfallele-seq: 5′-GACACAGGACCAATAACA ATTTTGGGA-3′. Genotyping of the gf^3 mutant allele was achieved through amplification of a 423 bp fragment of *GF* using the primers gfdeln-f: 5′-GCTCAA CAATGAACAACAGAGCTC-3′ and gfdeln-r: 5′-GAT AGACCTCGACTATTTCTCACAC-3′. The resulting fragments were sequenced using the nested primer gfdeln-seq: 5′-GCAAGAAGAATCAATCCATAGTAC-3′. PCR amplification of DNA fragments for genotyping was performed using GoTaq® DNA Polymerase (Promega Corporation, Madison, WI) and DNA sequencing and assembly was performed as described above.

Development of CAPS markers for the *gf* and gf^2 alleles

The potential for the *gf* mutant alleles to result in DNA sequence polymorphisms that can be resolved using CAPS markers, (Konieczny and Ausubel 1993), was investigated using the CAPS Designer tool (http://www.sgn.cornell.edu/tools/caps_designer/caps_input.pl). A 166 bp DNA fragment flanking the *gf* mutant allele was amplified from wild type (*GF/GF*) and stay-green (*gf/gf*) varieties using the primers gfallele-f: 5′-GTCC ATTGCCACATTAGTGGAGGCC-3′ and gfallele-seq: 5′-GACACAGGACCAATAACAATTTTGGGA-3′. A 358 bp DNA fragment flanking the gf^2 allele was amplified from wild type (*GF/GF*) and stay-green (gf^2/gf^2) varieties using the primers gf^2 caps-f: 5′-ATCTTGAATCTGCATCTACATCGTA-3′ and gf^2 caps-r: 5′-CCTATGGTTGTGTGTTTGATCCTCC-3′. DNA fragments were digested overnight at 37°C with 0.1 units of either *Hpy188*I for the *gf* allele or *Mse*I for the gf^2 allele (New England BioLabs, Ipswich, ME) and the fragments were resolved by separation through 3% agarose gels run at 4°C.

Results

Isolation of a *GF* genomic clone

The *gf* mutation results in a single A → T nucleotide change at position 571 within the cDNA sequence (Barry et al. 2008). In order to facilitate subsequent genotyping analysis of the stay-green heirloom varieties, a genomic fragment comprising the exons and introns of *GF* was amplified from DNA extracted from the Ailsa Craig (AC) cultivar. Sequence analysis of this 2.55 kb fragment revealed the presence of four exons and three introns. The exon/intron boundaries of the *GF* genomic clone match those of the closest *Arabidopsis* homolog, *At4g22920*. However, the introns are larger in *GF* than in *At4g22920*, particularly intron 2 which is 1,118 bp in length in *GF* compared with 194 bp in *At4g22920*. Sequence analysis of the genomic clone revealed that the A → T nucleotide change, characteristic of the *gf* mutant allele, is located at nucleotide 1,789 within exon 3 of the genomic clone (Fig. 1).

Phenotypic evaluation of tomato heirloom varieties

Leaves of the *gf* mutant exhibit a stay-green phenotype when exposed to nutrient stress or when excised leaves are placed in the dark (Akhtar et al. 1999; Barry et al. 2008). Twenty-six heirloom tomato varieties reported to have a fruit phenotype similar to the *gf* mutant were evaluated for inhibition of chlorophyll degradation using a detached leaf assay (Table 1). Leaf samples from duplicate plants of each variety, together with Ailsa Craig (AC) (*GF/GF*) and an isogenic line homozygous at the *gf* locus (*gf/gf*) (LA3534), were excised and floated on water in petri dishes for 2 weeks at room temperature in the dark. The AC and *gf* lines displayed yellow and stay-green phenotypes, respectively, and all 26 heirloom varieties displayed a stay-green phenotype. The stay-green phenotype was also evident in the fruit of the heirloom varieties and all had a phenotype identical to fruit of the *gf* mutant.

