A Cost-Effective Strategy for Marker Assisted Selection (MAS) in Apple (*M. pumila* Mill.): The Experience from the Fondazione Edmund Mach Programme for Resistance and Quality Traits

E. Banchi, L. Poles, P. Magnago, M. Pindo, F. Costa, R. Velasco and D.J. Sargent
Research and Innovation Centre, Fondazione Edmund Mach
Via E. Mach 1, 38010 San Michele all'Adige (TN)
Italy

Keywords: breeding, SCAR, SSR, ASO, apple scab, powdery mildew, fruit texture, KAPA 3G

Abstract

Marker assisted selection (MAS) permits the selection of plants carrying genes that control interesting agronomic traits through the screening of associated markers; this allows the breeders to retain only germplasm conferring desirable traits from an early stage. Usually MAS involves the screening of hundreds or thousands of seedlings and thus the main challenge faced is the development of a pipeline that starts with a "lab friendly" and unambiguous seedling disposition in greenhouse, allows the rapid collection of samples avoiding cross contamination, implements a fast, cost-effective, high-throughput method of DNA extraction to be used for a small number of PCRs, and utilizes robust, reliable, and easy to interpret molecular markers. In this study ~6,500 seedlings from 24 apple crosses were genotyped in order to select seedlings conferring resistance traits, fruit quality traits or both. Lab work was performed using the KAPA 3G Plant PCR kit. The main problems encountered were associated with dirty extractions; however, following optimization less than 10% of the seedlings had to be re-sampled and in total about 70% of the samples tested carried desirable traits.

INTRODUCTION

Marker assisted selection (MAS) is used to identify seedlings from controlled crosses carrying alleles conferring superior phenotypes through the screening of markers tightly linked to those traits. In practice, it is possible to select plants at the seedling stage to enable the breeders to retain only germplasm conferring desirable traits from an early stage in the breeding pipeline. In recent years, MAS has become a valuable tool in breeding programs around the world. Usually MAS involves the screening of hundreds or thousands of seedlings and thus the main challenge faced is the development of a pipeline that begins with a "lab friendly" and unambiguous seedling configuration in greenhouse, which then allows the rapid collection of samples avoiding cross contamination, performs a fast, cost-effective, high-throughput method of DNA extraction to be used for a small number of PCRs, and utilizes robust, reliable, and easy to interpret molecular markers.

In this work we report on the development of a MAS pipeline at the Fondazione Edmund Mach (FEM) to enable the implementation of a time and cost effective MAS strategy for apple (*Malus pumila* Mill.).

MATERIALS AND METHODS

In 2013, ~6,500 seedlings sown in a greenhouse from 24 apple crosses between parental lines conferring disease resistance characteristics, fruit quality traits, or both. The seedlings were selected based on the genotypes associated to scab resistance, powdery mildew resistance and fruit quality (texture and storability) (Table 1).

The choice of the markers (seven for scab, two for powdery mildew, three for fruit quality) was based on published markers and previous experience in our institute (Table 2). The first step in the molecular analysis was the use of fluorescently labeled primers in order to multiplex multiple markers into single PCR reactions.

Lab work was performed using the 3G Plant PCR kit (Kapa Biosystems).

Pipeline:

1. "Quick'n'dirty" DNA extraction – KAPA 3G Plant PCR "Reducing contamination protocol":
   15 mm leaf disc + 200 µL TDS (50 mM Tris_HCl, 0.1 mM EDTA, 0.2x Kapa Enhancer); Boil 5 min at 95°C
2. DNA amplification (KAPA 3G Plant PCR kit – optimized)

PCR Mix		**PCR Cycle**	
KAPA Plant PCR Buffer 2X	0.5X	Initial denaturation	95°C _5 min
Forward Primer (10 µM)	0.3 µM	Denaturation*	95°C _0.20 s
Reverse Primer (10 µM)	0.3 µM	Annealing*	58°C _0.45 s
KAPA3G DNA Pol. 2.5U/µL	½U/50 µL	Extension*	72°C _1 min
H_2O	As required	Final extension	72°C _1 min
Extract DNA	1 µL	Hold	4°C _∞
Reaction volume	25 µL	* = 35 cycles	

3. Capillary electrophoresis of the PCR products on ABI 96-capillary 3730xl
   1 uL per PCR
   For each sample 9.35 µL Hi-Di formamide and 0.15 µl LIZ1200 (Life Tech.)
   For each sample 9.45 µL Hi-Di formamide and 0.05 µl LIZ500 (Life Tech.)
   Denaturation 3 minutes at 95°C.
4. Data Analysis with GeneMapper v.4 Software.

