

# Genetic Mapping of the *evergrowing* Gene in Peach [*Prunus persica* (L.) Batsch]

Y. Wang, L. L. Georgi, G. L. Reighard, R. Scorza, and A. G. Abbott

In temperate locations, terminal apices on evergrowing (also called evergreen) peach trees keep growing in winter until killed by low temperatures, while the lateral buds go into dormancy. A recessive allele of a single gene (*evergrowing* or *evg*) controls this trait in peach. The amplified fragment length polymorphism (AFLP) technique and bulked segregant analysis were applied to construct a local genetic linkage map for the *evg* gene from the cross Empress op op dwarf  $\times$  Evergrowing (P.I. 442380). This map, comprising nine AFLP markers and the *evg* locus, covers a total genetic distance of 79.3 cM. Four dominant AFLP markers (EAT/MCAC, ETT/MCCA2, EAT/MCTA, and ETT/MACC) were linked to the *evg* locus at distances of 1, 5.3, 6.7, and 11.7 cM, respectively. EAT/MCAC and EAT/MCTA were converted into polymorphic sequence-tagged sites. Microsatellite markers in the *evg* region were developed from peach bacterial artificial chromosome (BAC) clones that hybridized to the AFLP marker fragments. Using three microsatellite anchor markers (pchgms12, pchgms17, and pchgms19), the local genetic linkage map was integrated into one minor linkage group of a previously constructed peach rootstock genetic linkage map. Three AFLP markers from the rootstock genetic linkage map were found linked to the *evg* locus.

Evergreen genotypes of peach [*Prunus persica* (L.) Batsch] have been identified in Mexico, in areas where killing frosts do not occur. In Mexico, terminal growth on evergreen trees is continuous under favorable environmental conditions and leaves are retained until they are lost due to drought and/or disease (Diaz 1974). We initiated the procedure of changing the name from "evergreen" to "evergrowing" because evergrowing describes the continuous growth of terminal apices better than evergreen does. Evergrowing peaches can be harvested twice per year in Mexico, but only once per year in temperate regions. In temperate locations, the terminal apices on evergrowing trees keep growing in winter until killed by low temperatures, while the lateral buds go into dormancy (Rodriguez et al. 1994). Cold hardiness develops in both evergrowing and deciduous genotypes, but the maximum hardiness level in deciduous trees is more than twofold that of evergrowing trees (Arora and Wisniewski 1994, 1996). Flower buds on evergrowing trees require less chilling hours and bloom earlier than those on deciduous trees. An evergrowing genotype P.I.

442380 was introduced into the United States as seeds. Hybridization of the evergrowing germplasm with deciduous parents produced F<sub>1</sub> trees that were deciduous and F<sub>2</sub> seedlings that segregated as 3 deciduous:1 evergrowing, suggesting that a recessive allele of a single gene, *evergrowing* or *evg*, controls the evergrowing trait (Rodriguez et al. 1994). Therefore, evergrowing peach can be used for peach breeding programs and provides a useful system for studying both cold hardiness and dormancy where these two processes develop independently.

Peach is an important member of the Rosaceae family, which contains many fruit, nut, and ornamental species. Currently many research efforts have been focused on building saturated genetic linkage maps from which agronomically important traits can be located. Several genetic linkage maps of intraspecific and interspecific crosses in *Prunus* L. have been constructed (Abbott et al. 1998; Chaparro et al. 1994; Dirlewanger et al. 1996, 1999; Foolad et al. 1995; Joobeur et al. 2000; Quarta et al. 1998; Rajapksse et al. 1995; Sosinski et al. 1998; Viruel et al. 1998). DNA markers and genome regions

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**Table 1. Primers used for the PCR amplification of STS and SSR markers**

Markers	Fragment sequence GenBank accession no.	Primer sequences	Ta (°C)
EAT/MCAC STS	AF516359	F: 5'-AGCATTGAAGACTTTCAGCAG-3' R: 5'-AGCATGTATTCATGGCAAAA-3'	55
EAT/MCTA STS	AF516360	F: 5'-TGCCCTCAGATTAGATTGGGA-3' R: 5'-TTTGGACTCTGGGAATTGACC-3'	56
pchgms12	AF516361	F: 5'-CGACACTTAGCTAGAAAGTTGCCTTA-3' R: 5'-TCAAGCTCAAGGTACCAGCA-3'	55
pchgms13	AF516362	F: 5'-CACAGCACCTAGAAATGGAA-3' R: 5'-ACTATATAGTGGAGAATGTC-3'	49
pchgms17	AF516363	F: 5'-ATGCACTCAAGTGGCAAGC-3' R: 5'-GGTTTTGAGCAAAGATGCAC-3'	55
pchgms19	AF516364	F: 5'-GAAGCAACAAGTTGGTGCAAA-3' R: 5'-GCACAACATGGACCAATGA-3'	55

that are conserved among species are very useful in map-based cloning of genes, marker-assisted selection (MAS) in breeding programs, and comparative mapping among related species. Lu et al. (1998) developed a peach genetic linkage map for the K62-68 family of the rootstock cross Nemared × Lovell. As one of the longest whole genome genetic linkage maps for *Prunus* species, this map has 15 linkage groups, covering 1297 cM, with an average interval of 9.1 cM.

