A DNA test for routine prediction in breeding of peach blush, Ppe-R$_f$-SSR

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Abstract Blush, the proportion of red overcolor on the skin surface of fruit, is highly variable in peach breeding germplasm and is important in the marketing of peach fruit. The fresh market peach industry demands a high level of blush to entice consumers, while the processing peach industry requires minimal blush. Therefore, blush is a major selection criterion in breeding programs. The use of DNA-based information could improve breeding efficiency and accuracy for fruit blush coverage, but a predictive DNA test is required. The objective of this study was to develop a DNA test for the prediction of blush coverage by targeting the major locus, R_f, associated with blush variation. Initially, haplotypes were developed based on five SNP markers associated with variation in blush coverage. To convert the 5-SNP haplotype test into a single, simple PCR-based assay, 11 simple sequence repeat markers were designed and used to screen individuals representing all SNP haplotypes. The most informative assay, named Ppe-R$_f$-SSR, was chosen to screen 200 individuals of the RosBREED peach reference germplasm set that incorporated germplasm from four breeding programs. Ppe-R$_f$-SSR accurately differentiated individuals with high-, medium-, and low-blush coverage in most lineages. Outcomes highlighted that DNA tests can be quite predictive for some breeding programs or specific germplasm sets, while for others the predictiveness can falter. Therefore, the confirmation of genotype effects for any DNA test is recommended in new germplasm before routine use. The prediction accuracy and breeding utility of Ppe-R$_f$-SSR in the University of Arkansas breeding program were subsequently confirmed by screening 443 seedlings, independent of the initial DNA test development process, derived from 18 cross-combinations of 28 parents. Ppe-R$_f$-SSR can be used to efficiently and accurately predict fruit blush coverage, especially in fresh market germplasm, and has been deployed for routine use in the University of Arkansas peach breeding program.

Keywords Anthocyanin · Fruit skin color · Marker-assisted selection · *MYB10* · *Prunus persica*

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Introduction

The peach, *Prunus persica* L. Batsch, has been cultivated and enjoyed worldwide for centuries (Janick 2005), ranking in the top 10 in total world fruit production in 2013, with US annual production of almost one million tons (FAO 2015). The allure of the fruit and consumers' initial fruit purchase is based primarily on external

appearance and aroma (Olmstead et al. 2015). A high degree of blush, the red pigmentation on the fruit skin surface, entices consumers, and contributes to the flavor and nutritional quality of fruit because of the associated anthocyanin compounds (Frett et al. 2014; Rahim et al. 2014). For retailers, blush, along with size and firmness, have historically been the principal components of fruit quality (Crisosto and Costa 2008). Furthermore, skin color is an important factor in determining fresh market peach prices at the producer (Parker et al. 1991) and retail levels (Jordan et al. 1986). In the USA, the USDA has established specific standards for evaluating and differentiating quality grades of fresh market peaches and the proportion of fruit skin "blushed, pink or red" color is grade-defining; a minimum of one-third surface coverage is required for USA. Fancy and one-fourth surface coverage on 50% of fruit in any lot is required for US Extra No. 1, the two highest quality grades (USDA 2004).

The proportion of blush on fruit of standard peach breeding germplasm is independent of skin ground color (yellow or white) (Bassi and Monet 2008) and can range from negligible to complete coverage (Fig. 1). Although extreme variation is easily observed, standard fresh market cultivars produce fruit with a high proportion of blush coverage while the processing types produce fruit with minimal blush coverage. Processing peach breeders have targeted development of new cultivars with minimal blush coverage, as red pigmentation causes flesh staining and peeling problems during fruit processing (Gradziel and McCaa 2008). In contrast, US and European fresh market breeders have primarily focused on developing cultivars with considerable levels of blush (Scorza and Sherman 1996; Sansavini et al. 2006; Okie et al. 2008) because of the preference of European and US consumers for high (~80%) skin coverage (Byrne et al. 2012). However, even with this targeted focus, low blush levels, along with poor texture, low sweetness, and small size are likely responsible for the recent stagnancy in US fresh market peach consumption (Crisosto and Costa 2008;

Olmstead et al. 2015). Development of fresh market peach cultivars with elevated blush levels could enhance fruit appearance, flavor, and health properties, thereby encouraging increased peach consumption (Frett et al. 2014).

Early research (Blake 1932; Blake 1940; Weinberger 1944) studied the inheritance of blush, but no firm conclusions were made (Scorza and Sherman 1996). More recently, estimates of blush heritability have ranged dramatically, with de Souza et al. (1998) reporting a high heritability (~70%) only 12 years after Hansche (1986) reported the heritability to be three times lower. The greater genetic variability and improved evaluation and estimation methodology of de Souza et al. (1998) likely provided for a more accurate estimation, yet the inconsistencies in estimation and observed influence on blush development of environmental factors such as light exposure and nutrient availability (Crisosto and Costa 2008; Luchsinger et al. 2002; Byrne et al. 2012), uncontrolled in genetic studies, provide evidence for the proposed moderate heritability, and polygenic control of the trait (Byrne et al. 2012). Additionally, there are several known lineage-specific exceptions to the quantitative nature of blush, including the recessive complete blush coverage phenotype associated with the dominant allele of the "full red" (Fr) locus found in "UF Queen" and related University of Florida germplasm (Beckman and Sherman 2003) and the complete suppression of blush caused by the recessive allele of the "highlighter" (h) locus found in South American "criollo" and Italian germplasm (Beckman et al. 2005).

In standard US peach breeding germplasm, blush is a quantitative trait that is expressed during the final stage of fruit development and is maximized in high light environments (Bassi and Monet 2008; Frett et al. 2014). Since the early 2000s, at least seven independent studies have reported QTLs associated with peach blush (Yamamoto et al. 2001; Verde et al. 2002; Quilot et al. 2004; Yamamoto et al. 2005; Cantín et al. 2010; Eduardo et al. 2011; Frett et al. 2014). Although

Fig. 1 Variation in proportion of blush coverage observed within a single segregating family of the University of Arkansas peach breeding program

multiple QTLs were reported on linkage groups 2, 3, 4, 6, and 7, Frett et al. (2014) described a major effect QTL, labeled *Blush.Pp.ZC-3.1*, on peach chromosome 3 that explained up to 70% of blush phenotypic variance in an F_2 population of "Zin Dai" × "Crimson Lady." The candidate skin color gene, *PprMYB10* (Lin-Wang et al. 2010), was located within this QTL region, and its activation is likely the major physiological determinant of red skin color in peach (Ravaglia et al. 2013; Rahim et al. 2014; Zhou et al. 2014). The orthology of *PprMYB10* to *MdMYB1* and *MdMYB10* underlying the "red fruit" (R_f) locus of apple (*Malus × domestica* Borkh.), associated with red flesh and foliage as well as red skin overcolor (Takos et al. 2006; Chagné et al. 2007; Espley et al. 2007), and *PavMYB10* underlying the R_f locus of sweet cherry (*Prunus avium* L.), associated with red skin overcolor (Sooriyapathirana et al. 2010; Sandefur et al. 2016), provides strong evidence that extant genetic variation in *MYB1/10* is a major causal agent of observed fruit color phenotypic variation within many crops across the Rosaceae family (Arús et al. 2006; Lin-Wang et al. 2010). Therefore, R_f was adopted herein as the locus name, as this is the accepted name of the orthologous locus in apple and sweet cherry (Sandefur et al. 2016) to replace the QTL label of *Blush.Pp.ZC-3.1* from Frett et al. (2014).