RESULTS AND DISCUSSION

Table 3 gives a brief description of the results for each marker used.

Both the microsatellite (SSR) and gene specific markers related to fruit quality performed well, producing scoreable, reproducible signals (Fig. 1) even for larger fragments.

Regarding the markers for resistance we found the major difficulties with the SCAR marker (sequence characterized amplified region) because of low signals (Fig. 3), monomorphic profile or intense electrophoretic smear (Fig. 4). Better results were obtained with an SSR marker designed for the same trait. In fact most SSRs resulted with good amplification profiles, apart from Ch02c02a (which produced a "hedgehog" profile) (Fig. 2).

The main problems encountered were associated with poor quality "quick-and-dirty" extractions, which are a particular problem for plants like apple, which are rich in polyphenols and polysaccharides; however, following optimization less than 10% of the seedlings had been re-sampled.

With the proposed protocol, we also reduced time and cost of the MAS analysis: there are no step of samples dilution from the extraction to the capillary electrophoresis, and the reduced volume of the reagents permit to perform more reactions without increased costs.

MAS involves large numbers of seedlings that have to be screened in a time efficient manner so only selected genotypes can be transplanted.

The numbers of reactions for the ~6,500 plants was around 8,000, from which 69.51% (about 4,500) of the seedlings carried resistance and/or quality traits.

We have demonstrated that marker assisted selection for apple fruit quality and disease resistance can be applied to the breeding program ongoing at FEM and in future years will improve in time and cost effectiveness.

Literature Cited

Bus, V.G.M., Rikkerink, E.H.A., Van de Weg, W.E., Rusholme, R.L., Gardiner, S.E., Bassett, H.C.M., Kodde, L.P., Parisi, L., Laurens, F.N.D., Meulenbroek, E.J. and Plummer, K.M. 2005. The *Vh2* and *Vh4* scab resistance genes in two differential hosts derived from Russian apple R12740–7A map to the same linkage group of apple. Mol. Breed. 15:103-116.

Costa, F., Stella S., Weg, W., Guerra, W., Cecchinel, M., Dallavia, J., Koller, B. and Sansavini, S. 2005. Role of the genes Md-ACO1 and Md-ACS1 in ethylene production and shelf life of apple (*Malus domestica* Borkh). Euphytica 141:181-190.

Dunemann, F., Peil, A., Urbanietz, A. and Garcia-Libreros, T. 2007. Mapping of the apple powdery mildew resistance gene *Pl1* and its genetic association with an NBS-LRR candidate resistance gene. Plant Breeding 126:476-481.

Longhi, S., Moretto, M., Viola, R., Velasco, R. and Costa, F. 2005. Comprehensive QTL mapping survey dissects the complex fruit texture physiology in apple (*Malus* × *domestica* Borkh*)*. J. Exp. Bot. 63(3):1107-1121.

Patocchi, A., Bigler, B., Koller, B., Kellerhals, M. and Gessler, C. 2004. *Vr2*: a new apple scab resistance gene. Theor. Appl. Genet. 109:1087-1092.

Tartarini, S., Gianfranceschi, L., Sansavini, S. and Gessler, C. 1999. Development of reliable PCR markers for the selection of the *Vf* gene conferring scab resistance in apple. Plant Breed. 118:183-186.

Vinatzer, B.A., Patocchi, A., Tartarini, S., Gianfranceschi, L., Sansavini, S. and Gessler, C. 2004. Isolation of two microsatellite markers from BAC clones of the *Vf* scab resistance region and molecular characterization of scab-resistant accessions in *Malus* germplasm. Plant Breeding 123:321-326.

Tables

Table 1. List of FEM apple crosses.