Amplified fragment length polymorphisms (AFLPs), based on selective polymerase chain reaction (PCR) amplification of restriction fragments from digested genomic DNA, are widely utilized markers for constructing genetic linkage maps (Vos et al. 1995). AFLP markers are reliable, high throughput, and cost effective (Ballvora et al. 1995; Hansen et al. 1998; Jones et al. 1997). However, because they are not always transferable among crosses and species, it is desirable to convert AFLP marker locations into markers such as sequence tagged sites (STSs) or SSR markers (Wang et al. 2002). In order to find DNA markers that are tightly linked to a target qualitative gene, bulked segregant analysis (BSA) is a very effective approach, especially for species in which it is hard to develop near isogenic lines (NILs) (Baranger et al. 1997; Eastwood et al. 1994; Michelmore et al. 1991; Quarrie et al. 1999; Yang et al. 1997). In fruit trees, coupling AFLP technology with BSA provides a rapid approach for obtaining markers linked closely to a trait of interest.

For breeding applications in perennial species, molecular markers, closely linked to target traits, have special advantages in MAS. They are highly efficient for screening large populations, and depending on the marker type, highly reproducible across laboratories (Abbott 2002). In the case of *evg*, field

evaluation of the evergrowing trait requires 2–3 years after seed germination. The knowledge of linked molecular markers can dramatically reduce the labor and time required to develop cultivars and improve the accuracy of selection in peach breeding programs.

Since the exact role of this *evergrowing* gene and the gene products were unknown in peach, a map-based cloning strategy was taken for isolating this gene. Molecular markers were also developed during this process for MAS in peach breeding programs. In this article we present the construction of a local genetic linkage map for the *evg* gene, using AFLP and BSA, the characterization of linked AFLP markers, and the integration of this local map into the peach rootstock genetic linkage map.

## Materials and Methods

### Plant Material and DNA Extraction

At the ARS-USDA Appalachian Fruit Research Station, Kearneysville, WV, deciduous Empress op op dwarf and Evergrowing peach (P.I. 442380) were crossed to produce F<sub>1</sub> hybrids. The female parent Empress op op dwarf was a dwarf seedling obtained after two generations of open pollination of Empress dwarf, with a high chilling requirement (about 850 h). The pollen parent is an ARS-USDA germplasm accession P.I. 442380, collected in 1979 as seed from a local market in Tupachula, Chiapas, Mexico (latitude 15°N, longitude 92°W). Growth types of the F<sub>2</sub> trees were classified as evergrowing if terminal growth continued at the end of the fall until killed due to freezing and/or if terminal growth initiated earlier in spring than lateral growth (Rodriguez et al. 1994). One hundred nine trees with consistent phenotypes in 1997 and 1998 were randomly selected from the F<sub>2</sub> population of 314 trees for genetic mapping of the *evg*

gene. Because the original parent and F<sub>1</sub> trees were dead, the same P.I. 442380 clone from Byron, GA, and Empress trees bought from a wholesale nursery (L. E. Cooke Company, Visalia, CA) were used for this study. Fifty-five F<sub>2</sub> trees from a Nemared × Lovell peach cross (the K62-68 family), previously used for constructing a peach rootstock genetic linkage map (Lu et al. 1998) and mapping SSR markers, were used for map merging.

Genomic DNA was extracted from young leaves of the 109 F<sub>2</sub> trees and the parent trees from the cross Empress op op dwarf × Evergrowing, and 55 F<sub>2</sub> trees from the cross Nemared × Lovell using a modified 2× CTAB method (Eldredge et al. 1992). Working solutions of genomic DNA at 100 ng/μl and 10 ng/μl in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) were prepared for AFLP and SSR analyses, respectively.