Based on a single SNP (SNP_IGA_341962) located upstream to *PprMYB10*, a cleaved amplified polymorphic sequence (CAPS) marker, CAPS_341962, was developed for use in marker-assisted selection (Frett et al. 2014). The developed CAPS marker was shown to differentiate three levels of blush within the 93 progeny used in the initial QTL discovery, and the predictiveness of the marker was confirmed in modern peach germplasm (Gasic et al. 2014). However, CAPS markers are incompatible with multiplexing and genotyping with CAPS markers requires additional steps (restriction enzyme digestion and incubation) and higher quality DNA extracts than do simple PCR markers, which can limit their use for routine breeding applications (Ramkumar et al. 2015). Nonetheless, this work showed that the R_f locus containing *PprMYB10* is a promising target for the development of a DNA test for the prediction of peach fruit skin blush coverage.

Over recent decades, peach breeders in the USA and internationally have released approximately 100 new cultivars annually (Sansavini et al. 2006; Byrne et al. 2012). While many of these cultivars have been widely adopted, traditional breeding is challenged by long generation cycles, large land requirements, and intensive phenotyping (Scorza and Sherman 1996). For most breeding programs, generation of new seedling families is easy, but their management, evaluation, and selection limit cultivar development (Byrne et al. 2012). In addition, establishing a parent's genetic potential to produce high- or low-blush offspring has traditionally required progeny testing (Byrne et al. 2012), which is highly resource intensive because of the protracted juvenility period and associated phenotyping. The addition of DNA-based information to traditional breeding operations (i.e., marker-assisted selection) in horticultural crops has been discussed since the early 1990s as a means to overcome such obstacles. However, the lack of DNA tests for breeding-relevant traits (Byrne 2007), lack of understanding of the technology (Bliss 2010), and the perceived high cost of genotyping (Luby and Shaw 2001; Edge-Garza et al. 2015) have limited its broad application in horticultural breeding programs (Ru et al. 2015). One of the major limitations is the conceptual gap between genomics researchers and plant breeders, resulting in a failure to ensure that QTL information is converted into robust DNA tests (Collard and Mackill 2008; Iezzoni et al. 2010; Ru et al. 2015). DNA tests have been developed for several peach breeding-relevant traits such as pubescence (Vendramin et al. 2014), flat shape (Picañol et al. 2013), slow ripening (Eduardo et al. 2015), flesh color (Falchi et al. 2013), texture (Peace and Norelli 2009), and acidity (Eduardo et al. 2014), and the development and deployment of DNA tests for skin blush in particular has been successful in apple (Yuan et al. 2014) and sweet cherry (Sandefur et al. 2016).

A DNA test to predict peach fruit skin blush coverage could eliminate the need for extensive progeny testing and improve selection efficiency of this breeding-relevant trait. For wide use in breeding, such a genetic test must be simple to deploy and reliably predictive across diverse germplasm. The objective of this study was to develop a DNA test targeting the R_f locus that can be used routinely in peach breeding for the prediction of fruit skin blush coverage.

Materials and methods

Germplasm

Three sets of germplasm were used to develop a DNA test to predict peach fruit blush coverage (Fig. 2 and below).
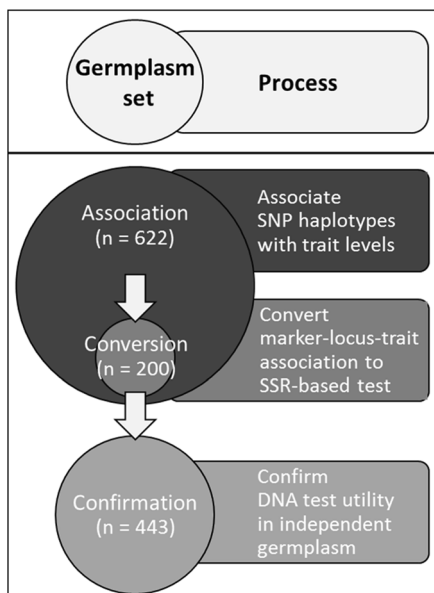
Fig. 2 Experimental approach to develop a trait-predictive, locus-specific DNA test for routine breeding use. A three-step process established an association with SNP markers, converted the association to a single PCR assay, and then confirmed the DNA test's utility in a target breeding program

Association set The germplasm phenotyped and SNP-genotyped (*n* = 622) and used to establish the SNP haplotype-blush coverage association, termed the "Association set," was part of the RosBREED US Peach Crop Reference (CR) Set (*n* = 308) and Breeding Pedigree (BP) Sets (*n* = 314) that used material from the University of Arkansas, University of California—Davis, Clemson University, and Texas A&M University peach breeding programs (Fig. 2; Online Resource 1). The sets were originally chosen to contain a diversity of important cultivars, ancestors, founders, and progeny representative of North American breeding germplasm (Peace et al. 2014). In total, 175 germplasm individuals (50 from the CR Set and 125 from BP Sets) were historically selected for commercial performance, while 447 (258 from the CR Set and 189 from BP Sets) were unselected seedlings in numerous small bi-parental families (8–63 seedlings per family). BP Set individuals were evaluated along with other germplasm but are not specified because this germplasm is privately held. Individuals were maintained as described by Frett et al. (2012).

Conversion set A subset, termed "Conversion set" (*n* = 200), of the Association set (*n* = 114 CR Set individuals and *n* = 86 individuals of the BP Sets) for which DNA extracts from the original SNP genotyping (Verde et al. 2012) were available and that represented all haplotypes observed was used for marker conversion (Fig. 2; Online Resource 2). The conversion set consisted of 35 germplasm individuals (23 from the CR Set and 12 from BP Sets) historically selected for commercial performance and 165 (91 from the CR Set and 74 from BP Sets) unselected seedlings in numerous small bi-parental families.

