Ppe.XapF: High Throughput KASP Assays to Identify Fruit Response to Xanthomonas Arboricola pv. pruni (Xap) in Peach

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Research Article

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Abstract

Background

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*), is a serious peach disease with symptoms that traverse severe defoliation and black surface pitting, cracking or blemishes on peach fruit with global economic impacts. A management option for control and meeting consumer demand for chemical-free, environmentally friendly fruit production is the development of resistant or tolerant cultivars.

Results

We developed simple, accurate, and efficient Ppe.XapF DNA tests based on SNP genotyping with KASP technology to quickly test for bacterial spot resistance alleles in peach fruit and cull at the greenhouse stage. The objective of this research was to validate newly developed Ppe.XapF DNA tests that target the two major QTLs for fruit resistance in peach with diagnostic utility in predicting fruit response to bacterial spot infection.

Conclusion

Our study confirms that only two Ppe.XapF DNA tests, Ppe.XapF1-1 and Ppe.XapF6-2, are needed to distinguish between susceptible and resistant haplotypes. Use of these efficient and accurate Ppe.XapF KASP tests resulted in 44% reduction in seedling planting rate in the Clemson University peach breeding program.

Background

Peach (*Prunus persica* L. Batsch) belongs to the *Prunus* genus of the Rosaceae family and is one of the most economically important fruit tree crops worldwide [1]. It is extensively grown throughout the temperate zone for its delicious and healthy fruit [2, 3]. China is the world's largest producer of peaches and nectarines, followed by Spain, Italy, Greece, Turkey and the U.S with a global annual production of ~ 25 million tons [4].

One of the major obstacles in growing peaches and nectarines worldwide is fruit susceptibility to diseases. No peach cultivar resistant to major peach diseases currently occupies any substantial U.S. market share, and despite the hundreds of existing peach cultivars used for fresh market, there is continuing need to develop new peach cultivars as the requirements of the industry and preferences of consumers change [5]. Diseases such as bacterial spot create an extra pressure on breeding programs to incorporate high genetic tolerance or resistance into new high-quality varieties for three main reasons: existing cultivars are not resistant [6–8]; chemical spraying does not provide adequate protection during pathogen-favorable years [9, 10]; and new races of the existing pathogen that are resistant to active components used in chemical spraying programs, continue to emerge [11].

Bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni* (*Xap*), is a serious disease that affects nearly all cultivated *Prunus* species and their hybrids [10, 12]. The most severe infections have been reported on Japanese plum (*P. salicina*), Korean cherry (*P. japonica*) and plum hybrids, as well as on peach and nectarines (*P. persica*) and their hybrids [6]. Disease etiology of bacterial spot on the peach tree includes fruit spots, leaf spots, and twig cankers. Symptoms on the peach fruit include pitting, cracking, gumming, and water-soaked tissue, which in turn can increase the susceptibility of the fruit to other fungal infections, such as rhizopus and brown rot. Eventually,

severe leaf spot infections can cause early tree defoliation, resulting in reduced vigor and winter hardiness [6]. Conventional methods of control include antibacterial sprays, such as oxytetracycline, or copper-based compounds, but these are only effective in years with low to medium disease pressure. There is also concern both with excessive antibiotic use and adding heavy metals to the environment, which has increased the focus on durable genetic resistance.

Peach is highly susceptible to *Xap* and most peach cultivars exhibit great levels of variation in disease susceptibility [7]. The most effective control for *Xap* is by incorporation of resistant alleles into the host plant through breeding. *Xap*-tolerance was introgressed from 'Elberta' into the popular commercial cultivar 'J.H. Hale' [13], resulting in a few resistant cultivars such as Clayton and Candor (Okie 1998; Okie et al. 2008). Unfortunately, many resistant cultivars, such as these, lack desirable fruit and marketing characteristics [13]. Most cultivars currently in production are susceptible to *Xap* with origins that trace back to the high-quality cultivar O'Henry, which is highly susceptible to both leaf and fruit *Xap* infections. Therefore, a need is still present to introgress *Xap* resistance into high-quality varieties that span the ripening season and are adaptable to various environmental conditions.

Initial success in developing bacterial spot resistant peach cultivars suggested that resistance might be conferred by only a few dominant genes [14]. Inconsistent levels of leaf and fruit resistance in the same peach cultivar also indicated involvement of separate genetic factors in leaf and fruit resistance [8, 15]. Several controlling loci in the peach genome conferring quantitative resistance have been reported [15]. Using peach 9K SNP array data [16], haplotypes in two quantitative trait loci (QTLs) with the largest effects on bacterial spot resistance in peach fruit, *Xap.Pp.OC-1.2* and *Xap.Pp.OC-6.1*, were determined and their frequency and effect were evaluated in a large collection of U.S. peach breeding germplasm [17]. Using this information, simple sequence repeat diagnostic tests for fruit response to bacterial spot infection in peach, Ppe-Xap-LG1 and Ppe-Xap-LG6, were developed within the RosBREED project (www.rosbreed.org). However, the tests could not distinguish between all *Xap* haplotypes and only had partial accuracy and agreement with 9K SNP data.

Great urgency has been associated with discovering efficient ways to incorporate disease resistance with high fruit quality and productivity into newly developed peach varieties [8, 17–19]. Despite vast genetic and genomic resources for peach [20, 21], most efforts stopped at revealing loci in the peach genome associated with traits of interest and have rarely provided the breeding community with tools for DNA-informed breeding (marker-assisted breeding) [22].

Kompetitive Allele Specific PCR (KASP) assays are a rapid and robust way to genotype SNPs of interest [23, 24]. These PCR-based assays use two forward primers in the same reaction: the final (3') base of each primer is complementary to one of the alleles at the SNP locus. Assay conditions encourage allele-specific primer binding, so amplification only occurs for the SNP allele present in the template DNA. Amplification is detected as fluorescence, where each SNP allele is associated with unquenching of either the FAM or HEX fluorophore by co-amplification of a tag sequence at the 5' end of each forward primer. The number of PCR cycles required to achieve maximal separation between HEX and FAM fluorescence signals varies depending on genomic DNA concentration and purity. The appropriate cycle can be easily selected by running real-time PCR assays, making the assay highly flexible [25].