Identification of novel alleles at the *GF* locus

Following isolation of the *GF* genomic clone, a 304 bp fragment flanking the *gf* mutant allele was

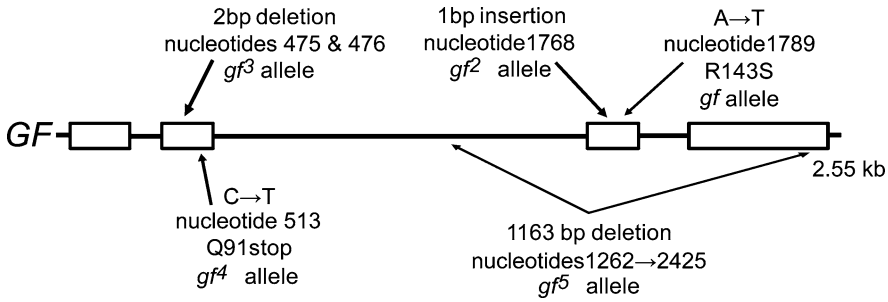

Fig. 1 Genomic structure of *GF*. The 2.55 kb structure of the genomic clone of *GF* is shown with exons depicted as *boxes* and introns as *lines*. The nucleotide positions and changes that give rise to the five *gf* mutant alleles are shown

amplified from AC, *gf*, and 26 stay green heirloom varieties. Sequence analysis of this fragment revealed that only eight of the heirloom varieties possessed the A → T polymorphism characteristic of the *gf* mutant (Table 1). This suggested that the other heirloom varieties may represent new alleles of *gf* or mutations at an additional locus or loci that lead to a stay-green phenotype. In support of the hypothesis that the heirloom varieties contain additional *gf* alleles, sequence analysis of the 304 bp fragment revealed that the Black Plum variety contained a single adenine insertion following nucleotide 1,768 of the *GF* genomic clone (Fig. 1). This additional base is located within exon three and causes a frame shift mutation following leucine 136 that leads to the incorporation of four spurious amino acids followed by a premature stop codon. Therefore, this single base insertion effectively truncates the GF protein in the Black Plum variety by 136 amino acids, resulting in a null allele that has been designated gf^2. This insertion was confirmed in an independent DNA sample isolated from a separate individual. In addition, sequence analysis of the full-length genomic sequence of *GF* isolated from two Black Plum individuals failed to reveal any additional sequence polymorphisms.

The remaining seventeen lines exhibited a stay-green phenotype but did not carry the *gf* or the gf^2 alleles. In order to continue the search for additional mutant alleles of *GF*, the 2.55 kb *GF* genomic fragment, was amplified and cloned from the Paul Robeson variety (Table 1). Comparison of four independent clones from the Paul Robeson variety to the genomic sequence isolated from the AC cultivar revealed a 2 bp deletion within exon 2 removing nucleotides 475 and 476 of *GF* in the stay-green variety (Fig. 1). This deletion removes the first two nucleotides encoding histidine 78 causing a frame shift mutation that results in the incorporation of a glutamine at position 78 immediately followed by a stop codon. This stop codon truncates the GF protein by 194 amino acids in the Paul Robeson variety. These data suggested that the Paul Robeson variety carries a null mutation in *GF* that we have designated gf^3. Following the discovery of the gf^3 allele in the Paul Robeson variety, a 424 bp fragment surrounding this region was amplified from genomic DNA of all 26 heirloom varieties. Sequence analysis of this fragment revealed that ten additional heirloom varieties also contained the deletion corresponding to the gf^3 allele (Table 1). In addition, DNA sequence analysis of the 424 bp fragment also revealed that three of the varieties, Black Cherry, Purple Calabash, and Purple Prince did not contain the 2 bp deletion but did contain a single nucleotide polymorphism (C → T) at nucleotide 513 of the *GF* genomic clone (Fig. 1). This mutation converts glutamine 91 into a stop codon that truncates the predicted GF protein by 181 amino acids. Therefore, these three varieties carry a null allele of *GF* that we have designated gf^4 (Table 1). The fidelity of the gf^3 and gf^4 alleles was confirmed by sequencing of this region from different individuals and in the case of the gf^4 allele sequence analysis of the entire 2.55 kb *GF* genomic region from the Black Cherry, Purple Calabash, and Purple Prince varieties.