### Bulked Segregant Analysis

Bulked segregant analysis was employed to quickly identify AFLP markers linked to the *evg* gene. Four bulks were established using different F<sub>2</sub> individuals from the same cross: bulk 1 (B1) and bulk 2 (B2) were formed by pooling DNA from 10 and 15 F<sub>2</sub> evergrowing individuals, respectively, and bulk 3 (B3) and bulk 4 (B4) were formed by pooling DNA from 10 and 15 F<sub>2</sub> deciduous individuals, respectively. AFLP analysis was first conducted with B1 and B3 to screen the possible primer combinations. Primer pairs amplifying bands that were in the deciduous bulk and parent, but absent from the evergrowing bulk and parent, were defined as potential primer combinations. In order to include all possible primer combinations, those with very faint bands present in the evergrowing bulk were also selected. These potential primer combinations were confirmed by AFLP analysis of B2 and B4, and were then used for AFLP analysis of individual progeny plants.

### AFLP Analysis

AFLP core reagent and starter primer kits for small genomes were obtained from Life Technologies (Invitrogen, Carlsbad, CA) except for the following: AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), γ-<sup>33</sup>P [ATP] (NEN, Boston, MA), and additional primers (Integrated DNA Technologies, Coralville, IA). AFLP reactions were conducted using procedures modified from Vos et al. (1995). The sequences of the adapters and

preamplification primers are shown in Table 1. The selective primers included 12 *EcoRI* primers, 10 *PstI* primers, 4 *KpnI* primers, and 26 *MseI* primers, with two, two, two, and three selective bases, respectively. Polymerase chain reaction (PCR) products were mixed with tracking dye, denatured at 94°C, and electrophoresed on 6.0% denaturing gels (20:1 acrylamide-bisacrylamide, 7.5 M urea) using 1× TBE buffer on a BioMax STS-451 vertical sequencing gel electrophoresis unit (Kodak, Rochester, NY). Gels were run at constant power (70 W) for 2.5 h, vacuum dried, and exposed to BioMax MR film (Kodak, Rochester, NY) for 2–4 days.

Designation of individual AFLP markers was based on the primers used. All markers were scored twice independently, and the final data were used for further analysis. All AFLP markers were evaluated by chi-square tests for goodness-of-fit against a 3:1 ratio ( $P \leq 0.05$ ). A local genetic linkage map for the *evg* gene was built using MapMaker software (version 2.0) for Macintosh (Lincoln et al. 1992), using a minimum logarithm<sub>10</sub> of odds (LOD) score of 4.0, maximum  $\theta$  of 0.25 (recombination frequency), and the Kosambi mapping function.

#### Conversion of AFLP Markers to STS Markers

AFLP marker bands were cut from dry acrylamide gels and dissolved in 100  $\mu$ l ddH<sub>2</sub>O overnight at 4°C to extract the DNA. Solubilized DNA from the AFLP bands was amplified with preamplification primers in 25  $\mu$ l containing: 10  $\mu$ l template DNA, 50 ng of each primer, 1.5 units of *Taq* DNA polymerase, 1× PCR buffer, 5 mM MgCl<sub>2</sub>, and 200  $\mu$ M of each dNTP. The PCRs were amplified for 35 cycles of 1 min at 94°C, 1 min at 56°C, and 2 min at 72°C. The PCR products were purified using Centricon 100 columns (Millipore, Bedford, MA), then cloned into a pGEM easy vector using a TA cloning kit (Promega, Madison, WI). The insert DNA was sequenced using a Dye Terminator Cycle DNA sequencing kit (Perkin-Elmer/Cetus) and M13 forward and reverse primers. Pairs of 20-mer oligonucleotide primers for STSs were designed according to sequences of the AFLP markers using the online program Primer 3 (Rozen and Skaletsky 1998) and purchased from Integrated DNA Technologies. The STS markers were amplified from the Evergrowing and Empress DNAs and the F<sub>2</sub> progeny using the designed primers (Table 2).

**Table 2. Adapters and preamplification primers used in the AFLP analysis**

Primers and adapters	Sequences
<i>MseI</i> adapter	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
<i>EcoRI</i> adapter	5'-CTCGTAGACTGCGTACC-3' 3'-CTGACGCATGGTTAA-5'
<i>KpnI</i> adapter	5'-ACACTCGTAGACTGCGTAC-3' 3'-TGTGAGCATCTGACG-5'
<i>PstI</i> adapter	5'-CTCGTAGACTGCGTACATGCA-3' 3'-CATCTGACGCATGT-5'
Preamplification primers	
M-C	5'-GATGAGTCCTGAGTAAC-3'
M-G	5'-GATGAGTCCTGAGTAAG-3'
M-A	5'-GATGAGTCCTGAGTAAA-3'
E-0	5'-GACTGCGTACCAATTC-3'
K-A	5'-TCGTAGACTGCGTACCA-3'
P-0	5'-GACTGCGTACATGCAG-3'

#### SSR Development and Linkage Analysis

SSR sequences were identified from peach BAC clones according to the strategy in Wang et al. (2002). Primers for amplifying the SSR markers were designed according to sequences flanking the SSRs using the online program Primer 3 (Rozen and Skaletsky 1998). SSR assays were carried out as in Sosinski et al. (2000), using optimal annealing temperatures according to the primers' melting temperatures. SSR markers were amplified from 109 F<sub>2</sub> individuals of the Empress op op dwarf × Evergrowing family and 55 F<sub>2</sub> trees from the K62–68 family. Linkage analysis for each population was performed with combined SSR and AFLP datasets for the F<sub>2</sub> progenies using MapMaker (version 2.0) for Macintosh (Lincoln et al. 1992).