A high throughput DNA test to predict bacterial spot phenotypes in breeding germplasm and segregating progeny at the seedling stage can significantly reduce screening and selection costs, while increasing the efficiency of the

breeding program. The objective of this study was to develop a rapid and unambiguous DNA test, using SNPs identified within the *Xap.Pp.OC-1.2* and *Xap.Pp.OC-6.1* QTL, that can routinely be used in peach breeding for prediction of bacterial spot fruit resistance.

Results

Genotyping results from seven SNPs

Five haplotypes on chromosome 1, in *Xap.Pp.OC-1.2*, and six haplotypes on chromosome six, in *Xap.Pp.OC-6.1*, can be uniquely identified with four SNPs each (Table 1). Haplotypes and their associated bacterial spot phenotypes [susceptible (S), almond (A), intermediate (I), resistant-1 (R1) and resistant-2 (R2)] are presented in Table 1. One SNP, which differentiates between the heterozygous haplotypes S|A and I|R2 on chromosome 6, was not suitable for a KASP assay.

Table 1
Ppe.XapF SNPs, haplotypes and 9K IPSC peach array (Verde et al., 2012) codes and their associated haplotypes. S – susceptible; R1 and R2 – resistant; I – intermediate; alm – allele observed in almond; - allele with unknown bacterial spot phenotype; *SNP that could not be used in KASP. Bolded SNPs used for culling susceptible seedlings.

QTLs	SNPs	Nucleotides					9K IPSC codes						Ppe.XapF
		S	R1	I	R2	alm	S	R1	R2	I	alm	-	
Xap.Pp.OC-1.2	SNP_IGA_39717	T	C	C	C	C	A	B	B	B	B	A	**1–1**
	SNP_IGA_40295	A	G	G	G	A	A	B	B	B	A	B	1–2
	SNP_IGA_43384	T	T	T	C	T	A	A	B	A	A	B	1–3
	SNP_IGA_46754	G	A	G	G	G	B	A	B	B	B	B	1–4
Xap.Pp.OC-6.1	SNP_IGA_680615	T	G	T	G	T							
	SNP_IGA_680882	C	T	C	C	C							
	SNP_IGA_680889*	C	C	T	T	T	B	B	A	A	A		
	SNP_IGA_680909	T	C	T	C	C	A	B	B	A	B		**6 – 2**
	SNP_IGA_680953	A	G	G	A	G	A	B	A	B	B		6 – 3
	SNP_IGA_681081	G	G	G	A	A	B	B	A	B	A		6 – 4
	SNP_IGA_681113	T	T	T	G	G							
	SNP_IGA_681119	G	G	G	A	A							

The KASP assays designed for each of the remaining seven SNPs successfully amplified and distinguished between AA, AB, and BB genotypes (Figs. 1, 2). Four clusters were observed for each KASP assay: high FAM fluorescence and low HEX fluorescence, high HEX fluorescence and low FAM fluorescence, intermediate FAM and HEX fluorescence, and ~ 0% FAM or HEX fluorescence. These clusters indicate genotypes of, respectively, AA, AB, BB, and no amplification.

By collapsing FAM and HEX fluorescence values into the one-dimensional measurement, delta, we could easily assign samples to the appropriate genotype. We designed a template spreadsheet that assigns genotypes automatically based on default or user-specified parameters (Supp. Table 1). No-template controls have delta values right at 0, while AB samples all fall slightly above or slightly below 0. All BB samples have delta values above a certain threshold, and all AA samples have delta values below a certain threshold. These thresholds can be set manually so that automatic genotype assignments reflect what is observed in the scatterplot of relative FAM and HEX fluorescence values. For example, for Ppe.XapF6-3, the AB and AA clusters were quite close together on the scatterplot (Fig. 2C), but the threshold delta value was set so that the genotypes assigned by the delta plot (Fig. 2D) corresponded to the clusters observed on the scatterplot (Fig. 2C). On occasion, the delta value of samples falls very close to the threshold. These samples were marked if they fell within two units of the threshold value, and this margin can be adjusted to suit the assay and experimenter's needs. It is easy to then inspect the relative fluorescence scatterplot to see how ambiguous these calls are. Assays of all seven SNPs only produced two marginal calls, one in the Ppe.XapF6-2 assay and one in the Ppe.XapF6-4 assay (Figs. 2B, 2F). However, upon inspection of the scatterplot, the genotypes were assigned correctly by the delta plot.

In some cases, the positive controls failed, either by not amplifying (Ppe.XapF6-3) or by having an unexpected genotype (Ppe.XapF1-3, Fig. 1E-F). As long as the four clusters (described above) were observed, genotypes could still be assigned. For Ppe.XapF1-3, the B

Table 2
Peach germplasm used for development and validation of the Ppe.XapF DNA test. Ppe.XapF phenotypes predicted with KASP and 9K IPSC SNP array. Bold accessions in which KASP and array data disagree.

Name	Type	Ppe.XapF			
		KASP		Array	
		F1	F6	F1	F6
ArcticBelle	cultivar	R1|R1	S|S		
ArcticBlaze	cultivar	R1|S	R1|S		
ArcticGold	cultivar	R1|R1	R1|S		
ArcticPride	cultivar	R1|S	S|S		
ArcticStar	cultivar	R1|R1	S|S		
ArcticSweet	cultivar	R1|S	R1|S		
Arrington	cultivar	S|S	R2|R2	S|S	R2|R2
Autumnflame	cultivar	R1|S	S|S		
Autumnprince	cultivar	R1|S	S|S		
Autumnred	cultivar	S|S	S|S		
Blazeprince	cultivar	S|S	S|S	S|S	S|S
Blazingstar	cultivar	R1|S	R2|S	R1|S	R2|S
Bounty	cultivar	R1|R1	R2|S	R1|R1	R2|S
Bradley	cultivar	S|S	R2|R2	S|S	R2|R2
BY00P4945	breeding material	S|S	R1|R1		
BY00P6346u	breeding material	R1|R1	R1|S	R1|R1	R1|S
BY01P9169c	breeding material	I|I	R1|R2		
BY01P9239	breeding material	S|S	S|S		
BY07n3500	breeding material	**I**|S	R2|S	**R1**|S	R2|S
BY99P4366	breeding material	S|S	R1|R1		
Caroking	cultivar	R1|R1	R2|S	R1|R1	R2|S
Carored	cultivar	S|S	R2|S	S|S	R2|S
CaryMac	cultivar	R1|R1	R2|R2		
China Pearl	cultivar	I|-	R2|S	I|-	R2|S
Chinese cling	cultivar	R1|S	R2|1	R1|S	R2|1
Clayton	cultivar	R1|S	R1|R2	R1|S	R1|R2

