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Peach Rootstock Identification by DNA-Fingerprinting with Microsatellite (SSR) Markers

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Abstract

Peach (*Prunus persica* [L.] Batsch) rootstocks are usually propagated from seed. Seedlings are difficult to distinguish morphologically, and once grafted, no above ground material is available for visual identification. To avoid misidentification and to protect plant varieties and patents, DNA fingerprinting was investigated as a robust rootstock identification tool. The objective of this study was to distinguish progeny from among seven peach seedling rootstocks: Bailey, Halford, Lovell, Nemaguard, Nemared, Guardian® (selection 3-17-7) and S-37. We initially screened 102 *Prunus* microsatellite (SSR) markers on Lovell, Nemaguard, Nemared and selection 3-17-7. Seventy-five markers showed polymorphism among these rootstocks. The polymorphic markers were then used to screen Bailey, Halford and S-37. Based on the patterns of amplified DNA fragments (two seedlings from each rootstock were tested), eight SSR-markers reproducibly divided the seven rootstocks into as many as five groups. It was necessary to use a multiplex approach to uniquely identify each rootstock because no single SSR locus evaluated thus far was able to differentiate all seven genotypes. To confirm the identity of the SSR markers, we cloned the polymorphic DNA fragments amplified by one of the eight polymorphic SSR primers, which was developed for an AC-enriched sequence isolated from almond. DNA sequence analysis showed that the amplified fragments shared a common AC-enriched repeat with copy number ranging from 5 to 14. Taken together, these results demonstrate that this microsatellite-based DNA fingerprint system has great potential for peach rootstock identification.

Peach (*Prunus persica* [L.] Batsch) is an economically important fruit tree species in the Rosaceae. The annual world peach production is approximately 10 million metric tons (5), with 1.3 metric tons produced in the United States alone. In commercial production, peach trees are actually composed of two genotypes, the scion and the rootstock. Scion cultivars are selected and released for their agronomic traits such as fruit size, taste and skin color. In contrast, rootstocks are selected and released for traits such as biotic or abiotic stress resistance or tree vigor in specific environments.

There are five or six peach seedling rootstocks commonly used in the United States. These are Lovell, Halford, Nemaguard, Nemared, Bailey and Guardian® (selection 3-17-7). Another former peach rootstock that is a distant parent of Guardian® is S-37. All of these rootstocks have compatibility with many scion cultivars and some possess specific pest or disease resistance to nematodes and/or peach tree short life. Our research efforts focused on these seven rootstocks.

Clearly, rootstocks play an important role in commercial peach production. Unfortunately, peach rootstock seedlings are very difficult to identify using morphological traits. Also, once grafted, any characteristic leaf, floral or fruit traits of the rootstock phenotype will not be visible. However, DNA fingerprinting could provide evidence to demonstrate that apparently identical rootstocks are in fact genetically distinct. Rootstock identification is important for peach breeders and growers. It provides evidence to protect plant variety protection (PVP) patents for breeders, and growers can be more confident in their purchases since there is a method to identify and confirm rootstocks in their orchards.

Many DNA-based marker systems can be used for fingerprinting. Restriction fragment length polymorphism (RFLP) has been used for cultivar identification in rose (11) and tall fescue (2). Amplified fragment length poly-

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morphism (AFLP) has been used successfully to identify apricot (7) and mango (13) cultivars. Randomly amplified polymorphic DNA (RAPD) has been used to identify strawberry (8) and calla lily (9) cultivars.

Microsatellites (Simple Sequence Repeats, SSRs), another PCR-based system like RAPDs and AFLPs, have been used frequently in recent years for linkage map construction and DNA fingerprinting. SSRs are DNA fragments consisting of 1 to 6 nucleotide repeats distributed throughout the genome. SSRs show variation in fragment length based on the repeat copy numbers in one genotype compared to another. This variation can be used for molecular characterization. In contrast with the other marker systems described above, SSRs have high reproducibility and are easily detectable. Hundreds of SSR markers have been developed in the Rosaceae and used widely to characterize *Prunus* species such as apricot (16) and almond (1).

In this study, we used SSR markers to identify seedlings from seven peach rootstock genotypes. Our results demonstrated that this SSR marker system had the potential to unambiguously identify peach seedling rootstocks at the molecular level.

Materials and Methods

Peach rootstock accessions and genomic DNA isolation. Leaf tissue from seven peach rootstock cultivars (Lovell, 3-17-7, Nemaguard, Nemared, S-37, Halford and Bailey), and from two additional seedlings of each rootstock was collected during the summers of 2005 and 2006. All samples were collected from Musser Fruit Research Center near Clemson University (Clemson, SC). Five grams of young leaf tissue of each rootstock accession were frozen in liquid nitrogen and stored at -80°C.

Genomic DNA was isolated from frozen leaf tissue (1g fresh weight) using a modified sodium dodecyl sulfate (SDS) method (3). DNA concentrations were measured using picogreen dye (Invitrogen, Carlsbad, CA) on a TBS-380 fluorometer (Turner BioSystems, Sunnyvale, CA). For each sample, the genomic DNA was then diluted to 10 ng/ul.