Confirmation set To confirm allele effects in the target breeding program, a different germplasm set was used. This "Confirmation set" (Fig. 2) comprised 443 seedlings from 18 cross-combinations among 28 parents made in 2010 at the University of Arkansas Fruit Research Station, Clarksville, AR.

Phenotypic data

All germplasm sets were phenotyped following the protocol of Frett et al. (2012). Blush coverage was determined by visual estimation on a 0–5 scale (0 = no blush, 1 = 1–20%, 2 = 21–50%, 3 = 51–80%, 4 = 81–99%, 5 = 100%) on each of five fruits, with the average per individual recorded. Phenotypic data were collected on the Association set (including the Conversion set) in 2010 and 2011. However, only 46% (*n* = 143) of individuals phenotyped in 2011 (*n* = 308) were phenotyped in 2010 and additional individuals were phenotyped in 2011. Because of the high congruence between 2010 and 2011 data (r^2 = 0.78) considering the expected heritability of the trait (~70%; de Souza et al. 1998) and the substantially greater amount of data available in 2011, only phenotypic data collected in 2011 (Online Resource 1) was used in this study. The Confirmation set was independently phenotyped in 2014.

To facilitate later estimation of the general effects of SNP haplotypes, SNP diplotypes, SSR alleles, and SSR genotypes on blush coverage (below), the original 0–5 rating scale was converted into a 1–3 scale: 1 = 0–20% coverage (scores of 0 and 1 on the previous 0–5 scale), 2 = 21–80% coverage (scores of 2 and 3 on 0–5 scale), 3 = 81–100% coverage (scores of 4 and 5 on 0–5 scale).

Association

SNP haplotype construction All association set individuals were genotyped using the International Peach SNP Consortium 9 K SNP array v1 (Verde et al. 2012). SNP

array data was used to confirm the parentage of the Association set families (L. Antanaviciute, L. Cai, and C. Peace, manuscript in preparation). Subsequently, five SNP markers were chosen for haplotype construction because they flanked three peach *MYB10* homologs, *MYB10.1* (ppa026640m; *PprMYB10* candidate gene), *MYB10.2* (ppa016711m), and *MYB10.3* (ppa020385) (Zhou et al. 2014) (Fig. 3; Online Resource 1). The *MYB10* homologs and the five SNPs were located in the *P. persica* Whole Genome Sequence v1.0 (Verde et al. 2013) using the GBrowse tool on the Genome Database for Rosaceae (Jung et al. 2014; www.rosaceae. org). For all individuals, haplotypes were initially constructed using FlexQTL™ software (Bink et al. 2008) and subsequently confirmed based on inheritance analysis (allelic flow through pedigreed individuals) in the association set. A chi-squared test of independence was performed to examine the relation between the 5-SNP haplotypes and proportion of blush coverage and between the 5-SNP diplotypes and proportion of blush coverage ($P < 0.05$).

Haplotype and diplotype effects The general effect of each haplotype and diplotype was expressed as "high"

(H) (mode of 3 on 1–3 phenotypic scale), "medium" (M) (mode of 2 on 1–3 phenotypic scale), or "low" (L) (mode of 1 on 1–3 phenotypic scale). As each individual carried two haplotypes, each haplotype's general effect was influenced by the frequency of the specific diplotype observed. Therefore, when a haplotype was principally observed in combination with just one or a few others, this was noted and the likely impact on the blush association was reported. Diplotype effects were calculated using the same method described above to provide an estimate of the associated effect of paired haplotypes.

The non-parametric Kruskal-Wallis test with post hoc Kruskalmc multiple comparisons test (Giraudoux 2015) ($P < 0.05$) was used to evaluate differences among haplotypes and diplotypes for their proportion of blush coverage, with all analyses conducted using a modified R script (R Core Team 2013). Six seedlings from University of California—Davis and one from Clemson University were not included in the analyses because of the DNA source used for SSR genotyping not matching that used for SNP haplotyping based on observations of SSR alleles not matching parentage.

Conversion

Candidate assays The five SNP markers were converted to a single PCR-based simple sequence repeat (SSR) marker assay that best captured the desired allelic information by the following process.

Using the BatchPrimer3 SSR screening tool (You et al. 2008; http://probes.pw.usda.gov/batchprimer3/), microsatellite motifs were detected in the ±100 kb flanking sequence around peach *MYB10.1* in the *P. persica* Whole Genome Sequence v1.0 (Verde et al. 2013) accessed via the Genome Database for Rosaceae (Jung et al. 2014). As 90 microsatellites were detected within this interval, only the 11 microsatellites that were closest to the *MYB10* homologs had the largest number of tandem repeats and were well-spaced throughout the 200 kb region were targeted. Primer design followed criteria described by Stegmeir et al. (2014) using Primer3Plus software (Untergasser et al. 2012; www.primer3 plus.com). All primer pairs were verified for specific amplification in only the genomic region of the R_f locus using the NCBI BLAST software on the *Prunus persica* Whole Genome Sequence v1.0 (www.rosaceae. org/tools/ncbi_blast). Suitable primer pairs were considered candidate assays (Online Resource 3).
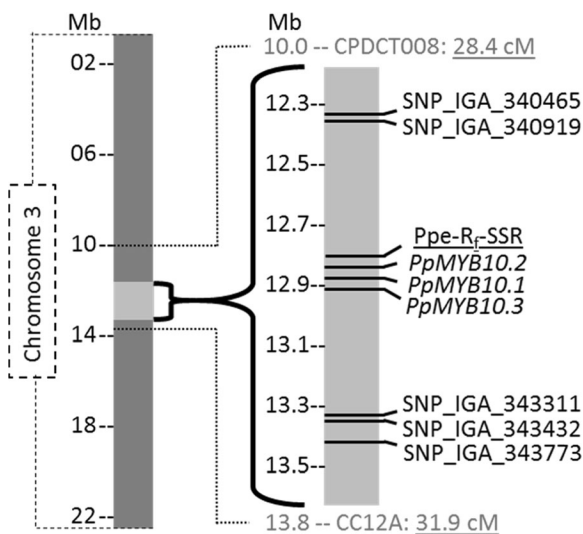

Fig. 3 Graphical representation of *Prunus persica* L. Batsch chromosome 3 with the enlarged location of the ~700 kb flanking the three *PpMYB10* transcription factors, including the 5-SNP markers used for haplotyping and Ppe-R$_f$-SSR. Physical locations were sourced from the *Prunus persica* Whole Genome Sequence v1.0 (Verde et al. 2013) housed on the Genome Database for Rosaceae (Jung et al. 2014). Two flanking markers and their estimated genetic positions based on the *Prunus*-TE-F$_2$ reference map are included in gray font