Name	Type	Ppe.XapF			
		KASP		Array	
		F1	F6	F1	F6
Clemson Lady	cultivar	R1|S	S|S		
Contender	cultivar	R1|S	S|S	R1|S	S|S
Coronet	cultivar	R1|S	S|S		
Crimson Lady	cultivar	R1|S	S|S	R1|S	S|S
CVN-1	cultivar	S|S	S|S		
Dixired	cultivar	R1|S	R2|S		
Elberta	cultivar	R1|S	R1|S	R1|S	R1|S
Empress	cultivar	S|alm	R2|S		
Fireprince	cultivar	R1|S	S|S	R1|S	S|S
Flameprince	cultivar	R1|S	R2|S	R1|S	R2|S
Flavorich	cultivar	R1|alm	S|S		
FlavorTop	cultivar	R1|S	R1|S		
GlacierWhite	cultivar	S|S	S|S		
Glenglo	cultivar	R1|R1	R2|R2		
Goldcrest	cultivar	S|S	R2|S	S|S	R2|S
Hakuto	cultivar	S|S	S|S	S|S	S|S
Harrow Diamond	cultivar	S|S	R2|R2		
Harvester	cultivar	S|S	S|S	S|S	S|S
Honey Blaze	cultivar	R1|R1	R2|S		
Intrepid	cultivar	S|S	R1|R2	S|S	R1|R2
Jayhaven	cultivar	S|S	R2|R2		
Joanna Sweet	cultivar	R1|S	S|S		
Julyprince	cultivar	R1|S	R1|S	R1|S	R1|S
Loring	cultivar	R1|R1	R2|R2	R1|R1	R2|R2
O'Henry	cultivar	S|S	S|S	S|S	S|S
Raritan Rose	cultivar	R1|I	R1|R1	R1|I	R1|R1
Redhaven	cultivar	R1|S	R2|S		
Reliance	cultivar	**-|-**	R2|S	I|I	R2|R2

Name	Type	Ppe.XapF			
		KASP		Array	
		F1	F6	F1	F6
Rich Joy	cultivar	R1|S	R2|S	R1|S	R2|S
SC08_02_012	breeding material	S|S	S|S	S|S	
SC08_09_006	breeding material	R1|R1	S|S	R1|R1	
SC08_13_001	breeding material	R1|S	R2|S	R1|S	
SC08_16_005	breeding material	S|S	S|S	S|S	
SC08_16_070	breeding material	R1|S	R1|S	R1|S	
SC08_17_059	breeding material	R1|S	R2|R2	R1|S	
Scarletprince	cultivar	R1|S	R2|S	R1|S	
September Snow	cultivar	R1|S	S|S	R1|S	
Snowbrite	cultivar	R1|S	S|S	R1|S	
Snowprince	cultivar	S|S	S|S	S|S	
Stark Saturn	cultivar	R1|S	R1|R1	R1|S	
Sugar Lady	cultivar	R1|S	S|S	R1|S	
Summergold	cultivar	R1|S	R2|S	R1|S	
Summerprince	cultivar	R1|S	R2|R2	R1|S	R2|R2
Summer Sweet	cultivar	S|S	S|S		
Sunbrite	cultivar	S|S	S|S		
Suncrest	cultivar	S|S	S|S		
SuziQ	cultivar	S|alm	R2|S		
Sweet Blaze	cultivar	R1|S	S|S		
Sweet Dream	cultivar	R1|R1	S|S		
Topaz	cultivar	R1|R1	R2|R2		
UF Gold	cultivar	R1|S	R2|R2	R1|S	R2|R2
Vulcan	cultivar	R1|S	R2|R2		
Westbrook	cultivar	R1|S	R2|R2	R1|S	R2|R2
White Lady	cultivar	S|S	R2|S		
Wild Rose	cultivar	R1|I	R2|S		
Winblo	cultivar	S|S	S|S	S|S	S|S

Name	Type	Ppe.XapF			
		KASP		Array	
		F1	F6	F1	F6
Yukon King	cultivar	R1|R1	S|S		
Zephyr	cultivar	R1|R1	S|S		

allele was rare in germplasm used for validation, but a single BB genotype and two AB genotypes were identified by the KASP assay (Table 2). These individuals could be used as positive controls for future assays.

All 32 samples for which SNP genotyping was previously done using peach 9K SNP array [16] and *Xap* alleles were known, matched KASP-obtained genotypes for all 7 reactions except for 2 samples, breeding material BY07n3500x and cultivar Reliance, providing 94 % accuracy in assigning haplotypes (Table 2). KASP results from previously genotyped samples resulted in a change in haplotype assignment, from R1 to I for Ppe.XapF1 for By07n3500x and from I to unknown for 'Reliance'. The predicted genotype of 'Reliance' for Ppe.XapF6 KASP assay was heterozygous R2|S while array data suggested homozygous R2.

Endpoint assays

Realtime PCR ensures that an appropriate final cycle is chosen but limits assay throughput. The same high-quality DNA was tested with endpoint KASP assays for Ppe.XapF1-1 and F6-2, using the number of cycles determined from realtime PCR. Endpoint assays performed similarly to realtime assays (Fig. 3). Four distinct clusters were observed for both SNPs. One sample (SC08_16_005) successfully amplified for Ppe.XapF1-1 with the realtime assay (genotype AA) but failed to amplify with the endpoint assay. However, the same sample successfully amplified for the endpoint Ppe.XapF6-2 assay.