In total these analyses resulted in the identification of mutant alleles of *GF* in all of the heirloom varieties examined with the exception of Black Prince, Nyagous, and Purple Russian. Therefore, PCR was performed with the aim of amplifying the entire 2.55 kb genomic fragment of *GF* from these three varieties. Gel electrophoresis of the amplified fragments revealed that they were ~1 kb smaller than the corresponding fragment obtained from the AC cultivar (Fig. 2). Sequence analysis of these fragments indicated the presence of a 1,163 bp deletion between nucleotides 1,262 and 2,425 that is identical

Table 1 Genotyping at the *GREEN-FLESH* locus in 26 stay-green heirloom varieties of tomato

Variety	Genotype	Allele
Ailsa Craig	*GF/GF*	–
Green flesh (LA3534)	A → T[c]	*gf*
Ananas Noire[a]	A → T	*gf*
Black Brandywine[a]	2 bp deletion[d]	gf^3
Black Cherry[a]	C → T[e]	gf^4
Black From Tula[a]	2 bp deletion	gf^3
Black Krim[a]	2 bp deletion	gf^3
Black Pear[a]	2 bp deletion	gf^3
Black Plum[a]	A insertion[f]	gf^2
Black Seaman[a]	2 bp deletion	gf^3
Black Zebra[a]	A → T	*gf*
Cherokee Purple[a]	A → T	*gf*
Indian Dark Violet Beefsteak[a]	2 bp deletion	gf^3
Paul Robeson[a]	2 bp deletion	gf^3
Purple Passion[a]	2 bp deletion	gf^3
Black Crimson[b]	A → T	*gf*
Black Ethiopian[b]	2 bp deletion	gf^3
Black Prince[b]	1,163 bp deletion[g]	gf^5
Carbon[b]	A → T	*gf*
Cherokee Chocolate[b]	A → T	*gf*
Chocolate Stripes[b]	2 bp deletion	gf^3
Indische Fleish[b]	A → T	*gf*
Japanese Black Trifele[b]	2 bp deletion	gf^3
Nyagous[b]	1,163 bp deletion	gf^5
Purple Calabash[b]	C → T	gf^4
Purple Prince[b]	C → T	gf^4
Purple Russian[b]	1,163 bp deletion	gf^5
Schwarze Sarah[b]	A → T	*gf*

[a] Seeds obtained from Tomato Bob's Heirloom Tomatoes

[b] Seeds obtained from Gary Ibsen's TomatoFest

[c] A → T nucleotide change at position 1,789 of the *GF* genomic clone

[d] 2 bp deletion at nucleotides 475 and 476 of the *GF* genomic clone

[e] C → T nucleotide change at position 513 of the *GF* genomic clone

[f] Single A insertion following nucleotide 1,768 of the *GF* genomic clone

[g] 1,163 bp deletion between nucleotides 1,262 and 2,425 of the *GF* genomic clone

in each of the three varieties. The start of this deletion occurs within the second intron and removes the entire third exon and the coding region of the fourth exon and is therefore predicted to severely disrupt GF function (Fig. 1). The allele corresponding to this large deletion has been designated gf^5 (Table 1).

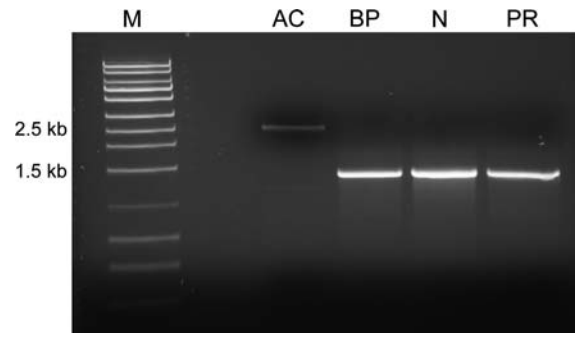

Fig. 2 Identification of a large deletion in *GF* from three stay-green heirloom varieties. PCR amplification of a 2.55 kb *GF* genomic clone from the AC cultivar together with amplification of a 1.4 kb fragment from the Black Prince (*BP*), Nyagous (*N*), and Purple Russian (*PR*) heirloom varieties. The 1.4 kb is the result of an internal 1,163 bp deletion in the stay-green varieties. Fragments were resolved through a 1% agarose gel together with a 1 kb DNA ladder (*M*)