For candidate assays, PCRs were performed in a final mix total volume of 10.0 μl containing 1 μl DNA (~20 ng μl^{-1}), 5.70 μl of autoclaved double-distilled water, 2.0 μl Taq PCR buffer [×5; GoTaq® buffer (Promega Corp., Madison, WI)], 0.20 μl deoxyribonucleotide triphosphates (dNTP) (10 mmol^{-1} for each dNTP), 0.50 μl of each forward and reverse primer (10 mmol^{-1}; Integrated DNA Technologies, Coralville, IA), and 0.10 μl Taq polymerase [5 U μl^{-1}; GoTaq® (Promega Corp., Madison, WI)]. PCR conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 60 °C for 45 s, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 7 min.

Visualization of PCR results for candidate assays was completed with fragment separation on denaturing gels containing 4% acrylamide and 7.5 M urea in 1× TBE buffer run on a 50-cm Sequi-Gen GT system (BioRad, Hercules, CA) for ~2 h at 85 W. Gels were stained with Promega Silver Sequence™ Staining System (Promega Corp., Madison, WI) as per manufacturer's instructions. Amplified fragments from each primer pair for 20 individuals representing the most common SNP haplotypes were compared, and primer pairs producing fragments matching SNP haplotype patterns were shortlisted for subsequent use on a high-throughput platform to check suitability for routine breeding use. Visualization of shortlisted assay outcomes and the chosen candidate DNA test were completed with fragment separation on an ABI Prism 3730xl DNA Analyzer (Applied Biosystems by Life Technologies, Grand Island, NY) and size calling with GeneMarker® software (SoftGenetics, LLC, State College, PA). An internal size standard (Integrated DNA Technologies, Coralville, IA) and control samples with known allele sizes were used to ensure accurate size-calling.

Chosen assay The Ppe-R$_f$-SSR DNA test (SSR#2) that was subsequently developed had a forward primer sequence of 5′ CGG ACA ATA TTC TTG TGA TGC 3′ (melting temperature 58.1 °C and GC content 43%) and a reverse primer sequence of 5′ GAT CTG AGC CAT TAC ACA GTG C 3′ (melting temperature 59.8 °C and GC content 50%).

DNA test genotypic effects A chi-squared test of independence was performed to examine the relation between the Ppe-R$_f$-SSR alleles and genotypes and the proportion of blush coverage ($P < 0.05$). To represent the general effect of each allele and genotype, an

associated phenotype was calculated using the same method as that described above for calculating SNP haplotype and diplotype general effects. The non-parametric Kruskal-Wallis test with post hoc Kruskalmc multiple comparisons test (Giraudoux 2015) ($P < 0.05$) was used to evaluate phenotypic differences between Ppe-R$_f$-SSR alleles and genotypes, with all analyses conducted using a modified R script (R Core Team 2013).

Confirmation

To confirm the genotypic effects of the DNA test developed in the target breeding program, the Confirmation set was screened with Ppe-R$_f$-SSR. DNA extraction was conducted for the "Confirmation set" ($n = 443$) according to the method of Edge-Garza et al. (2014).

Visualization of the PCR outcomes and calculation of DNA test genotypic effects were conducted as described above for the conversion process.

Results

Association

Phenotypic variation Individuals with extreme phenotypes in the Association set ($n = 622$) were rare: only 4% of germplasm ($n = 25$) had no blush (score of 0) and only 2% ($n = 12$) had complete blush coverage (score of 5). Germplasm with no blush consisted of two selections and 23 unselected seedlings from the CA breeding program and germplasm with complete blush coverage were all unselected seedlings from either the AR or TX breeding programs. Frequencies of intermediately blushed individuals (scores of 1 to 4) were higher (1 = 28%, 2 = 16%, 3 = 30%, 4 = 20%). The mode of the Association set was 3, indicating the germplasm set was slightly skewed toward higher proportions of blush coverage. The 1–3 scaling classified 32% of individuals as low (0–20% coverage), 46% as medium (21–80% coverage), and 21% as high (81–100% coverage).

SNP haplotypes Among the 622 individuals of the Association set, 11 unique haplotypes were observed, with frequencies ranging from 2% (for H1 and H8) to 37% (for H7) (Table 1; Online Resource 1). In germplasm having undergone historical selection for commercial performance (cultivars and selections), haplotype

Table 1 Unique 5-SNP haplotypes associated with proportion of blush coverage within the Association set, including their frequencies, ancestral sources, associated phenotypes, and Ppe-Rf-SSR alleles

| 5-SNP haplotype | | No. of haplotype observed[a] | Frequency[b] (%) | | | | | | Phenotype associated[c] | | | No. of sources detected[d] | No. of sources investigated | No. of individuals observed[e] | SSR alleles associated |
Haplotype	Label		Total	Sel	AR	CA	SC	TX	mode	mean ± SD	1–3 scale				
BBBBB	H1	25	2	5	0	6	0	0	1	0.96 ± 0.45	L	6	3	5	395, 399, 401
ABABB	H2	38	3	1	0	3	5	0	1	1.11 ± 1.03	L	3	1	13	401
BAABB	H3	167	13	19	0	40	0	0	1	1.29 ± 0.67	L	20	12	85	399, 401
AAABB	H4	39	3	7	7	6	0	0	1	2.11 ± 1.17	M	10	6	21	399
BBBAB	H5	59	5	7	0	11	3	0	1	1.19 ± 0.83	L	9	4	5	399, 401
ABBAB	H6	86	7	8	0	14	6	0	1	1.39 ± 0.88	L	11	6	17	399, 401
BABAB	H7	459	37	24	53	6	62	24	3	3.03 ± 1.03	M	33	27	133	395, 397
AABAB	H8	26	2	2	2	0	4	1	2	1.88 ± 1.31	M	5	1	4	399
BBABA	H9	46	4	7	16	0	3	0	3	3.36 ± 0.61	M	5	4	21	395, 397
ABABA	H10	92	7	7	20	8	2	6	1	2.29 ± 1.35	M	10	8	38	395, 397
AAABA	H11	207	17	13	1	4	14	69	3	2.95 ± 0.89	M	22	16	58	395, 397

[a] Number of times each haplotype was observed in the Association set (n = 622)

[b] Sel = cultivars and selections, AR = University of Arkansas, CA = University of California–Davis, SC = Clemson University, TX = Texas A&M University (TX)

[c] SD = standard deviation; mode, mean, and SD using 0–5 phenotyping scale (0 = 0%, 1 = 1–20%, 2 = 21–50%, 3 = 51–80%, 4 = 81–99%, 5 = 100% proportion of blush coverage); 1–3 scale L = mode of 1, M = mode of 2, H = mode of 3

[d] Number of earliest pedigree-traceable individuals with SNP haplotype; only CR Set source cultivars listed; sources listed in Online Resource 4

[e] Total number of individuals observed with the CR Set haplotype sources investigated

frequencies ranged from 1% (for H2) to 24% (for H7). In unselected germplasm (seedlings), haplotype frequencies ranged from 2% (for H1) to 39% (for H7). All haplotypes, including the rarest (H2), were derived from more than one pedigree-traceable cultivar or selection (Online Resource 4).