SSR markers and PCR amplification. The 102 SSR markers investigated were developed from four *Prunus* spp. (i.e. almond, apricot, cherry and peach) (4, 14, 18, 19). The primer sequences were obtained from the Genome Database for Rosaceae (GDR) (6). The final 13 polymorphic markers selected for fingerprinting were named Plm1 to Plm 13 (Plm for PoLyMorphic marker). The forward primer of each marker pair was radiolabeled with [γ-P^{33}] ATP by 5'-end labeling reaction using a modified version of the process found in Promega technical bulletin # 519 (15).

Denaturing polyacrylamide gel electrophoresis. Samples were size fractionated in a 6% denaturing polyacrylamide gel on a vertical gel electrophoresis rig. After 2 hours at 80 watts, the gel was transferred to 3MM Whatman filter paper and dried for 90 minutes using a FB-GD-45 gel dryer vacuum system (FisherBiotech, Wembley, West Australia, Australia). The dried gel was exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY) at room temperature with the exposure time varying from 1 to 5 days.

Sequencing PCR-amplified polymorphic fragments. Polymorphic DNA fragments amplified by one SSR marker, Plm11, were cloned and sequenced to confirm their identity as SSRs. The amplified DNA fragments were separated in 3% Nusieve (Cambrex, Rockland, ME) agarose gel and stained with ethidium bromide. The polymorphic fragments were cut from the gel, and purified using a rapid gel extraction system (Marligen Biosciences, Ijamsville, MD).

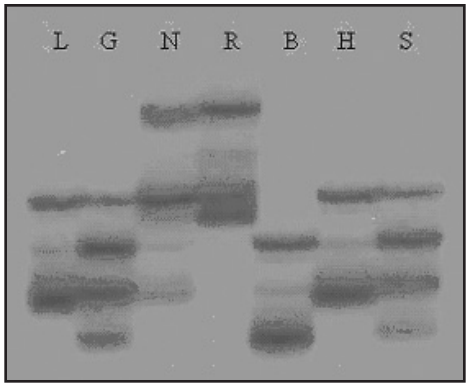
The fragments were ligated into a TA cloning vector, pGEM-TEasy (Promega, Madison, WI), following the manufacturer's instructions. Ligated plasmids were transformed into *E. coli* strain DH5αMCR (12) by a heat shock protocol (10). Plasmid DNA from putative transformants was isolated using an alkaline lysis plasmid miniprep protocol (17).

Sequencing reactions were set up using a SequiTherm Excel™ II DNA sequencing kit (Epicentre® Biotechnologies, Madison, WI).

Sequencing products were analyzed using a LI-COR 4200 automated sequencer (LI-COR, Lincoln, NE).

Results and Discussion

The 102 SSR markers were initially screened against four rootstocks: Lovell, 3-17-7, Nemaguard and Nemared. Twenty-seven markers amplified monomorphic patterns and thus, these markers did not differentiate among the rootstocks. Seventy-five markers showed polymorphisms among the four rootstocks and divided the four rootstocks into two to four groups. Based on the amplification patterns, twenty of the seventy-five polymorphic markers appeared to divide the four rootstocks into four groups. These twenty were screened against all seven rootstocks. Seven of the twenty SSR markers were less informative because they did not amplify a new pattern from the three additional rootstock genotypes tested. The remaining thirteen polymorphic SSR markers divided the seven rootstocks into groups of five, six or seven. To illustrate, Fig. 1 shows the polymorphic pattern amplified by SSR marker Plm1. Nemaguard, Nemared and Bailey each had unique patterns. In addition, Lovell and Halford had a common, but distinct pattern and 3-17-7 and S-37 shared a pattern but it differed from that of all the others. Thus, Plm1 divided the seven rootstocks into five groups.



L: Lovell; G: 3-17-7; N: Nemaguard; R: Nemared; B: Bailey; H: Halford; S: S-37

Figure 1. Polymorphic pattern amplified by SSR Plm 1 from the DNA of 7 different peach rootstocks.

The reproducibility of the patterns amplified by the thirteen polymorphic SSR markers was tested. Two additional seedling accessions of each rootstock were screened with the thirteen SSR primer pairs. Five of the thirteen markers did not produce consistent patterns between the seedlings of each rootstock and each original accession. Thus, these five markers were not helpful to this study and were no longer

Table 1. Reproducibility of amplification patterns for the eight consistent polymorphic SSR markers used to differentiate the 7 peach rootstocks and seedling accessions.