Seedling genotyping and culling

Seedlings were screened with KASP endpoint assays for Ppe.XapF1-1 and F6-2. A template spreadsheet was designed that could import data from multiple plates and SNP assays, automatically assign genotypes, and recommend culling or keeping seedlings based on genotype (Supp. File 1). Results from seedling screening assays that used crude DNA were more variable than from high-quality DNA: a number of samples failed to amplify, and samples right at the upper or lower boundaries of the AB genotype occurred more frequently. Nevertheless, it was possible to assign genotypes to all samples that did amplify (Fig. 4, Supp. Table 2, 3). Problems with sample amplification due to low DNA concentration occurred in ~ 9% of samples, leading to ~ 379 assays being repeated with DNA at a higher concentration (5x dilution instead of 40x dilution), which was sufficient to solve the problem. Crude DNA extracted from leaf tissue collected from seedlings already planted in the field (1,774 hybrid seedlings from crossing years 2016–2017) had expected concentration of 180–400 ng μL^{-1} and successfully amplified. However, crude DNA extracted from 2,401 seedlings in the greenhouse from crossing seasons 2018–2020 exhibited variability in DNA concentration directly associated with the age of plantlets, as younger plantlets had lower DNA concentration and required lower dilution for final PCR set up. In total 2,340 (56%) seedlings were kept, resulting in a 44% reduction in the number of seedlings planted in the field. Assays Ppe.XapF1-1 and F6-2 are sufficient to cull individuals with undesirable S haplotypes on either chromosome 1 or chromosome 6 (Tables 1 and 3).

Table 3
Parameters for keeping or culling seedlings based on Ppe.XapF1-1 and Ppe.XapF6-2 KASP assays in the Clemson University peach breeding program.

F1-1 genotype	F6-2 genotype	Seedling fate
AA	BB	keep
AB	AB	keep
AB	BB	keep
BB	AB	keep
BB	BB	keep
AA	AA	cull
AA	AB	cull
AB	AA	cull
BB	AA	cull

Discussion

Developed KASP assays successfully identified bacterial spot resistance haplotypes in breeding stock germplasm including F1 individuals. Early screening for genetic resistance prior to field planting led to a dramatic decrease in the number of seedlings planted, as well as the number to be screened at maturity for productivity and fruit quality traits. This strategy also allows for an increase in annual seedling production from ~ 3–4,000 hybrids (the target for hybrid production in the Clemson University peach breeding program), as only the small number hybrids with desired *Xap* haplotype combinations require planting. Nearly complete haplotype identification is possible using seven SNPs, although two haplotypes for the QTL on chromosome 6 are indistinguishable without genotyping an eighth SNP that does not work in KASP assays.

In the development and validation of this DNA breeding tool, both the realtime and endpoint PCR results were in agreement, indicating that endpoint PCR can be adopted to increase throughput after optimizing reaction parameters with realtime PCR. A high-throughput workflow was established, using a rapid and simple crude DNA extraction method, a robotic liquid handler for assay setup in 96-well plates, and endpoint assays followed by bulk plate reading on a real-time machine. The post DNA extraction workflow can easily process 32 96-well plates per typical workday with a single technician with very low cost. The workflow is limited only by the number of end point PCR units one has access to, as the approximate time to run an endpoint PCR assay is less than 2 hours and reading the result on a real time unit takes less than 3 minutes. Using automation to dilute samples and prepare DNA for PCR reactions eliminated human pipetting errors and ensured reproducibility of results. The stability of crude DNA is limited to a few days at + 4 ºC or longer at -20 ºC.

Although Bio-Rad's software can produce genotype calls, when clusters aren't clearly separated, the software's calls can disagree with the user's perception of what the call should be. Using the delta method, the user can easily change genotype assignments and determine which wells are associated with which genotype. The user can also adjust the sensitivity of the method to their needs.

PCR was not inhibited by any compounds co-extracted with DNA with the crude method. All DNA extracted with the same method was diluted by the same factor, regardless of actual DNA concentration, in the interests of increasing throughput speed. Samples that did not amplify generally failed in several assays, indicating these particular samples needed to be repeated with a more concentrated DNA solution. We observed a correlation between the plant age and low DNA concentration when a 3mm disc from the first true leaf was harvested. A plausible reason for low DNA yield could be low nuclei content at this early stage of plant development. The adequate dilution of crude DNA is essential for successful PCR, and a balance must be struck between diluting away inhibitors and maintaining appropriate DNA concentration for amplification within 40 cycles, as was suggested by Noh et al. [26] for strawberry.

Conclusions

KASP assays for SNPs associated with *Xap* resistance were developed and validated on crude DNA extractions of individuals with known and unknown genotypes. These assays were highly accurate, identifying the correct genotype for 94 % of samples with known genotypes. Of the seven assays developed, only two are needed to identify the susceptible haplotype. End-users have control over genotype assignment by using the delta method, which also highlights any problematic samples or assays. These assays provide a rapid, cheap, accessible method to cull seedlings with undesirable genotypes before field planting, and flag seedlings with desired genotypes for further genotyping and phenotyping.

Methods

Plant material

The peach material (84 cultivars and advanced selections), used in development and validation of the Ppe.XapF DNA test (Table 2), is part of the Prunus germplasm collection maintained at the Clemson University Musser Fruit Research Center, Oconee county in Seneca, SC (Latitude:34.639038, Longitude: -82.935244, Altitude 210msl), under standard commercial practices for irrigation, fertilization, and pest and disease control. The original source of the material is Adams County Nursery (Aspers, PA, USA). The trees were at least 5 years old, grafted on Guardian® rootstock, planted in duplicate at 1.5 m × 4 m spacing and trained to a perpendicular V system. In addition, the Ppe.XapF KASP assay was used on 4,175 seedlings generated from crosses in the Clemson University peach breeding program to evaluate *Xap* fruit response using a crude DNA extract [26].

DNA extraction

Development and validation of the Ppe.XapF KASP assay used high-quality DNA extracted from young leaf tissue following a protocol modified from [27]. Approximately 100 mg of fresh leaf tissue was collected per well of a 96-well plate (Ab-Gene AB-0661) and a stainless-steel bead was added. The tissue was lyophilized in a LABCONCO LYPH-LOCK 6 freeze dryer and ground to a fine powder using a Geno-grinder (SPEX). Extraction buffer [100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 1% SDS, 1% PVP40, 500 µg/mL proteinase K, and 1% DTT] was prepared prior to extraction and heated to 65ºC before adding 0.5 mL to each well of the plate. The plate was sealed and inverted to mix. The plate was incubated at 65ºC for 30 min with occasional agitation, then cooled at -20ºC for 15 min. After cooling, 250 µL of cold (4ºC) 6M ammonium acetate was added to each well and the plate was resealed, inverted, and returned to -20ºC for an additional 15 min. The plate was centrifuged in a swing-bucket bench top centrifuge for 20 min at 1,976 g at 4ºC. After centrifugation, ~ 400 µL of supernatant was

transferred from each well to a combination filter/receiver plate (Pall Filter# 8130; Ab-gene receiver AB-0859) and centrifuged at 1,147 g for 7 min. The filter was discarded and 240 µL of chilled isopropanol was added to each well. The plate was sealed, mixed and placed at 4ºC overnight to precipitate DNA. Next day the plate was centrifuged at 1,792 g for 30 min at 4ºC and decanted. Pelleted gDNA in each well was washed twice with 450 µL of 70% ethanol and resuspended in 50 µL of DNase/RNase free water (Gibco). DNA was RNase treated with 1 U of RNase A (ThermoFisher) for 1 hour at 37ºC.