Haplotypes H1, H2, H3, H5, and H6 were associated with low blush (mode of 1 on 1–3 scale), haplotypes H4, H7, H8, H9, H10, and H11 were associated with medium blush (mode of 2 on 1–3 scale), and no haplotypes were associated with high blush (mode of 3 on 1–3 scale) (Table 1). The above low blush haplotypes, when grouped, had a mean blush level of 1.3 ± 0.8 and mode of 1 while the medium blush haplotype group had a mean blush level of 2.9 ± 1.1 and mode of 3. The low-blush haplotypes H1, H3, and H5 had a significantly lower blush coverage than each of the medium-blush haplotypes; however, haplotypes H2 and H6 were not significantly different from H4, possibly owing to H4 being paired with an allele associated with low blush in approximately 70% of individuals. The medium-blush haplotypes H7, H9, H10, and H11 had a significantly higher blush coverage than each of the low-blush haplotypes. Although haplotypes H8 and H10 were associated with medium blush, they were not significantly different from the assumption of allele-proportion of blush independence (chi-square; $P < 0.05$). However, haplotype H10 had a significantly higher proportion of blush coverage than each of the low-blush haplotypes and haplotype H8 had a significantly higher proportion of blush coverage than each of the low-blush haplotypes except for H2 and H6. For H10, approximately half of the sources ["Carson," "China Pearl," "Greensboro," and a single breeding program selection (D62-193)] were associated with low blush, while the other sources, which included several University of Arkansas and Texas A&M selections, were associated with medium blush. The only other haplotype to be inconsistently associated with a simplified blush phenotype was H7, where all but a single source, a *P. persica × Prunus davidiana* hybrid (P × D), were associated with medium blush, whereas P × D was associated with low blush. However, due to the low frequency of the P × D source of the H7 haplotype, this haplotype had a significantly higher blush coverage than each of the low blush haplotypes.

SNP diplotypes In total, 38 of 66 possible diplotypes were observed in the Association set (Online Resource 5). Diplotype frequencies ranged from <1% (for 13 diplotypes) to 21% (for H7 | H7), with a mean of 2.6%. For all common L | L diplotypes (which consisted of two low-blush haplotypes, H1, H2, H3, H5, and H6 on the 1–3 scale), represented by a minimum of six individuals, their association with a low proportion of blush coverage matched the expected association based on the haplotype combination. All remaining diplotypes (L | M and M | M), except for H3 | H10 and H5 | H10, were associated with a medium blush level. When averaged, the L | M haplotype combinations had a higher frequency of individuals scored as 1 than as 3 and the M | M haplotype combinations had a higher frequency of individuals scored as 3 than as 1 (Online Resource 5). The three diplotype groups, L | L, L | M, and M | M, each had significantly different levels of blush coverage. As 95% of H3 | H10 and 90% of H5 | H10 individuals' H10 haplotypes came from the low-blush sources of the haplotype ('China Pearl' and D62-193) instead of the medium-blush sources, the H3 | H10 and H5 | H10 diplotypes were associated with a low proportion of blush coverage. The only L | M diplotypes significantly different from M | M diplotypes were those where the M haplotype was H4. In all other cases, only L | L and M | M diplotypes were significantly different. When all diplotypes were grouped using the 1–3 scale as L | L, L | M, or M | M and H10 was considered L or M depending on haplotype source, M | M had an average blush level of 3.3 ± 0.8 and mode of 3, L | M had an average of 2.2 ± 1.0 and mode of 2, and L | L had an average of 1.1 ± 0.6 and a mode of 1 based on the original 0–5 rating scale.

Conversion

Candidate assays A total of 24 di-nucleotide microsatellite motifs, each with at least 10 repeats, and 15 tri-nucleotide motifs and nine tetra-nucleotide motifs, each with at least four repeats, were detected in the ±100 kb flanking sequence around peach *MYB10.1*. The di-nucleotide microsatellites contained up to 26 repeats, whereas the tri- and tetra-nucleotide microsatellites each contained only four repeats. Eleven di-nucleotide microsatellites were subsequently chosen as targets for primer design (Online Resource 3). After screening of the 20 of individuals representing the most common SNP haplotypes, six of these 11 candidate assays for R_f (#2, #3, #4, #6, #7, and #9; Online Resource 3) were deemed polymorphic. Of these, only

SSR#2, located 36 kb upstream of peach *MYB10.2* and 76 kb upstream of peach *MYB10.1*, was able to clearly differentiate the two haplotype groups of medium and low proportion of blush coverage based on the 1–3 scale. This SSR was chosen for additional evaluation and subsequently named Ppe-R$_f$-SSR.

Ppe-R$_f$-SSR alleles and effects Ppe-R$_f$-SSR (SSR#2) was observed to have four unique SSR alleles (395, 397, 399, and 401 bp in length; Fig. 4) within the 200 Conversion set individuals screened (Table 1; Online Resource 2). A fifth allele, 403, was observed only in one University of Arkansas breeding program selection and 31 seedlings derived from this selection in the Confirmation set. Among the 200 individuals of the Conversion set, allele frequency in germplasm having undergone historical selection for commercial performance (*n* = 70) ranged from 7% (for 397) to 56% (for 395) (Online Resource 6). In unselected germplasm, the trend was similar, with 10% of alleles being 397 and 52% being 395.