SSR marker	No. of groups	Amplification patterns of rootstocks[z]					Inconsistent patterns between original and new accessions[y]
		a	b	c	d	e	
Plm1	4	R	B	H		S	L^{H}, G^{S}, N
Plm3	3	R	B, H	S			$L^{B/H}$, G, N
Plm4	4	L, H	G	B	S		N, R^{G}
Plm6	5	L, B, H	G	N	R		S
Plm7	4	L, H	G, S	N, R	B		
Plm9	4	L, N, H	G, B	R	S		
Plm11	3	L, H	R, B	S			G, N
Plm12	4	L, N, S	G, R	B	H		

[z] L: Lovell; G: 3-17-7; N: Nemaguard; R: Nemared; B: Bailey; H: Halford; S: S-37. Pattern "a" amplified from one marker is different from pattern "a" amplified from any other markers (similarly for patterns b, c, d or e)

[y] Rootstocks with a superscript(s) share a common pattern with the corresponding rootstock(s) for that particular SSR marker

used. The other eight markers showed consistent patterns between some of the original rootstocks and their corresponding seedlings. The results are summarized in Table 1.

Four of the eight SSR markers (i.e., Plm6, Plm7, Plm9 and Plm12) amplified consistent patterns between the original and its additional two accessions among all seven rootstocks (Table 1). These four markers can be used to subgroup all seven rootstocks. For example, marker Plm6 amplified five patterns among all seven rootstocks. Lovell, Bailey and Halford share the same pattern and, therefore, group together. On the other hand, Nemared, Nemaguard, 3-17-7 and S-37 each have their own unique patterns, and thus group separately.

The other four markers (i.e., Plm1, Plm3, Plm4 and Plm11) amplified consistent patterns among the original accession and its seedlings for four or five of the rootstocks, but produced inconsistent patterns for the remaining rootstock accessions. Thus, these four markers can be used only to subgroup the rootstocks with consistent patterns. For example, Plm1 showed consistent patterns only among the accessions of Nemared, Bailey, Halford and S-37, but inconsistent patterns among the Lovell, 3-17-7 and Nemaguard accessions (Table 1, last column). Furthermore, Nemared, Bailey, Halford and S-37 each had a unique Plm1 pattern and could be grouped separately. Based on our overall results, the eight selected markers could divide the seven rootstocks into as many as five groups.

At the present time, these seven rootstocks could not be uniquely identified by a single SSR marker. Nonetheless, combinations of SSR markers can be used to differentiate each of the seven rootstocks. At least two markers must be selected in order to uniquely identify each of the seven rootstocks. For example, Plm6 identifies 3-17-7, Nemaguard, Nemared and S-37 because each of these rootstocks has a unique pattern for this SSR marker. Then Plm12 can be used to identify Lovell, Bailey and Halford, each with their own unique pattern. In addition to SSR combination Plm6/Plm12, other marker combinations can be used to confirm the results (e.g., Plm7/Plm12).

These eight selected markers were developed from almond, cherry and apricot. Although these markers amplify polymorphic fragments in peach rootstock, an additional SSR marker developed from peach might be the single perfect marker. Furthermore, an additional 10 seedlings of each rootstock from independent sources will be used to corroborate the results obtained in the initial study. To confirm that the amplified polymorphic DNA fragments originated from microsatellites, we cloned DNA fragments amplified by SSR marker Plm11 (an AC-enriched sequence, approximately 160 bp in length that was initially developed from an

Table 2. Sequences of the fragments amplified by Plm 11 and subsequently cloned, showing the selection from which the fragment originated, its length, and the number of AC repeats it contained.

DNA fragment origin	Fragment length (bp)	Number of AC repeats
Bailey	133	6
Halford	135	7
Lovell	137	7
Nemaguard-1[z]	143	11
Nemaguard-2[z]	134	5
Nemared	133	6
S-37	133	6
3-17-7-1[y]	157	14
3-17-7-2[y]	143	12

[z] Suffixes 1 and 2 are separate fragments amplified from Nemaguard rootstock

[y] Suffixes 1 and 2 are separate fragments amplified from rootstock 3-17-17

almond genomic library). Two DNA fragments from 3-17-7 and Nemaguard and one DNA fragment from each of the other five rootstocks were sequenced (Table 2). Sequencing results showed that these 9 cloned fragments varied in length from 133 bp to 157 bp. All 9 clones contained the AC-repeat. The large 157 bp fragment cloned from 3-17-7 had the greatest number of AC repeats (copy number = 14). Thus, as expected, the 134 bp fragment cloned from Nemaguard had the least number of AC repeats (copy number = 5). These results confirm that the amplified DNA fragments are in fact SSRs, and the amplified fragments showed variation in fragment length based on difference in the number of repeat copies, which can be used to help identify the different rootstocks.

Conclusion. With the exception of Nemared, which bears red leaves, the other six peach rootstocks are difficult to identify morphologically. Each of the eight selected markers can divide the seven rootstocks into subgroups. Up to this point, no single SSR could uniquely distinguish all seven rootstocks. However, choosing marker combinations based on the alleles they detect can distinguish each rootstock from the other six. Our initial study demonstrates that the SSR marker system used here has the capability to differentiate mislabeled rootstock seedlings, identify unknown rootstocks and to provide evidence for plant variety protection or patent protection.

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