#### Integration of the Local Genetic Linkage Map into the Rootstock Map

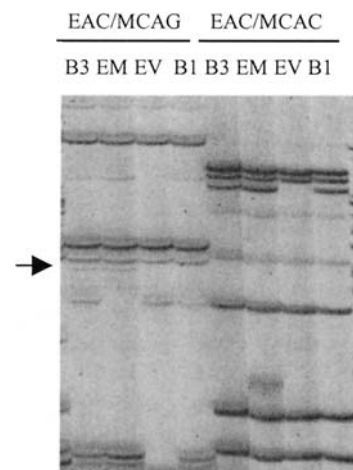
SSR markers polymorphic in both the K62-68 family and the Empress op op dwarf × Evergrowing family were used as anchor SSR markers to integrate the *evg* local genetic linkage map into the peach rootstock map. Joinmap 2.0 (Stam 1993) was used to construct the combined map, with a minimum LOD score of 3.0, maximum  $\theta$  (recombination frequency) of 0.25, and Kosambi mapping function.

#### Results

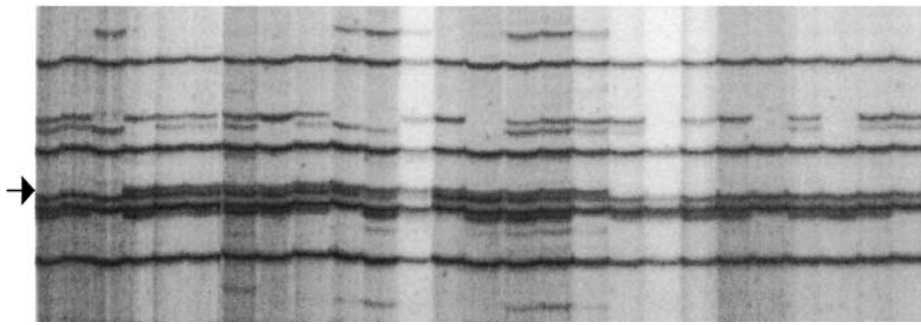
##### AFLP Analysis and Construction of the Local Genetic Linkage Map

Most of the primer combinations displayed distinct bands of varying sizes (Figures 1 and 2). However, some primer combinations (P+2/M+G+2, K+2/M+G+2, and P+2/M+A+2) produced abnormal patterns, such as double bands, faint bands, clustered bands, a few very strong bands, etc. Thus, they were not used. After screening 536

primer combinations in the B1 and B3 bulks and the Empress and Evergrowing (Figure 1), 64 primer combinations produced bands that were present in the Empress and deciduous bulk B3, but were absent from the evergrowing parent and absent or very faint in the evergrowing bulk B1. These candidate primer combinations were further tested in the B2 and B4 bulks. Overall 14 primer combinations (none of the 64 *KpnI/MseI* combinations, one of the 160 *PstI/MseI* combinations, and 13 of the 312 *EcoRI/MseI* combinations, respectively) had bands only in the B3 bulk and Empress, but not in the evergrowing parent and bulks, which suggested they were contributed from the female Empress op op dwarf parent. These 14 primer combinations were selected to run AFLP analysis in the 109 F<sub>2</sub> progeny (Figure 2). The *PstI/MseI* marker (PCG/MCAG) was not as-



**Figure 1.** AFLP gel autoradiogram of bulk segregant analysis from the first screening of primer combinations using the evergrowing bulk (B1), deciduous bulk (B3), evergrowing parent (EV), and deciduous Empress (EM). The arrow indicates AFLP fragment EAC/MCAG, which only appeared in the deciduous Empress and bulk (B3) but not in the evergrowing parent (EV) and bulk (B1).