The majority, four of five (H2, H3, H5, and H6), of the 5-SNP haplotypes associated with low blush coverage on the 1–3 simplified phenotype scale were represented by the 399 or 401 Ppe-R$_f$-SSR alleles, while most, four of the five (H7, H9, H10, and H11), haplotypes associated with medium blush coverage on the same scale were represented by the 395 or 397 allele in the Conversion set (Table 1). All sources of the H1 allele were represented by the 399 or 401 allele except for P × D which was represented by the 395 allele. All individuals with the H10 and H7 haplotypes including those from the low-blush sources had the 395 or 397 allele. Both the H4 and H8 alleles, which were associated with medium blush on the 1–3 scale, were represented by the 399 allele. All sources of the H1, H4, and H9 haplotypes were able to be investigated for association with Ppe-R$_f$-SSR alleles. One source of the H2, H3, and H8 haplotypes, two sources of the H10 and H11 haplotypes, three sources of the H5 and H6 haplotypes, and five sources of the H7 haplotype were not robustly tested because of access to too few individuals that carried these haplotypes. Although these sources of H2, H3, H5, and H6 were not tested, the other sources of these haplotypes were observed to be associated with the 399 or 401 allele. Similarly, the untested sources of H7, H10, and H11 were likely associated with the 395 or 397 as all other sources of these haplotypes were associated with these two alleles (Table 1).

Among the 200 individuals of the Conversion set, alleles 395 and 397 were associated with a medium proportion of blush coverage (mode of 2 on 1–3 scale)
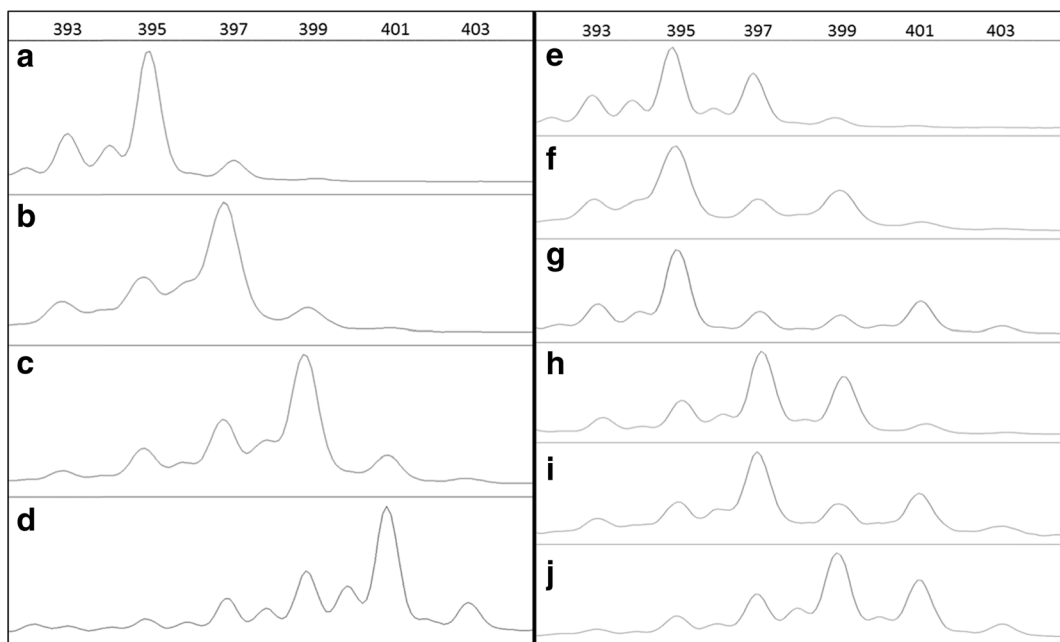

Fig. 4 High-throughput ABI output of Ppe-R$_f$-SSR genotypes: **a** 395 | 395, **b** 397 | 397, **c** 399 | 399, **d** 401 | 401, **e** 395 | 397, **f** 395 | 399, **g** 395 | 401, **h** 397 | 399, **i** 397 | 401, **j** 399 | 401. *Numbers* (e.g., 395) represent base pairs with the height of each peak relative to its fluorescence intensity

and alleles 399 and 401 were associated with a low proportion of blush coverage (mode of 1 on 1–3 scale) (Fig. 5a; Online Resource 6). The 395 allele had a significantly higher proportion of blush coverage than the 397 allele, and both 395 and 397 had a significantly higher proportion of blush coverage than the 399 and 401 alleles, which were not significantly different. When 395 and 397 were grouped as medium blush and 399 and 401 were grouped as low blush, the two allele groups were significantly different, with the mean blush level of the medium group being 3.1 ± 1.1 with mode of 3 and the mean blush level of the low group being 1.3 ± 0.8 with a mode of 1 (Online Resource 6). Although the 397 allele was observed to have a medium proportion of blush coverage (on 1–3 scale), it was not significantly different from the assumption of allele-proportion of blush independence (chi-square; $P < 0.05$) when considering all individual carrying it. When the source of the 397 allele was considered, two of the sources, "Carson" and D62-193, were associated with a low level of blush coverage, while all other sources were associated with a medium blush coverage (on 1–3 scale). The individuals with the "Carson" or D62-193 source of 397 were observed to have the form of the H10 allele associated with low blush. When these individuals were removed from the analysis, the frequency of individuals carrying the 397 allele and scored

as 1 (on 1–3 scale) decreased from 34 to 0%, and 397 no longer had a significantly lower blush coverage than 395. The only other allele to be inconsistently associated with a simplified blush phenotype was 395, where all but a single source, P × D, were associated with a medium level of blush coverage. The individuals with the P × D source of 395 all carried the H1 allele that was associated with the low-blush phenotype. The medium-blush allele group, on average and with the inconsistent sources accounted for, had a blush rating, on the 0–5 scale, 2.5 times greater than the average of the low-blush allele group (Online Resource 6).