Development of PCR based markers for detecting mutant alleles at the *gf* locus

Our genotyping data clearly indicates that four of the five alleles at the *gf* locus have been further propagated through selection, inadvertent crossing, and possibly targeted breeding (Table 1). The only exception to this appears to be the gf^2 allele that to date has only been identified in the Black Plum variety. In order to facilitate any future breeding strategies using these alleles, the potential to develop PCR based markers was explored. As shown above, the gf^5 allele is readily detectable by PCR amplification followed by agarose gel electrophoresis (Fig. 2). The sequences surrounding the mutated nucleotides in the other four *gf* alleles were examined using the CAPS Designer program (http://www.sgn.cornell.edu/tools/caps_designer/caps_input.pl). The single A → T nucleotide change in the *gf* allele was found to destroy an *Hpy188*I restriction enzyme site (TCNGA) that is present within the wild type allele. The utility of this polymorphism to design a CAPS marker was explored by amplifying the 166 bp fragment flanking the *gf* allele from AC and the *gf* mutant. Subsequent digestion of these PCR products with *Hpy188*I yielded fragments of 109 and 57 bp from the AC cultivar while the fragment amplified from the *gf* allele remained undigested (Fig. 3A).

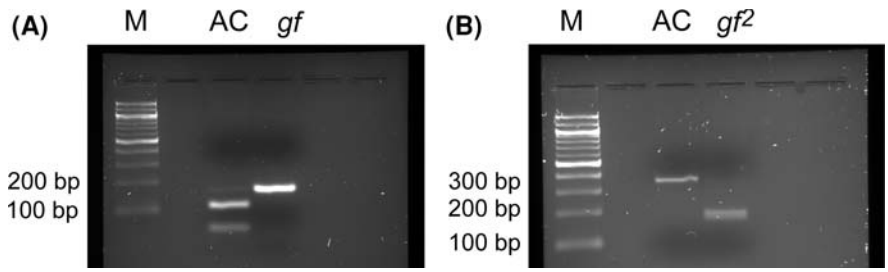

Fig. 3 Development of a CAPS marker to distinguish the *gf* and gf^2 mutant alleles. **a** A 166 bp DNA fragment spanning the *gf* allele was PCR amplified from *AC* (*GF/GF*) and LA3534 (*gf/gf*). Fragments were digested with *Hpy188*I which cuts once in the *AC* cultivar to yield fragments of 109 and 57 bp. **b** A 358 bp DNA fragment spanning the gf^2 allele was PCR amplified from *AC* (*GF/GF*) and the Black Plum (gf^2/gf^2) variety. Fragments were digested with *Mse*I which cuts once in the Black Plum variety to yield fragments of 187 and 171 bp. Fragments were resolved on 3% agarose gels together with a 100 bp DNA ladder (*M*)

The gf^2 allele found in the Black Plum variety is the result of the insertion of a single adenine following nucleotide 1,768 (Fig. 3b). This insertion creates an *Mse*I restriction site (TTAA) in varieties carrying the gf^2 allele that is not present in the wild type allele. A 358 bp fragment containing only the single *Mse*I site flanking the insertion site was amplified from AC and the Black Plum variety. Following digestion with *Mse*I two fragments of 187 and 171 bp were detected in the Black Plum sample whereas the AC allele remained undigested.