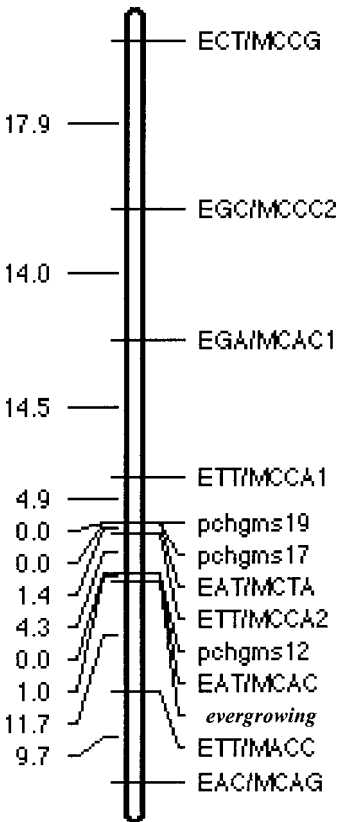


**Figure 2.** Autoradiogram showing the AFLP marker EAT/MCAC (indicated by the arrow) linked to the *evg* locus. Evergrowing bulks B1 and B2 have 10 and 15 F<sub>2</sub> evergrowing individuals, and deciduous bulks B3 and B4 have 10 and 15 F<sub>2</sub> deciduous individuals, respectively. EV is the evergrowing parent (Evergrowing), EM is the deciduous Empress (similar to female Empress op op dwarf parent), and lanes labeled from 53 to 18 are F<sub>2</sub> individuals of the Empress op op dwarf × Evergrowing cross.

signed to the same linkage group as the *EcoRI/MseI* markers using a maximum recombination value of 0.25.

In chi-square tests, the evergrowing phenotype segregated as 3:1 (deciduous:evergrowing). Four of the 13 AFLP markers had segregation patterns skewed away from a 3:1 ratio, two significantly at  $P = .05$  and

two significantly at  $P = .01$ , all of them skewed toward evergrowing. Although linked to the *evg* gene locus, these four markers were not used in the local map (Figure 3). The local genetic linkage map for this region included nine dominant AFLP marker loci and the *evg* locus, covering a total genetic distance of 79.3 cM. The AFLP marker EAT/MCAC was linked to *evg* at a distance of 1 cM. All deciduous F<sub>2</sub> progeny and Empress had this marker fragment, while it was absent from the evergrowing parent and all evergrowing F<sub>2</sub> progeny except one (tree no. 110). Two other AFLP markers, ETT/MCCA2 and EAT/MCTA, were also closely linked to *evg* on the same side as EAT/MCAC, at distances of 5.3 cM and 6.7 cM, respectively (Figure 3). On the other side, marker ETT/MACC was mapped 11.7 cM from *evg*.



**Figure 3.** Local linkage map of the *evg* gene region, constructed from the cross Empress op op dwarf × Evergrowing. Genetic distances are in centimorgans (cM) (left). AFLP markers (right) are named after the digestive enzymes and the selective nucleotide bases. Loci for SSR markers pchgms12, pchgms17, and pchgms19 are shown.

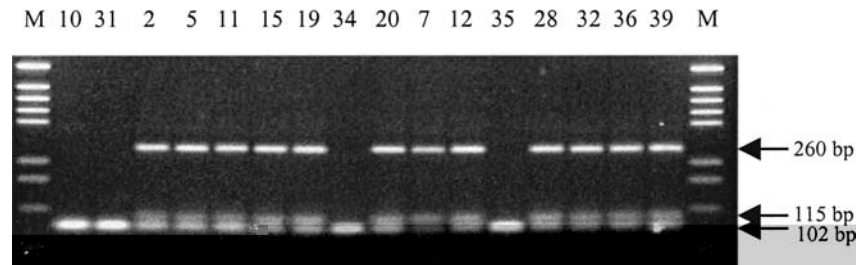
102 bp) were amplified from the deciduous Empress and F<sub>2</sub> individuals, while only the 102 bp fragment was amplified from the evergrowing parent and F<sub>2</sub> individuals (Figure 4). Comparison with the largest fragment showed that the 102 bp fragment resulted from a 158 bp deletion and the 115 bp fragment resulted from a deletion of 145 bp. Nucleotide substitutions also existed in these two small fragments (Figure 5). Because heterozygous individuals could not be distinguished from deciduous homozygotes, this AFLP marker was converted into a dominant STS marker.

The EAT/MCTA AFLP marker was sequenced and internal primers were designed to amplify a single fragment from each individual (Figure 6). Compared to the fragment from the evergrowing individual, the fragment amplified from the deciduous individual had a single nucleotide insertion (position 30), an A/C conversion (position 43), and a C/T conversion (position 301). A *DraI* recognition sequence (TTTAAA) present in the fragment from the evergrowing individual was absent in the fragment from the deciduous individual. After *DraI* digestion, the fragment from the evergrowing individual could generate two fragments, 325 bp and 40 bp. Before digestion, all F<sub>2</sub> individuals displayed similar-size fragments (Figure 7A). After digestion (Figure 7B), evergrowing individuals only had the small band (325 bp), since 40 bp fragments could not be visualized on the gel, homozygous deciduous individuals only had the large band (366 bp), and heterozygous deciduous individuals had both bands. Therefore, the EAT/MCTA AFLP marker was successfully converted into a codominant STS marker.

