Genetic Control of Internal Breakdown in Peach

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Abstract
Internal breakdown (IB), also known as chilling injury, is the collective term for various disorders that occur during prolonged cold storage and/or after subsequent ripening of stone fruit. Symptoms include mealiness, flesh browning, loss of flavor, and red pigmentation (bleeding). The problem is usually not noticed until fruit reaches consumers, reducing demand. Some cultivars tend to be more susceptible than others, indicating a significant genetic component underlying expression of the symptoms. With an improved understanding of this genetic component, strategies could be implemented for breeding new cultivars with low IB susceptibility. We have undertaken a classical and molecular genetics approach to search for controlling genes, using two related progeny populations of peach. Each IB trait had a high heritability in the populations, with relatively small seasonal or rootstock effects. Endopolygalacturonase (endoPG) was determined to play a qualitative role in the development of mealiness, as a functional endoPG gene must be present for the expression of other genes that influence mealiness susceptibility. Despite the control by endoPG of the Melting flesh trait in the late stages of normal fruit ripening, its enzymatic action leading to mealiness may instead occur during cold storage. The F-M/endoPG locus also had a large effect on the development of bleeding. A major QTL was identified for browning elsewhere in the genome. These results, combined with discontinuous trait distributions, suggest that there may be only a few major genes controlling each of the IB symptoms. EndoPG is the first of these genes for mealiness and probably bleeding also. Finding further genes and developing marker-assisted selection for IB in peach appear to be achievable goals.

INTRODUCTION
Internal breakdown (IB) is a major problem affecting the peach and nectarine fruit industry. Symptoms include mealiness, flesh browning, flesh bleeding, leatheriness, a loss of flavor, and development of off flavors, which appear during prolonged cold storage and/or after subsequent ripening (Crisosto et al., 1999). While certain treatments can be used to reduce the incidence of IB, the underlying mechanisms of genetic control are unclear. To date, our program has evaluated approximately 120 peach and nectarine (Prunus persica [L.] Batsch) cultivars for their susceptibility to IB. Certain cultivars appear more susceptible than others, indicating that the IB symptoms have a significant genetic component, though the genetic differences between low and high susceptible genotypes are not known. Peach breeding would benefit from the ability to identify at the seedling stage those genotypes not prone to IB, which marker-assisted selection (MAS) could provide. To develop this tool and gain a better understanding of the genetic control of IB, we have undertaken a classical and molecular genetics approach, using two related and genetically variable populations of peach. This research is currently focused on the symptoms of mealiness, browning, and bleeding.

To identify the various genes contributing to genetic variation in peach germplasm, the separate physiological components of each symptom need to be identified. Mealiness is a fruit flesh textural disorder, where affected ripe fruit have a dry grainy feel when chewed. In simple terms, mealy fruit are dry and soft when ripe, whereas leathery fruit are dry and firm when ripe, or remain firm because of a failure to ripen (Luza et al., 1992; Ju et al., 2000). Normal fruit are juicy and soft (melting flesh) or juicy...
and firm (non-melting flesh or other firm phenotypes such as stony hard). On a cellular level in mealy fruit, cell wall (particularly the pectin component) metabolism is altered, a gel is formed when pectic substances in intercellular spaces absorb free water, and intercellular adhesion is reduced (Brummell et al., 2004).

Browning is often seen in mealy fruit, though it can occur in the absence of mealiness (Crisosto et al., 1999; Brummell et al., 2004). Browning occurs when enzymes such as polyphenol oxidase act on phenolic substrates when they are brought into contact (Kader and Chordas, 1984). Genetic variation in browning susceptibility may therefore be within the genes coding for oxidizing enzymes or their regulators, in genes contributing to phenolic levels, and/or within genes affecting the degree of cellular decompartmentalization that may occur during cold storage or during subsequent ripening. Only genes in the latter category are likely to overlap with those involved in mealiness susceptibility, as the physiological mechanism of the common genetic factor appears to be through tissue deterioration or senescence processes following mealiness.

Bleeding results from a spread of red pigment, presumably anthocyanin, through the fruit flesh during cold storage or after subsequent ripening. Other forms of red pigmentation that also occur in peach germplasm may be related to IB flesh bleeding susceptibility. Red pigment occurs in the flesh close to the pit in many peach and nectarine cultivars, and bleeding in chilling-injured fruit may originate from this. Another form of flesh bleeding is visible in some fruit at harvest. Finally, all fruit of certain cultivars, such as ‘Indian Free’, have flesh that is mostly or entirely red. The genes causing IB bleeding genetic variation may be those involved in the anthocyanin biochemical pathway and/or those affecting the cellular decompartmentalization. As anthocyanin is a phenolic compound, bleeding may be controlled by similar genes to browning, and may involve cell wall metabolism genes in common with both browning and mealiness.