Crude DNA extraction from seedlings either in the greenhouse or in the field followed the protocol of Noh *et al*. [26] in a 96-well plate format, using one 3 mm leaf disc per plant per well. Plates were kept on ice during tissue collection. Directly following tissue collection, 50 µL of freshly prepared buffer A (100 mM NaOH, 2% Tween 20) was added to each well. The plate was sealed with foil tape, centrifuged at 1,792 g for 2 min, and heated at 95ºC for 10 min. An equal volume (50 µL) of buffer B [100 mM Tris-HCl (pH 8), 2 mM EDTA] was added to each well. The plate was centrifuged at 1,792 g for 2 min, sealed with fresh foil tape, and stored at 4ºC overnight. The next day each sample was diluted with addition of 100 µl of distilled water, sealed with fresh foil tape and stored for a week at 4 ºC or -20 ºC until use.

DNA concentration after either extraction protocol was checked on a subset of samples with a Nanodrop spectrophotometer. Observed concentrations ranged between 900 and 1000 ng μL^{-1} for standard extractions, or between 180 and 400 ng μL^{-1} for crude extractions. DNA was diluted 200x from all standard extractions and 40x from all crude extractions in nuclease-free water to achieve the recommended concentration for KASP assays of 5 ng μL^{-1}. Crude DNA extraction, dilution and PCR plate set up for both real time and endpoint reactions for both germplasm and seedling samples was conducted with the OT-2 robot from Opentrons (opentrons.com).

Primer design

Seven SNPs, four on *Xap.Pp.OC-1.2* and three on *Xap.Pp.OC-6.1*, were chosen to distinguish between five and six unique haplotypes on chromosomes 1 and 6, respectively (Table 1) [17]. Primer sets for the KASP assay were designed for each of the seven SNPs, identifying the presence of an “A” allele (A or T, assigned to the FAM fluorophore) or a “B” allele (G or C, assigned to the HEX fluorophore) (Supp. Table 4). Primers met the following criteria: GC content between 30–55%; Tm of ~ 64ºC ± 2; 21–30 bp long; product size of 50–100 bp; secondary structure more positive than − 9 kca/mole; no more than four di-nucleotides; no more than 4 or 5 identical nucleotides in a row; no more than 3 Gs and/or Cs in the last 5 bp of the primer.

KASP assay

A primer master mix of both forward primers and the reverse primer for a single SNP assay was assembled as follows, after resuspending primers in nuclease-free water at 100 µM: 18 µL of each forward primer and 45 µL of the reverse primer were combined with 69 µL of 10 mM Tris-HCl, pH 8.0. The reaction mixture for each assay included 432 µL of 2x PACE™2.0 genotyping master mix with no ROX (3CR Bioscience, Harlow, Essex, UK) (which includes polymerase, dNTPs, buffer, and HEX- and FAM-tagged oligonucleotides) and 11.88 µL of the appropriate primer master mix. Genotyping assays used 5 µL of reaction mixture and either 5 µL of water (no-template control) or 5 µL of genomic DNA at approximately 2.5 ng/µL. Three replicates of no-template controls and positive controls for AA, AB, and BB genotypes were always included, along with unreplicated samples of unknown genotype (“unknowns”). Positive controls for each assay were chosen from 18 accessions previously genotyped with the peach 9K SNP array [16] as part of the Peach Crop Reference Set [28] within the RosBREED

project (www.rosbreed.org) (Table 2). A total of 4,175 seedlings were organized in ~ 50 96-well plates using OT-2 robot, with row A containing no template control, AA, AB and BB controls in triplicate, respectively.

Reactions were performed in a Bio-Rad CFX Connect Real-Time PCR Detection System using the following program for all SNPs except Ppe.XapF1-3 and F6-2: 15 min at 95ºC (activation), followed by 10 touchdown cycles of 94ºC for 20s (denaturing), 61 – 55ºC for 60s (dropping 0.6ºC per cycle, for annealing and elongation), followed by 40 cycles of 94ºC for 20 s, 55ºC for 60s, 23ºC for 30s (for accurate plate reading). The Ppe.XapF1-3 and Ppe.XapF6-2 required higher temperatures for annealing/elongation (58ºC for XapF1-3, 57ºC for XapF6-2) to generate clearly separated genotype clusters, and consequently used a smaller temperature decrement during the 10 touchdown PCR cycles. For each plate, the cycle used for genotype assignment was chosen to maximize separation between genotype clusters and minimize background amplification, usually between cycles 22–28 of the 40-cycle period. Endpoint PCR reactions, which kept the step of 23ºC for 30s but omitted plate reading, were performed on Bio-Rad T100 thermal cyclers to assay Ppe.XapF1-1 and Ppe.XapF6-2 using 25 cycles identified from real-time PCR. High-quality DNA from previously genotyped samples was used first to validate the method, followed by crude DNA extracts from seedlings. Endpoint assays were read on the Bio-Rad Real-Time machine using Bio-Rad CFX Maestro™ software.

Data Analysis

To account for differences in fluorescence values between fluorophores and assays, HEX and FAM relative fluorescence units for each sample were transformed to reflect the percentage of the maximum fluorescence for each fluorophore within a plate

$$\%\ \text{fluorescence} = (\text{sample fluorescence} - \text{minimum fluorescence}) / (\text{maximum fluorescence} - \text{minimum fluorescence})$$

To assign genotypes to unknowns, the difference between % HEX fluorescence and % FAM fluorescence (“delta”) was calculated. Heterozygotes are expected to have approximately equal HEX and FAM fluorescence, yielding delta values close to 0. As primers were designed so that all A alleles were assigned to FAM fluorescence and all B alleles to HEX fluorescence, BB genotypes (high HEX fluorescence, low FAM fluorescence) are expected to yield positive delta values and AA genotypes (low HEX fluorescence, high FAM fluorescence) are expected to yield negative delta values. The exact cutoffs for genotype assignment were determined manually for each assay. Any samples with fluorescence values < 20% for both fluorophores were considered to have failed to amplify (Supp. Table 1).