Two other AFLP markers, ETT/MCCA2 and ETT/MACC, were also cloned and sequenced (data not shown). Internal primers were developed and STS frag-

**Conversion of AFLP Markers to STS Markers**

The EAT/MCAC AFLP marker was sequenced and internal primers were designed to amplify this fragment from deciduous and evergrowing F<sub>2</sub> individuals. Three DNA fragments (260, 115, and



**Figure 4.** EAT/MCAC STS marker fragments amplified from F<sub>2</sub> individuals of the cross Empress op op dwarf × Evergrowing (lanes labeled from 10 to 39) using designed primers. Evergrowing F<sub>2</sub> individuals (lanes 10, 31, 34, 35) only have one small fragment (102 bp), while deciduous F<sub>2</sub> individuals (other lanes) have three fragments (260 bp, 115 bp, and 102 bp). Marker (M) lanes contain the pGEM DNA marker.

DEC-1	<u>AGCATTGAAG</u>	<u>ACTTTCAGCA</u>	<u>GGAATATGCA</u>	TCCTTGCTCT	40
DEC-2	*****	*****	*****AT	***A*****	40
DEC-3	*****	*****	*****C**	*C*A*****	40
EVG	*****	*****	*****C**	*G*A*****	40
DEC-1	<u>CAAGTATGAG</u>	<u>AAAGAATTC</u>	<u>ATGAAATGGG</u>	GATAACATTA	80
DEC-2	*****	*****	*****	*****	46
DEC-3	*****	*****	*****	*****	42
EVG	*****	*****	*****	*****	42
DEC-1	<u>TTTTGTTATC</u>	<u>CCCGGCCAAT</u>	<u>AAAGTTATGA</u>	CACTCGTGAT	120
DEC-2	*****	*****	*****	*****	46
DEC-3	*****	*****	*****	*****	42
EVG	*****	*****	*****	*****	42
DEC-1	<u>AACACTTCTA</u>	<u>CGTGGCATA</u>	<u>TATTATTGGT</u>	TAGGGATAGC	160
DEC-2	*****	*****	*****	*****	46
DEC-3	*****	*****	*****	*****	42
EVG	*****	*****	*****	*****	42
DEC-1	<u>AAAACAATGC</u>	<u>TATCCTTGTT</u>	<u>GCAACTAAGT</u>	TTTTCTCCTC	200
DEC-2	*****	*****	*****	*****	55
DEC-3	*****	*****	*****	*****	42
EVG	*****	*****	*****	*****	42
DEC-1	<u>GAGTAGGGCA</u>	<u>ATGGGTGGTG</u>	<u>GAGCTATGAC</u>	CATAACATGT	240
DEC-2	A*****	*****	*****C*****T	*****	95
DEC-3	A*****	*****	*****	*****A*	82
EVG	A*****	*****	*****	*****A*	82
DEC-1	<u>TTTTGCCATG</u>	<u>AATACATGCT</u>			260
DEC-2	*****	*****			115
DEC-3	*****	*****			102
EVG	*****	*****			102

**Figure 5.** DNA sequence alignment of the EAT/MCAC STS marker fragments from deciduous F<sub>2</sub> tree 15 (DEC-1, DEC-2, and DEC-3) and evergrowing F<sub>2</sub> tree 16 (EVG). PCR primer sequences are underlined, identical nucleotide bases are indicated by a star (\*), and deletions are indicated by a slash (-).

ments were amplified from both the evergrowing and deciduous F<sub>2</sub> individuals, but no fragment size or restriction site differences were discovered between deciduous and evergrowing individuals. Nucleic acid and protein sequence database searches did not reveal significant homology between the AFLP marker sequences and other characterized sequences.

### SSR Marker Development and Map Merging

SSR markers were developed in this region to enable the merging of this local evergrowing linkage group with a rootstock peach genome map developed by Lu et al. (1998). Merging these maps would potentially provide greater marker saturation in the evergrowing region. Ten SSR markers were developed from the subclones of two BAC clones, PpN18F12 and PpN70H22, corresponding to AFLP markers EAT/MCAC and EAT/MCTA, respectively (Wang et al. 2002). After testing in the F<sub>2</sub> progenies of the Empress op op dwarf × Evergrowing and K62-68