CROSS	SELECTED FOR:	GENE	
12.1	disesase resistance	Vf	Pl1
12.3	texture	Md-PG1	
12.4	texture and ethylene	Md-PG1	Md-ACO1
12.5	disesase resistance	Vf	
12.6	disesase resistance	Vf	
12.8	disesase resistance	Vf	
12.9	ethylene	Md-ACO1	
12.10	disesase resistance	Vf	
12.11	texture	Md-PG1	
12.12	ethylene	Md-ACO1	
12.14	disesase resistance	Vh4	
12.15	disesase resistance	Vh2	
12.16	disesase resistance and texture	Vf	Md-PG1
12.17	disesase resistance	Vf	Vr2
12.18	texture	Md-PG1	
12.19	disesase resistance	Vf	
12.20	disesase resistance	Vf	Pl1
12.21	disesase resistance and ethylene	Vf	Md-ACO1
12.24	disesase resistance	Vf	Vr2
12.27	ethylene	Md-ACO1	
12.28	disesase resistance and texture	Vf	Md-PG1
12.29	disesase resistance	Vf	
12.30	ethylene	Md-ACS1	
12.31	ethylene	Md-ACO1	

Table 2. List of genes and associated markers.

	GENE	MARKER	Reference
Resistance	Vf (Scab)	Chvf1 AI07	Vinatzer *et al.* 2004 Tartarini *et al.* 1999
	Vh4 (Scab)	CH02c02a	Bus *et al.* 2005
	Vh2 (Scab)	CH02b10 OPL19	Bus *et al.* 2005
	Vr2 (Scab)	CH02c02a CH02f06	Patocchi *et al.* 2004
	PI1 (Powdery Mildew)	Hi07f01 AT20	Dunemann *et al.* 2007

	GENE	MARKER	Reference
Quality	Md-PG1 (Texture)	PG1	Longhi *et al.* 2011
	Md-ACS1 (Ethylene)	ACS1	Costa *et al.* 2005
	Md-ACO1 (Ethylene)	ACO1	Costa *et al.* 2005

Table 3. Type and results for each marker.

GENE	MARKER	DYE	TYPE	RESULTS
Vf1	Chvf1	[FAM]	SSR	Good signals
	AI07	[FAM]	SCAR	Smear in amplification
Vh4	CH02c02a	[HEX]	SSR	Good signals
Vh2	CH02b10	[FAM]	SSR	Good signals
	OPL19	[HEX]	SCAR	Monomorphic
Vr2	CH02c02a	[HEX]	SSR	Difficult to score
	CH02f06	[NED]	SSR	Good signals
PI1	Hi07f01	[NED]	SSR	Good signals
	AT20	[HEX]	SCAR	Low signals - monomorphic
Md-PG1	PG1	[FAM]	SSR	Good signals
Md-ACS1	ACS1	[HEX]	ASO	Good signals
Md-ACO1	ACO1	[FAM]	ASO	Good signals

SSR (Simple Sequence Reapeat)
SCAR (Sequence Characterized Amplified Region)
ASO (Allele Specific Oligonucleotide)

Figures

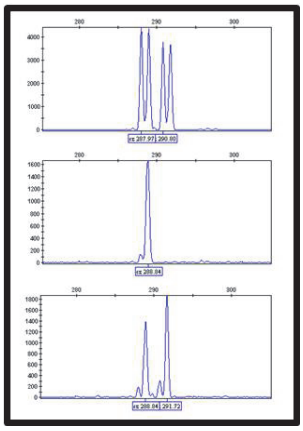

Fig. 1. Electropherogram of PG1 (SSR).

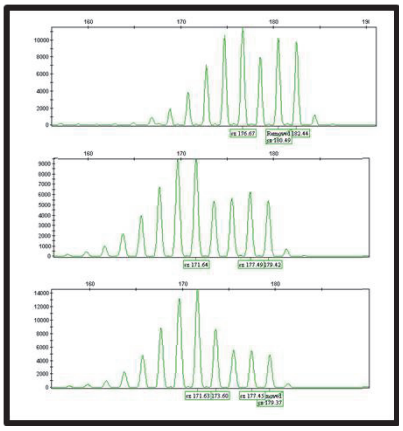

Fig. 2. Electropherogram of Ch02c02a (SSR)

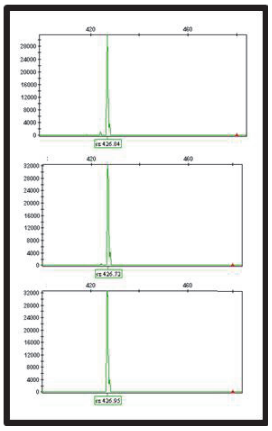

Fig. 3. Electropherogram of OPL19 (SCAR).

Fig. 4. Electrophoresis of Al07.