Abbreviations

A, Almond; I, Intermediate; KASP, Kompetitive Allele Specific PCR; QTL, Quantitative Trait Loci; R1, Resistant-1; R2, Resistant-2; S, Susceptible; SNP, Single Nucleotide Polymorphism; *Xap*, *Xanthomonas arboricola* pv. *pruni*

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files]. The peach 9K SNP array genotyping data for validation material can be found in online repository www.rosaceae.org under accession number tfGDR1048/b. Peach material used in validation is available from any fruit tree nursery.

Competing interests: The authors declare that they have no competing interests

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Authors' contributions: MBF: conceived and designed experiments, designed and tested primers, analyzed data, and wrote the paper. TM and ZL: prepared samples and collected data for validation. WF: prepared samples and collected data for greenhouse samples. KG conceived of and designed experiments, prepared samples and collected data, and wrote the paper. CS conceived of and designed experiments and wrote the paper. All authors read and approved the final manuscript.

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Figures

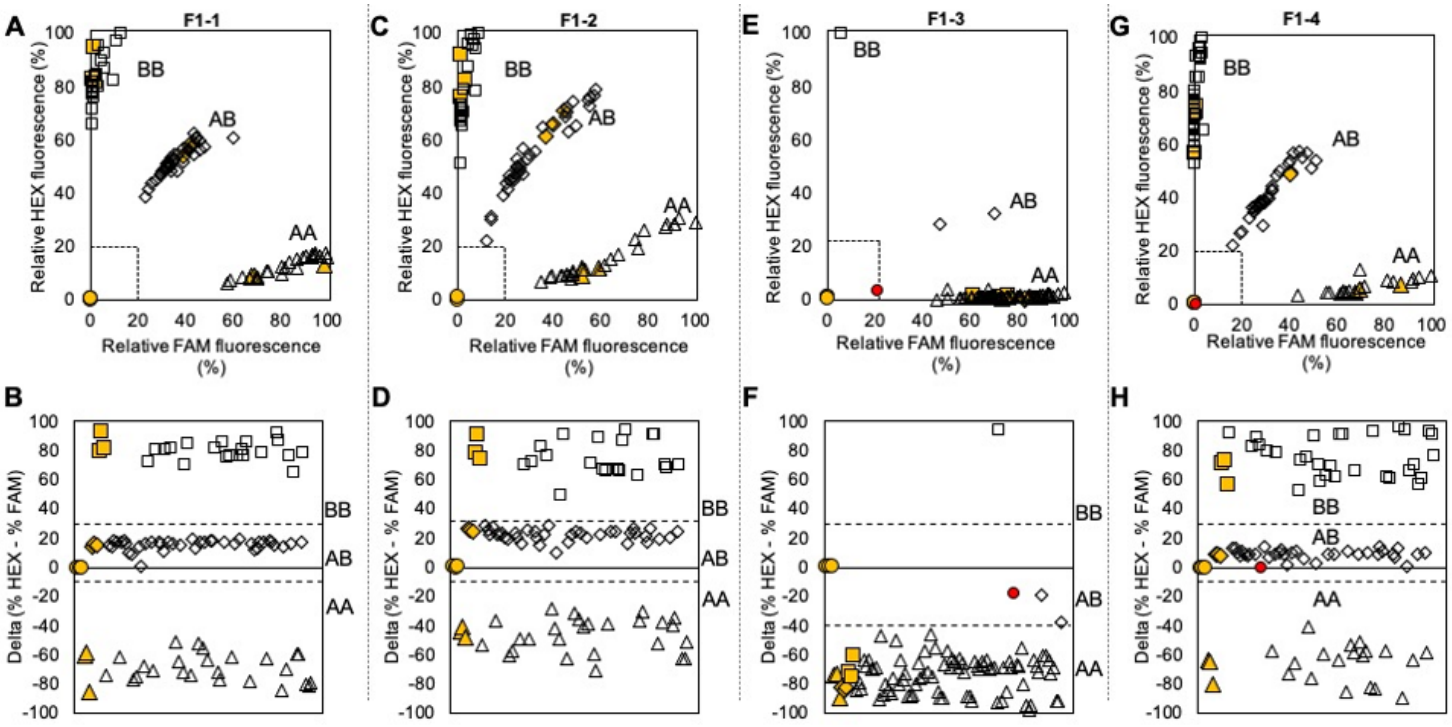

Figure 1

Results from KASP assay for SNPs on Ppe.XapF1 using clean DNA extraction and previously genotyped samples. Solid yellow shapes indicate positive controls, solid red indicates an unknown that failed to amplify, and empty shapes indicate unknowns, with the shape indicating the assigned genotype as follows: circle = no template/no amplification, triangle = AA, diamond = AB, square = BB. Dashed lines in plots in the top row indicate bounds for no amplification (% fluorescence < ~20% for both fluorophores). Dashed lines in plots in the bottom row indicate delta (% HEX - % FAM) values separating heterozygous and homozygous genotypes, adjusted to reflect assay conditions. A,B = F1-1, C,D = F1-2, E,F = F1-3, G,H = F1-4.