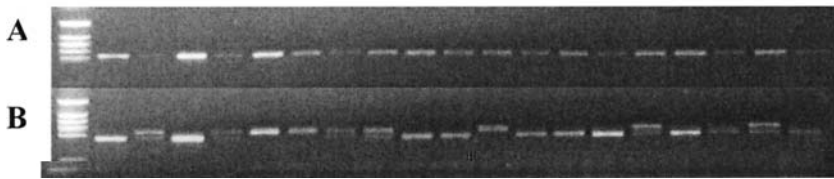
families, three SSR markers—pchgms12 (from PpN18F12), pchgms17, and pchgms19 (from PpN70H22)—were found to be polymorphic in both families. Chi-square test results ( $P \leq .05$ ) indicated that segregation of these three markers fit the 1:2:1 ratio for codominant markers in both families. Although SSR marker pchgms13 (from PpN18F12) was only polymorphic in the K62-68 family and not in the Empress op op dwarf × Evergrowing family, this locus was included in the combined map.

Mapping analysis in the cross of Empress op op dwarf × Evergrowing showed that pchgms12 cosegregated with the EAT/MCAC AFLP marker, and pchgms17 and pchgms19 cosegregated with the EAT/MCTA marker (Figure 3). Mapping analysis in the K62-68 family indicated that both pchgms12 and pchgms13 cosegregated, and both pchgms17 and pchgms19 cosegregated in a small linkage group with only three AFLP markers. With three anchor SSR markers—pchgms12, pchgms17, and pchgms19—the local genetic linkage map was integrated into the peach rootstock map (Figure 8). The integrated genetic linkage map covered 32.3 cM. Most markers from the local genetic linkage map followed the same order in

EVG	<u>TGCCCTCAGA</u>	<u>TTAGATTGGG</u>	<u>ACTTGGGAAG-</u>	AACCAAAATT	39
DEC	*****	*****	*****A	*****	40
EVG	<u>TAAAAGGACA</u>	<u>AAATACAAAT</u>	<u>ACATATAAAT</u>	TTGAAAAATC	79
DEC	**C*****	*****	*****	*****	80
EVG	<u>AATTATGGTA</u>	<u>CATTTACAG</u>	<u>AGCAGTAGAT</u>	GAGTGGTTAG	119
DEC	*****	*****	*****	*****	120
EVG	<u>GATGCATCTC</u>	<u>TTCAATGGCA</u>	<u>ATTCCTCTCT</u>	TATCTTTTTT	159
DEC	*****	*****	*****	*****	160
EVG	<u>GTTCTTTTTT</u>	<u>CTTTTTTGCA</u>	<u>TGACTCGCAA</u>	TTTCTGAAGA	199
DEC	*****	*****	*****	*****	200
EVG	<u>ATTGATATTT</u>	<u>CCATCTGCAG</u>	<u>TCGTACTGAA</u>	ACCCATCTTA	239
DEC	*****	*****	*****	*****	240
EVG	<u>GGTTACCAA</u>	<u>CAACAGCTCC</u>	<u>AAGCGATAAG</u>	CCGCGATGTG	279
DEC	*****	*****	*****	*****	280
EVG	<u>TGCGCCCTTT</u>	<u>CCGCGAATTA</u>	<u>CGCGTCTCTA</u>	CTTATCGCAT	310
DEC	*****	*****	*****	*****	310

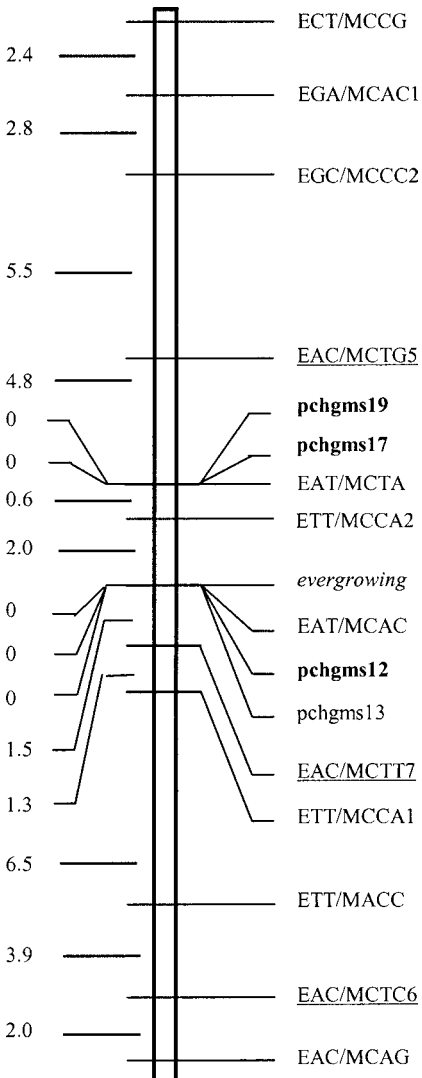
**Figure 6.** DNA sequence alignment of the EAT/MCTA STS marker fragments from deciduous F<sub>2</sub> individual 15 and evergrowing F<sub>2</sub> individual 16. PCR primer sequences are underlined, identical nucleotide bases are indicated by a star (\*), and the *Dra*I recognition site is indicated by bold letters.