When considering the 1–3 scale, the phenotypic effects of Ppe-R_f-SSR genotypes matched those expected based on additivity of allele effects, although the 395 | 397 and 397 | 399 genotypes had a higher than expected frequency of individuals scored as low blush (Fig. 5b). Of the individuals scored as 395 | 397, 25% had one or two alleles from the P × D source of 395 or the "Carson" and D62-193 source of 397, whereas 92% of individuals scored as 397 | 399 had 397 from either "Carson" or D62-193. When these sources were considered as different alleles, the mean of the 395 | 397 group increased from 2.5 to 3.1 on the 0–5 scale and the single 397 | 399 individual was rated as a 4 on the 0–5 scale in comparison to the 11 397b | 399 individuals that had a mean of 1.2, much more aligned with the other L | L allele
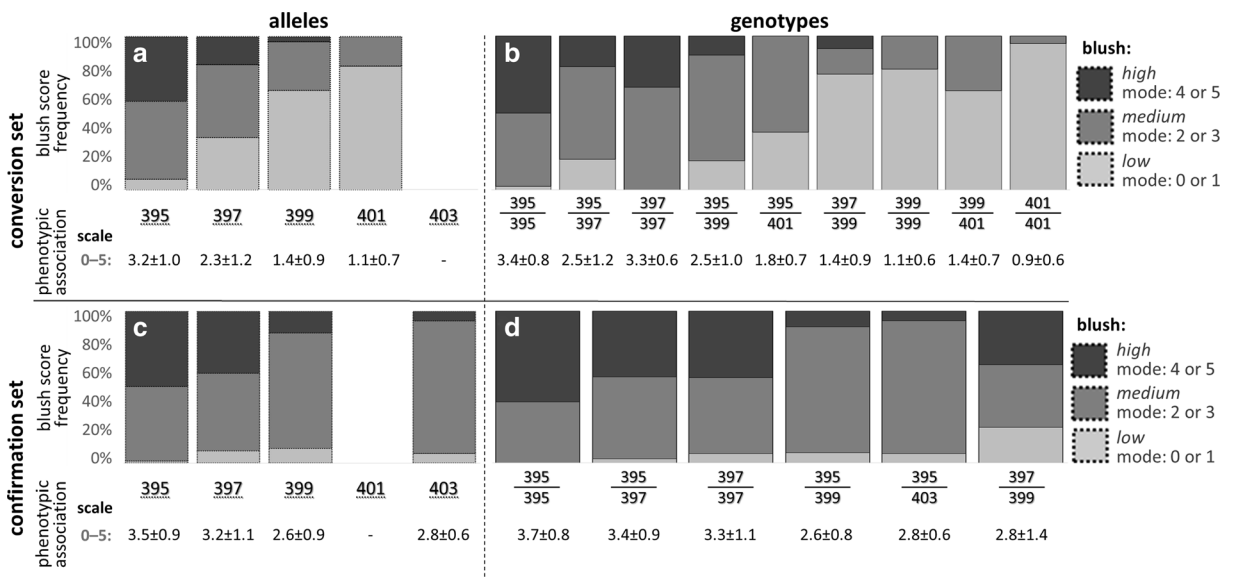

Fig. 5 Proportion of individuals rated as low (0–1), medium (2–3), or high (4–5) blush coverage, and 0–5 scale mean ± standard deviation for each Ppe-R_f-SSR allele (**a** and **c**) and genotype (**b** and **d**) within the Conversion (**a** and **b**; $n = 200$) and Confirmation (**c** and **d**; $n = 443$) sets. Individuals recorded with a 395 or 397 allele but with the low-blush source of the allele were not removed to mimic a situation where haplotype and pedigree information were unknown

combination groups. With this change, the mean of the generalized M | M group increased by 0.15, L | M increased by 0.34, and L | L decreased by 0.02, and the standard deviation of each group decreased (Online Resource 7). With and without the discrepant forms of 395 and 397 removed, the L | L, L | M, and M | M genotype groups had significantly different proportionate levels of blush coverage.

Confirmation

From the screening of the Confirmation set ($n = 443$) with Ppe-R_f-SSR, allele frequency ranged from 4% (for 403) to 75% (for 395) (Online Resource 8). The 403 allele was not detected in the Conversion set and was only observed in a single University of Arkansas peach selection and 32 seedlings derived from this selection. More than half of all seedlings had the 395 | 395 genotype which was associated with a high blush coverage having a mean of 3.7 and a mode of 4 (Fig. 5d). No seedlings had genotypes of 399 | 399, 399 | 403, or 403 | 403. The two alleles associated with a medium degree of blush coverage in the Conversion set, 395 and 397, were also associated with a medium (1–3 scale) level in the Confirmation set (Fig. 5c). The 399 allele, associated with a low degree of blush coverage in the Conversion set, was associated with a medium (1–3 scale) level in the Confirmation set, but 399 was always paired with either 395 or 397 in the Confirmation set, thereby skewing the effect of this allele toward a higher level of blush coverage (Fig. 5c; Online Resource 8). The 403 allele had a similar effect to the 399 allele, and although it was associated with a medium (1–3 scale) proportion of blush coverage, it was always paired with the 395 allele. Both the 399 and 403 alleles increased the frequency of individuals scored as low blush coverage when paired with 395 or 397, in contrast to 395 | 395, 395 | 397, and 397 | 397 (Fig. 5d; Online Resource 8). Using the 1–3 scale, the 395 and 397 alleles had a significantly higher blush coverage than the 399 and 403 alleles. When the alleles associated with medium blush (395 with 397) were grouped (M) and those associated with a lower degree of blush were grouped (L) (399 with 403), the medium-blush group had a mean blush level of 3.4 ± 0.9, 0.7 higher than the low-blush group (2.7 ± 0.8), with the groups being significantly different. On average, the M | M genotypic group had a mean blush level of 3.6 ± 0.8, 0.9 higher than the L | M group (2.7 ± 0.8) (Fig. 5d; Online Resource 8), with the

M | M group being significantly higher than L | M on all phenotypic scales.

Discussion

A trait-predictive, locus-specific DNA test for peach fruit skin blush coverage, Ppe-R_f-SSR, was successfully developed by converting the haplotypic information of five SNPs at the R_f locus into a single PCR-based assay. Ppe-R_f-SSR was able to differentiate alleles associated with low, medium, and high blush coverage in a diverse set of individuals representing North American breeding germplasm and was confirmed to differentiate breeding-relevant blush levels in University of Arkansas peach breeding germplasm. The quantitative nature of peach skin blush was confirmed and an additive genetic effect detected, as in most cases individuals carrying two high-blush alleles had a significantly higher proportion of blush coverage than those carrying only a single high-blush allele. Three particular germplasm sources, P × D, "Carson," and D62–193, introduced ambiguity into the DNA test's predictions. However, when parentage from these three sources was considered, Ppe-R_f-SSR was completely predictive of peach fruit skin blush coverage determined by the R_f locus.

The association of SNP haplotypes and SSR alleles with low, medium, and high levels of blush coverage observed within this study validated the utility in peach breeding germplasm of the major effect fruit skin color QTL that Frett et al. (2014) previously identified in this region. Many sources for each SNP haplotype were observed (3–33); however, many of the sources of the same haplotype likely trace to identical founding cultivars, although this hypothesis cannot easily be tested because of limited pedigree records that are often inaccurate owing to pollen contamination, labeling, and record-keeping errors (Doerksen and Herbinger 2010; Munoz et al. 2014). Because the phenotypic variance observed for proportion of blush coverage was greater than that explained by the R_f locus alone, other loci associated with fruit skin blush coverage might be responsible for the within-group differences observed within the germplasm studied here. Such loci could be those proposed by Cantín et al. (2010), Eduardo et al. (2011), and Frett et al. (2014), with a major locus reported on chromosome 4 and several minor loci reported on chromosomes 6 and 7. This hypothesis could be tested with experimental controls to minimize

environmental effects (e.g., differential exposure of developing fruit to sunlight) on blush development.