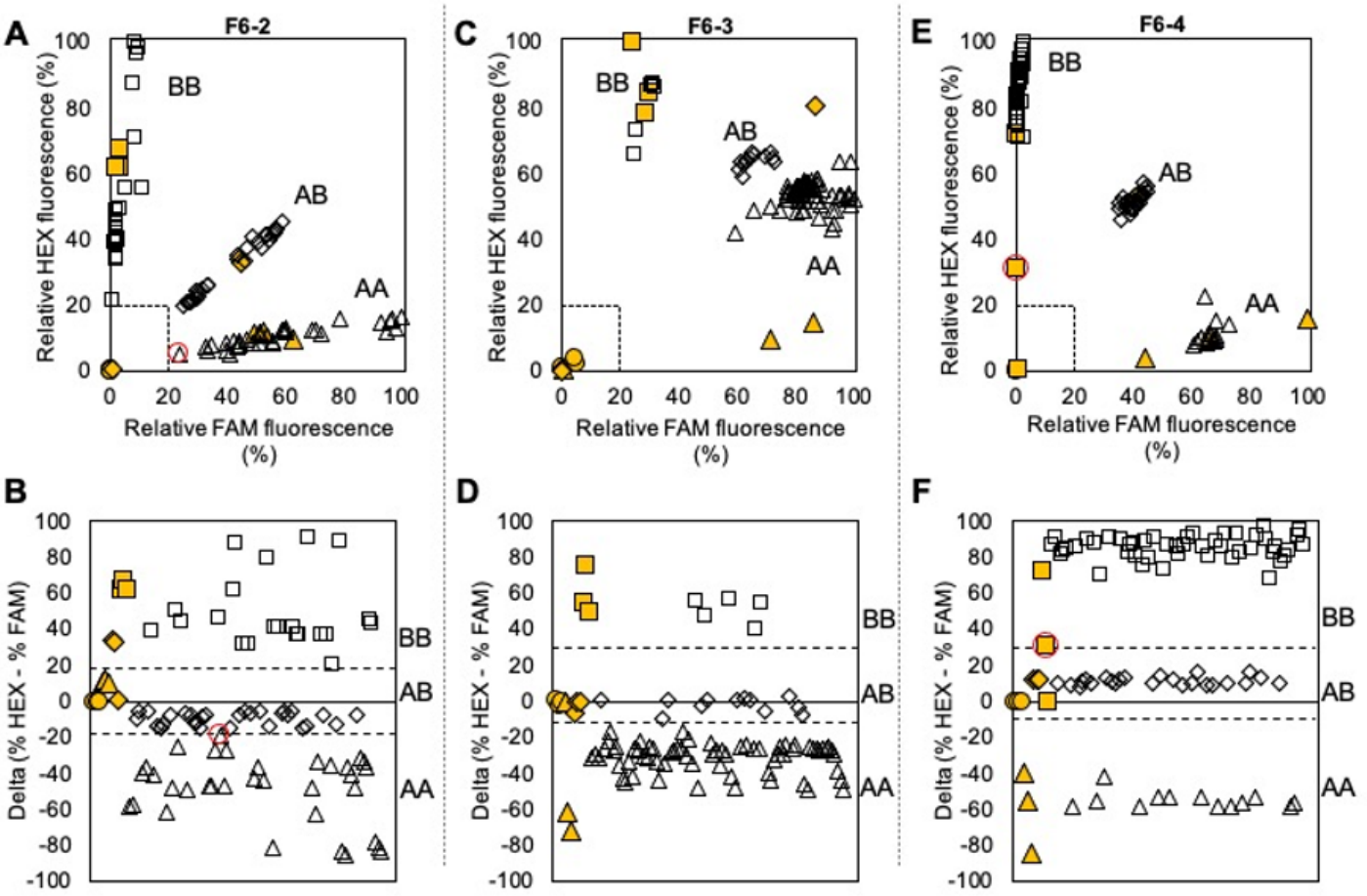

Figure 2

Results from KASP assay for SNPs on Ppe.XapF6 using clean DNA extraction and previously genotyped samples. Solid yellow shapes indicate positive controls, and empty shapes indicate unknowns, with the shape indicating the assigned genotype as follows: circle = no template/no amplification, triangle = AA, diamond = AB, square = BB. Dashed lines in plots in the top row indicate bounds for no amplification (% fluorescence < ~20% for both fluorophores). Dashed lines in plots in the bottom row indicate delta (% HEX - % FAM) values separating heterozygous and homozygous genotypes, adjusted to reflect assay conditions. Points are encircled in red when they are close enough to the dashed lines in the bottom row that manual inspection is advised. A,B = F6-2, C,D = F6-3, E,F = F6-4.