Our strategy combines phenotypic analysis, candidate gene analysis, and mapping/QTL (quantitative trait loci) analysis. Phenotypic analysis is employed to determine the degree to which the traits are genetically influenced, and to identify associations between traits. Candidate gene analysis is a potential shortcut to identifying important genes, through association of DNA sequence variation with trait variation in selected genes. This approach has thus far identified that a gene for endopolygalacturonase (endoPG) with three effective alleles lies at the Freestone and Melting flesh (F-M) locus, which determines whether fruit are freestone melting flesh (FMF), clingstone melting flesh (CMF), or clingstone non-melting flesh (CNMF) (Peace et al., 2005b). Mapping and QTL analysis is a broader approach that locates the location, number, and effect of genomic sites contributing to the phenotypic variation, without necessarily determining the actual genes at these sites.

**MATERIALS AND METHODS**

For this study, two peach populations were evaluated. The first was an F₁ cross between the cultivars ‘Dr. Davis’ and ‘Georgia Belle’, designated as Pop-DG. ‘Dr. Davis’ produces clingstone, non-melting flesh fruit used for canning, while ‘Georgia Belle’ produces freestone melting flesh fruit that are eaten fresh. ‘Georgia Belle’ is unusual in that it is known to be highly heterozygous (including at the F, M, and Y loci); however, this cultivar is particularly susceptible to IB. The second population, designated Pop-G, was derived from the selfing of ‘Georgia Belle’. Both populations consist of 70 progeny, with each of the progeny present twice in the orchard – once on its own roots and once on a common rootstock, ‘Nemaguard’. This replication allowed estimation of within-genotype variation and testing for rootstock effects on fruit quality traits. Selfs and outcrosses were subsequently detected in these populations with DNA markers, reducing the number of progeny to 51 and 64 for Pop-DG and Pop-G, respectively.

Fruit quality data were collected for the 2002 and 2003 seasons as described by Peace et al. (2005a), to which internal breakdown data for the 2004 season were added. IB was recorded after cold storage for 2 weeks for each year, and also after 3 weeks storage.
in 2003 only. Data were analyzed as described by Peace et al. (2005a) – summarized below. Effects of rootstock, year (within the 2 week storage duration), storage duration (within 2003), and the F-M and Y loci on quantitative traits were examined by two-tailed t-tests. Genotype effects on each quantitative trait were calculated by three-factor (genotype-year-rootstock or genotype-storage duration-rootstock) Analysis of Variance. Broad sense heritability was estimated as the proportion of genotypic to phenotypic variance. Spearman rank correlations between quantitative traits were performed on both an individual fruit and progeny basis.

Candidate genes for IB were nominated from literature surveys on genes potentially involved in their expression. Gene sequences were obtained primarily from CUGI (2004) and screened for polymorphism in Pop-DG as described by Peace et al. (2005a). Marker segregation data were obtained, and mapping and QTL analyses performed, as previously described by Peace et al. (2005a).

RESULTS AND DISCUSSION

The combined genetic approaches undertaken with two progeny populations have successfully begun to unravel the genetic control of internal breakdown in peach. A major step forward was made in determining the role of endoPG in the development of mealiness. Further genomic regions controlling IB traits were also identified. The high heritability of IB symptoms makes the prospect of finding the major controlling loci very promising, allowing objective DNA tests to be developed for use in marker-assisted selection.

Quantitative Variation for Internal Breakdown Traits

Considerable variation was present in both populations for IB symptoms, with bimodal and/or skewed distributions (particularly in Pop-G) suggesting oligogenic control (Fig. 1). The populations were also variable for other fruit quality traits recorded (results not shown). The single most influential factor identified contributing to phenotypic variation was genotype, i.e., the IB susceptibility for each of the progeny was quite consistent over the three years, on different rootstocks, and at different storage durations (Table 1). This was reflected in high heritability values for IB symptoms. While statistically significant for each IB trait and in each population, differences between years were very minor compared to differences between progeny. Rootstock differences were even smaller and were not consistently observed in both populations. Mealiness and browning were positively correlated, and both were negatively correlated with bleeding (Table 2). Mealiness and bleeding were positively correlated with flowering date (results not shown). Browning was positively correlated with harvest date (results not shown). Certain IB traits were also associated with the F, M, and Y loci (see below). Possible reasons for phenotypic correlations are shared controlling genes or linked controlling genes.

Effect of the Flesh Color Locus on Internal Breakdown

The Y locus had a non-significant effect, or was inconsistent between the populations, for mealiness and browning. However, significantly greater bleeding was observed in white-fleshed fruit (13% in Pop-DG and 23% in Pop-G) compared to yellow-fleshed fruit (7% in Pop-DG and 13% in Pop-G).