Discussion

An allelic series of mutations within a gene of interest represents an important tool for functional analysis. The *GF* gene encodes a novel protein that is required for chlorophyll degradation but contains no domains or motifs that are indicative of functional properties. The original *gf* mutant allele results in an amino acid substitution that converts an invariant arginine into a serine (Barry et al. 2008). This study was undertaken to determine whether the phenotypes of the stay-green heirloom varieties of tomato are the result of additional *gf* mutant alleles or novel stay-green mutants at unlinked loci. DNA sequence analysis of 26 heirloom varieties that display stay-green phenotypes in senescing leaves and ripening fruit revealed the identities of four new mutant alleles at the *gf* locus. However, unlike the *gf* mutant allele, all of the new alleles lead to truncated GF proteins as a result of either insertions (gf^2), deletions (gf^3 and gf^5) or the introduction of a premature stop codon (gf^4) (Fig. 1). Thus, all of the new alleles identified in this study are predicted to be null alleles that completely abolish GF function.

In general, the origin and pedigree of the brown fruited tomato varieties utilized in this study is poorly defined and the varieties analyzed in this study are likely to have originated through a combination of targeted breeding and adventitious pollination events. Furthermore, the lack of a curated germplasm collection increases the probability that seed sources may have become contaminated or the fidelity of variety names compromised over time. In addition, as these varieties are all commercially available, it is possible that their origin and histories may have been invented as a marketing tool. The data presented in this study, while not able to define the pedigree of these varieties, clearly identifies their relationship to one another with respect to fruit color and genotype at the *gf* locus. Prior to this study, only the *gf* mutant allele had been identified at the molecular level (Barry et al. 2008). The *gf* mutant allele was originally identified as a spontaneous mutant that arose in a commercial planting of the Philippine No. 2 variety and was backcrossed into the Ailsa Craig cultivar as part of a scientific program to introgress classical mutants into standard tomato varieties to enable meaningful scientific comparisons (Kerr 1956; Darby 1978). The genotyping data presented clearly shows that eight other varieties also carry the *gf* mutant allele, including Cherokee Purple and Cherokee Chocolate (Table 1). The relationship between these two varieties has previously been described with Cherokee Chocolate reported to have arisen as the result of a spontaneous mutation of Cherokee

Purple (Male 1999). However, the reported origin of Cherokee Purple as a variety of more than 100 years old originally grown by the Cherokee Indians is not supported by our genotyping data which indicates that this variety is more likely to be derived from the Philippine No. 2 variety. Similarly, the Ananas Noire variety that is reported to be from Belgium, likely has the same origin as other varieties carrying the *gf* allele.

The origin of the other *gf* alleles described in this study is less clear but reports from seed vendors and non-scientific literature suggest that a number of the varieties that carry these new *gf* alleles originated in Russia (Male 1999). Indeed, the genotyping data supports a common source of several of these varieties including Black from Tula, Black Krim and Black Pear, which are all reported to be of Russian origin and carry the 2 bp deletion characteristic of the gf^3 allele (Table 1). Similarly, Black Prince, Nyagous and Purple Russian are also reported to be of Russian origin and all three carry the 1,163 bp deletion that defines the gf^5 allele. Thus, the genotyping data would support the existence of at least two separate origins of these brown colored varieties within Russia. The only allele uncovered in this study that appears not to have been introduced into additional varieties is the gf^2 allele that was found solely in the Black Plum variety. Although we have uncovered four new alleles at the *gf* locus, our study is not comprehensive as additional brown-fruited varieties are available. It is therefore possible that other *gf* alleles await discovery or that the known alleles, including the gf^2 allele may be more widely distributed than this current study has revealed.

Many heirloom varieties of tomato and pepper exist that alter fruit color, size and shape. The origin of these varieties, together with their subsequent selection during domestication, is often unclear. Using the *GF* gene as a molecular tool, this study has investigated the relationship of 26 cultivated tomato varieties that display a stay green phenotype. Genotyping data indicates that mutations at the *gf* locus have arisen on at least five separate occasions and that these mutations have been subsequently dispersed into additional genetic backgrounds. This study clearly demonstrates the potential of cultivated varieties for identifying new alleles in genes of interest and provides new molecular markers for breeding these brown fruited tomato varieties.

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