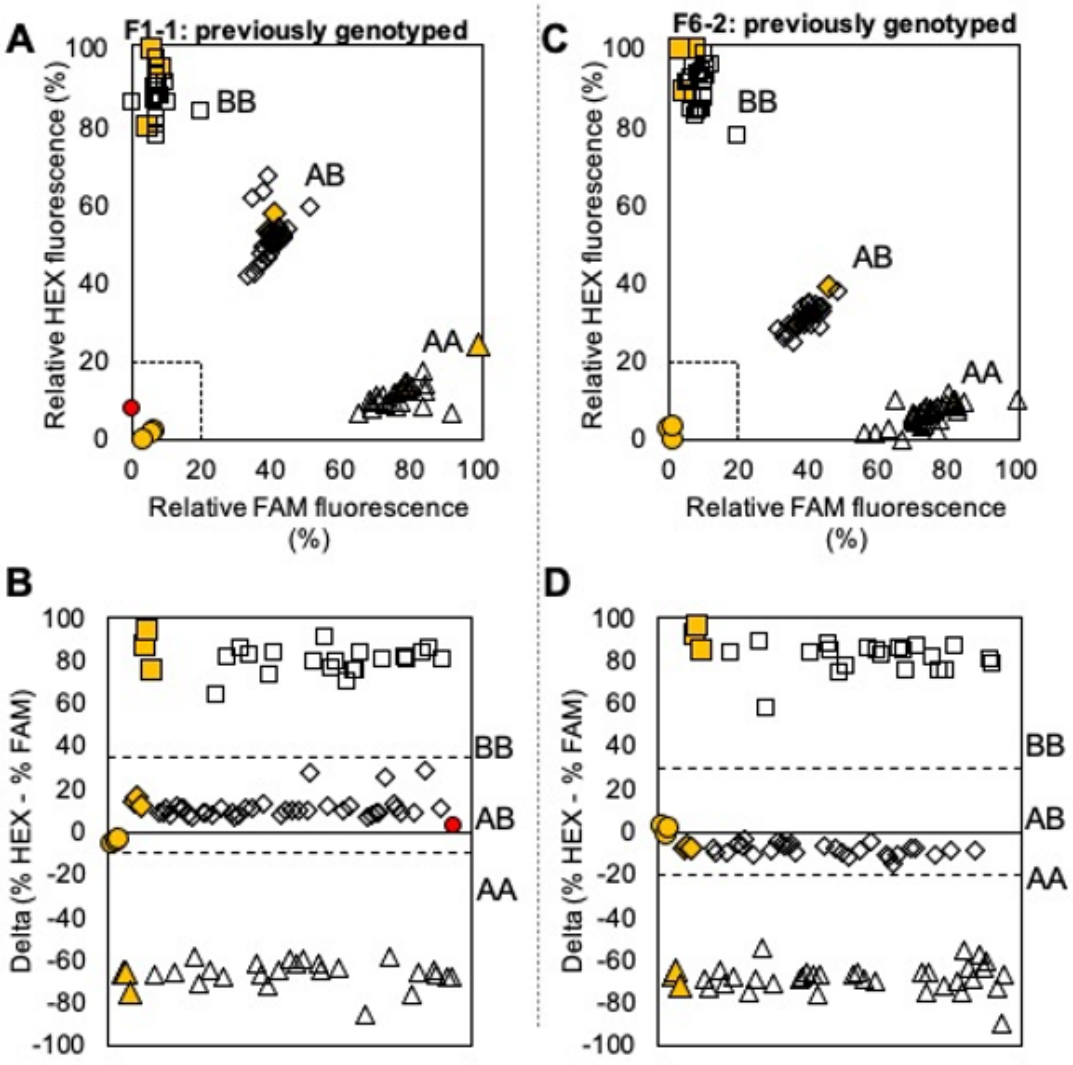

Figure 3

Results from KASP assay for two SNPs, Ppe.Xap F1-1 and Ppe.Xap F6-2, using endpoint PCR with previously genotyped samples. Solid yellow shapes indicate positive controls, solid red indicates an unknown that failed to amplify, and empty shapes indicate unknowns, with the shape indicating the assigned genotype as follows: circle = no template/no amplification, triangle = AA, diamond = AB, square = BB. Dashed lines in plots in the top row indicate bounds for no amplification (% fluorescence < ~20% for both fluorophores). Dashed lines in plots in the bottom row indicate delta (% HEX - % FAM) values separating heterozygous and homozygous genotypes, adjusted to reflect assay conditions.

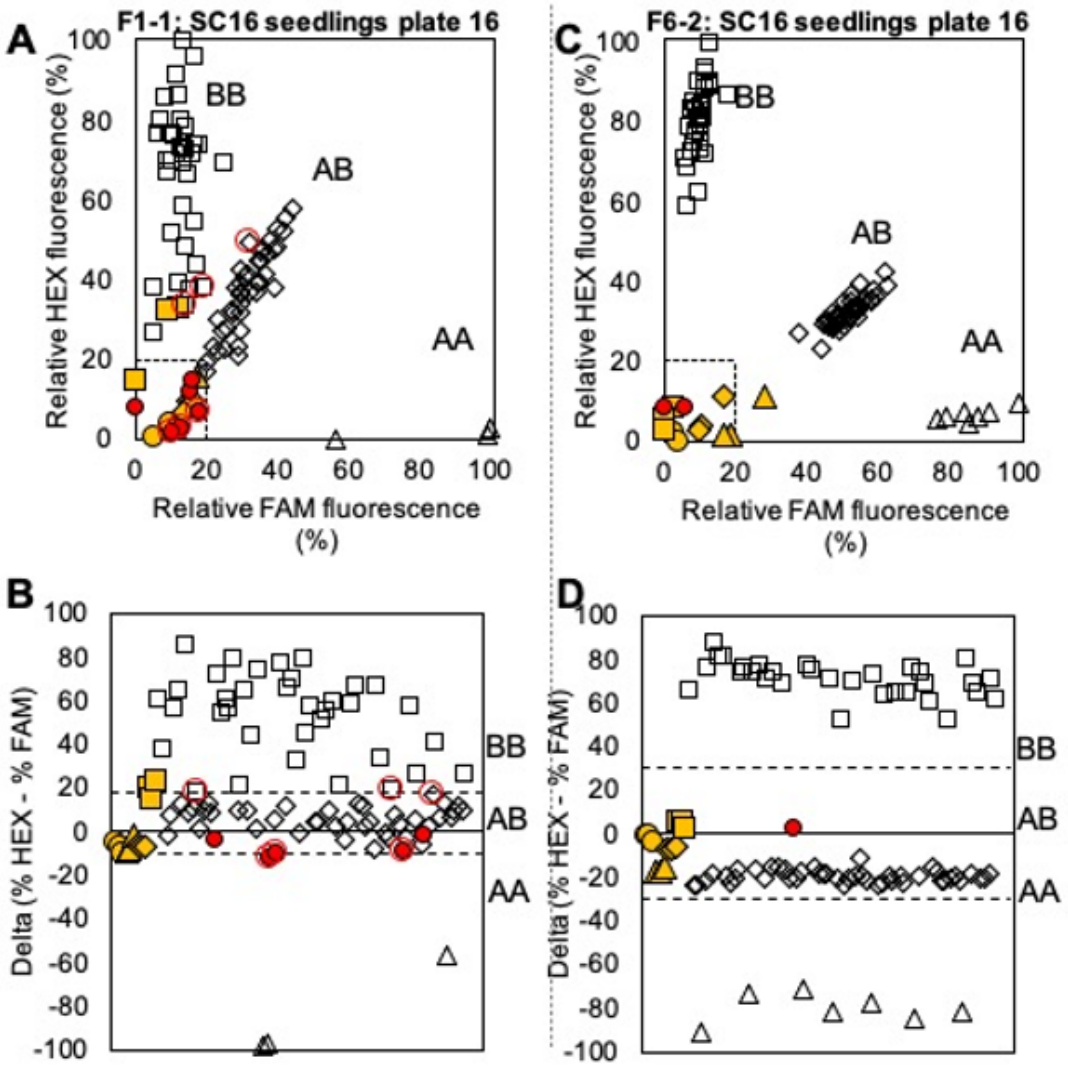

Figure 4

Results from KASP assay for two SNPs, Ppe.Xap F1-1 and Ppe.Xap F6-2, using endpoint PCR with crudely-extracted DNA from greenhouse seedling samples. Solid yellow shapes indicate positive controls, solid red indicates an unknown that failed to amplify, and empty shapes indicate unknowns, with the shape indicating the assigned genotype as follows: circle = no template/no amplification, triangle = AA, diamond = AB, square = BB. Dashed lines in plots in the top row indicate bounds for no amplification (% fluorescence < ~20% for both fluorophores). Dashed lines in plots in the bottom row indicate delta (% HEX - % FAM) values separating heterozygous and homozygous genotypes, adjusted to reflect assay conditions.

Supplementary Files

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