M 1 2 3 5 7 13 14 15 16 31 32 34 35 46 48 49 61 63 69



**Figure 7.** Comparison of DNA banding patterns from  $F_2$  individuals (A) before and (B) after *Dral* digestion of PCR products of the EAT/MCTA STS marker. Marker lanes (M) are the pGEM DNA marker.  $F_2$  individuals 1, 3, 16, 31, 34, 35, 46, 49, 61, and 69 are evergrowing trees.  $F_2$  individuals 2, 5, 14, 15, 48, and 63 are heterozygous deciduous trees.  $F_2$  individuals 7, 13, and 32 are homozygous deciduous trees.

the integrated linkage group, except ETT/MCCA1. AFLP markers EAC/MCTT7 and EAC/MCTC6 were mapped to the same side of the *evg* locus as ETT/MACC, at 1.5 cM and 13.1 cM, respectively. However,



**Figure 8.** Integrated genetic linkage map group, including the local genetic linkage map for the *evg* gene region. The SSR markers pchgms12, pchgms17, and pchgms19 (in bold) were anchor markers used for integrating the local map into the peach rootstock map. AFLP markers from the rootstock genetic linkage map are underlined. Genetic distances are in centiMorgans.

genetic distance estimates between markers in the combined map were smaller than those calculated by MapMaker.

## Discussion

Since its development (Michelmore et al. 1991), bulked segregant analysis (BSA) has been extensively used for quick identification of markers linked to genes of interest. BSA was particularly helpful in this study, since the trait of interest could be scored qualitatively and the original parents and  $F_1$  plants of the cross were not available for detection of DNA polymorphism. Using the first set of bulks, we rapidly narrowed down the potential primer combinations from 536 to 64. However, the frequency of false positives after the first selection was high (79.7%), with only 13 markers included in the final linkage map. Other researchers using BSA (Fang et al. 1997; Haley et al. 1993; Young and Kelley 1996) also found high rates of false positives, yet the theoretical probability of an unlinked locus being polymorphic between bulks of 10 individuals is only  $2 \times 10^{-6}$  (Michelmore et al. 1991). Variation in scoring by different individuals might lead to high false positives in BSA. In this study AFLP markers having very faint bands in the recessive bulk were considered as possible markers linked to the *evg* locus after selection using the first set of bulks, which dramatically increased the false-positive rate. The second set of bulks was very useful in reducing the false-positive rate to 7.14%. Therefore using two sets of bulks would be cost-effective for screening possible primer combinations in BSA.

In this study, the AFLP technique was successfully employed to generate the genetic linkage map for the *evg* gene, enabling the rapid development of numerous molecular markers. However, these AFLP markers could not be used directly for marker-assisted selection in breeding programs because of their low transferability among different crosses.

Therefore for MAS, AFLP markers EAT/MCAC and EAT/MCTA were converted into STS markers, which can be easily amplified from genomic DNA. Using these STS markers to preselect the evergrowing trait can significantly reduce the costs of maintaining undesirable trees in breeding programs targeting changes in chilling requirements of varieties.

The local genetic map for the *evg* gene has 10 loci and covers 79.3 cM. Besides developing molecular markers for the MAS, this map provides a solid foundation for further localization and cloning of the *evg* gene. This gene, in combination with transgenic technologies, has a great potential for manipulation of the winter dormancy and chilling requirements of other *Prunus* species. In addition, understanding the nature of this gene could greatly aid in discovery of other genes essential to the pathways of winter dormancy in perennial plant species. With knowledge of the *evg* gene's map position, comparative mapping of this region and similar regions in related species (such as apricot and cherry) will shed light on the evolution of this gene region in tree species.

When the local map was integrated into the rootstock map (using Joinmap), genetic distances between the linked AFLP markers (Figure 8) were different from those in the local map, which was constructed using MapMaker (Figure 3). This "shrinking" was due to different algorithms used in the two mapping programs (van Ooijen JW, personal communication). The ETT/MCCA1 marker was mapped between EAT/MCTA and EGC/MCC2 in the local map, but was mapped between the *evg* locus and ETT/MACC in the integrated map. This may suggest different recombination rates in the different crosses.

In summary, we developed molecular markers for MAS in peach breeding programs and positioned BAC clones in the *evg* gene region, then integrated the local genetic linkage map into a genome-wide map, thus facilitating construction of a physical map in this region and future cloning of the *evg* gene.

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