The 5-SNP haplotype and Ppe-R$_f$-SSR allele distributions within representative germplasm of four breeding programs used in this study reflects the target market of each program. Within the University of Arkansas, Clemson University, and Texas A&M University germplasm, Ppe-R$_f$-SSR alleles associated with a medium and high blush represented approximately 93% of alleles detected, consistent with their fresh market target. In contrast, 75% of alleles within the University of California—Davis program's germplasm were low-blush alleles. This breeding program is the only US program focused on developing cultivars for the processing peach market, for which cultivars with very little blush coverage are desired. The large proportion of market-targeting alleles in each breeding program is consistent with directional selection for favorable skin color alleles and/or minimal use of new parents with unfavorable alleles.

Ppe-R$_f$-SSR can determine an individual's zygosity at the R_f locus, differentiating those with the genetic potential to produce fruit with a low or high proportion of blush coverage. By selecting for homozygous high blush or heterozygous genotypes, high or medium blush coverage, respectively, could be targeted in breeding. With the CAPS marker developed by Frett et al. (2014), only three genotypes are possible to be distinguished, in contrast to the nine genotypes for Ppe-R$_f$-SSR. While predictiveness of the CAPS marker was confirmed in 69 peach cultivars (Gasic et al. 2014), Ppe-R$_f$-SSR was confirmed in 200 individuals representative of the North American peach breeding germplasm as well as more than 400 seedlings from 18 cross-combinations of 28 important parents of the University of Arkansas peach breeding program. Although CAPS marker alleles can be separated using regular agarose electrophoresis (Gasic et al. 2014), they cannot be readily multiplexed, reducing their utility for routine application in breeding programs. Therefore, compared to the previously available CAPS marker, the new DNA test, Ppe-R$_f$-SSR, is a robust genetic screening tool for peach skin blush coverage that is more technically amenable to routine breeding use and provides greater phenotypic resolution. Additionally, by establishing the SNP-based haplotypes and their association with fruit skin blush coverage across a diverse germplasm set, the use of the SNP set spanning the R_f locus could have broad applicability in peach breeding programs where SNP-based genotyping is preferred over PCR-based SSR assays.

The single source of the 395 bp Ppe-R$_f$-SSR allele that was associated with a low degree of blush coverage instead of a medium degree of blush coverage, P × D, was unique among the germplasm evaluated as it is an interspecific hybrid of commercial peach (*P. persica*) and its close wild relative *P. davidiana*. Using an advanced backcross breeding population derived from a cross of *P. persica* and *P. davidiana*, Quilot et al. (2004) found a QTL for red skin coloration on linkage group 5 that, in common peach breeding germplasm, is likely fixed for the red skin allele and therefore was not detected in other QTL mapping efforts that only used *P. persica* parents (Cantín et al. 2010; Eduardo et al. 2010; Frett et al. 2014). Therefore, the finding that the 395 allele of P × D and its offspring was apparently associated with low blush, rather than medium blush as in the majority of germplasm studied, could be due to the effect of the linkage group 5 locus segregating within this interspecific material. In addition, the "highlighter" attribute, representing a complete suppression of blush that was discovered in South American "criollo" and Italian germplasm, might already be present in processing peach germplasm (Beckman et al. 2005). Two of the three sources that did not match the expected medium-blush phenotype based on carrying the medium-blush allele were processing-type peaches with low blush levels that could be a result of an epistatic interaction between the "highlighter" and R_f loci. The hypothesis that such a second locus modifies the R_f locus effect is similar to that proposed by Sandefur et al. (2016) for sweet cherry fruit color and Decroocq et al. (2014) for apricot Plum Pox Virus resistance. A caveat regarding the R_f locus is that if individuals related to P × D, "Carson," or other such germplasm sources are used in the breeding program, pedigree information would be required to ensure appropriate interpretation of genotypic results. Continued investigation of Ppe-R$_f$-SSR genotype-phenotype associations on more diverse germplasm will help to ascertain the scope of the predictive power of this test across peach breeding germplasm.

The three germplasm sources with Ppe-R$_f$-SSR genotypes that did not have the expected phenotype compared to all other allele sources highlighted the need to confirm any DNA test's prediction accuracy in a target breeding program's germplasm prior to routine deployment. Independent confirmation of the predictive power of Ppe-R$_f$-SSR was successfully conducted here for the University of Arkansas peach breeding program, where

the homozygous medium-blush allele group of seedlings had a higher proportion of blush coverage than the heterozygous seedlings, with both a low- and medium-blush allele. The observations of a low proportion (15%) of low-blush alleles and no parental combinations used that could have resulted in homozygous low-blush offspring provides insight into how Ppe-R_f-SSR could be effectively deployed in the Arkansas program. With parent selection using R_f locus information, all offspring produced could be homozygous for medium-blush alleles, thereby eliminating the need to screen seedlings with this DNA test to increase the overall cultivar potential of seedlings produced.

The DNA test developed herein, Ppe-R_f-SSR, is based on a single microsatellite marker that uses a standard PCR protocol and is expected to be effective with low quality and quantity DNA extracts because of the simplicity of the PCR reaction. In addition, Ppe-R_f-SSR can be readily multiplexed with other DNA tests and its amplicons can be visualized on a range of genotyping platforms. This test has many potential uses, including determining if any medium-blush parent has the genetic potential to produce low-blush offspring (i.e., detecting R_f heterozygotes), predicting genetic potential for blush coverage in juvenile seedlings to facilitate early selection/culling, and enabling efficient sorting of seedlings into low-blush processing or high-blush fresh market groups to simplify within-group evaluation. Although Ppe-R_f-SSR can be directly applied in breeding programs using germplasm similar to that evaluated in this report, it is recommended that the test's prediction accuracy be confirmed in a target breeding program's germplasm prior to routine deployment. The University of Arkansas Peach Breeding Program has already adopted Ppe-R_f-SSR into routine breeding operations. In early spring 2015, ~350 peach seedlings were screened with this DNA test combined in multiplexed PCR reactions with DNA tests for other fruit quality and disease resistance traits. The successful adoption of Ppe-R_f-SSR demonstrates the breeding utility of this trait-predictive DNA test and the potential impact of such DNA-based diagnostic technologies in fruit breeding.

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