Effect of the Freestone-Melting Flesh Locus on Internal Breakdown

The F-M locus behaved as a simple Mendelian trait, with 29 FMF to 22 CNMF progeny (1:1 segregation) in Pop-DG, and 45:19 (3:1) in Pop-G. Internal breakdown symptoms were greatly influenced by this locus. Mealiness was the most dramatically affected, with CNMF trees exhibiting no mealiness, whereas FMF progeny ranged from 3% to 100% mealy fruit (averaging 45% in Pop-DG and 64% in Pop-G). Given that CNMF fruit do not soften substantially during ripening, a simple explanation for the lack of mealiness in CNMF fruit is that while they may develop “dry” flesh, it will not be soft,
and therefore cannot be classified as mealy. Brovelli et al. (1998) and Crisosto et al. (1999) reported that CNMF cultivars are less susceptible to internal breakdown. Such statements can confuse an understanding of the role of $F-M$ in mealiness, because IB includes other symptoms besides mealiness. A clearer statement is that CNMF cultivars cannot get mealy, but some may have a short market life due to high susceptibility to browning or leatheriness. On the other hand, FMF (and CMF) trees have the potential to develop mealiness in their fruit, depending on whether they carry further genes for susceptibility.

Browning was significantly lower in CNMF progeny (average score of 1.8 in Pop-DG and 2.0 in Pop-G) than in FMF progeny (2.1 in Pop-DG and 2.4 in Pop-G). However, the effect of the $F-M$ locus on browning was probably indirect, through the frequent occurrence of browning in mealy fruit after tissue senescence. The level of browning in low-mealiness FMF progeny (average score of 1.5 in Pop-DG and 1.2 in Pop-G) was actually lower than that in CNMF progeny. Considering only FMF fruit, the positive correlation between mealiness and browning remained (Table 2), indicating that loci other than $F-M$ account for the association between these two traits.

In contrast to mealiness and browning, bleeding was greatest in CNMF progeny (averaging 21% in Pop-DG and 62% in Pop-G) and almost non-existent in FMF progeny (1% in Pop-DG and 5% in Pop-G). In FMF fruit, the negative correlation between mealiness and bleeding was reduced or lost (Table 2), indicating that their inverse relationship was mainly due to the lack of mealiness but high level of bleeding in CNMF fruit. The reason for such a large $F-M$ effect on bleeding is not clear. Conceivably, the flesh softening of the melting phase or the loss of stone-flesh adhesion in fruit of the FMF progeny may retard the spread of the red pigment (anthocyanin) through the flesh from the red vascular rays close to the stone, assuming that these rays are the source of the pigment. Alternatively, another polymorphic gene closely linked to the $F-M$ locus may be part of the control mechanism for bleeding.

**Role of endoPG in the Development of Mealiness**

Considering that a gene for endoPG controls whether fruit are FMF, CMF, or CNMF, and that the genetic basis for the CNMF phenotype is a deleted or non-functional endoPG gene (Peace et al., 2005b), the large effect of the $F-M$ locus on mealiness can be investigated further. Previous research has constantly implicated a role for endoPG in mealiness. EndoPG depolymerizes and solubilizes large pectin molecules in cell walls following the action of pectin methylesterase (PME), which de-esterifies the pectin to allow endoPG action (Brummell and Harpster, 2001). The prevailing hypothesis is that low temperatures encountered during cold storage beyond a critical duration suppress endoPG but not PME activity during and after storage, resulting in altered pectic compounds that readily form gels, culminating in dry flesh (Buescher and Furmanski, 1978; Lill et al., 1989). However, this does not account for the “soft” component of mealiness and the lack of mealiness in CNMF fruit. Partial pectin depolymerization, presumably due to endoPG action, does occur during cold storage of FMF fruit, whether or not they will eventually become juicy or mealy (Brummell et al., 2004). When fruit that are to become mealy are ripened at ambient temperatures, the activity of many ethylene-regulated enzymes, including endoPG, remains low, and there is little further pectin depolymerization (Brummell et al., 2004). The typical climacteric production of ethylene does not occur, with lower production as mealiness severity increases (Zhou et al., 2001). Correspondingly, mealy fruit do not go through the melting phase, but soften gradually, developing an unusual texture with cell clumping and reduced cell fracture (Brummell et al., 2004; Crisosto et al., unpublished data). Partial pectin depolymerization in FMF fruit by endoPG during cold storage may allow enzymes other than endoPG that are not disabled to continue pectin metabolism and the softening process after cold storage, though in a manner unlike that of normal ripening fruit. The altered pectin metabolites and unusual texture result in dry, soft flesh, i.e. mealiness. In FMF fruit that become leathery, there is an even greater arrest of pectin depolymerization and an overall
inhibition of ripening enzyme activity (Ju et al., 2000; Brummell et al., 2004). The difference with CNMF fruit is that they would not have any endoPG-related depolymerization, and so they remain firm and mostly juicy following cold storage and ripening, or can become leathery if general ripening is disabled. Our results confirm that endoPG is involved in mealiness; however, it plays a qualitative role in that a functional endoPG gene must be present for the expression of other genes that influence mealiness susceptibility. Despite the control of the melting phase by endoPG in normally ripening fruit, its enzymatic action leading to mealiness may occur during cold storage rather than during subsequent ripening.

**Beyond endoPG: Resistance to Mealiness in FMF Fruit**

How do “resistant” (low-susceptible) melting flesh cultivars avoid mealiness? Within the FMF progeny of Pop-DG and Pop-G, there were certain progeny that were consistently resistant to mealiness and others with consistently high susceptibility, such that heritability was still moderate when considering only these progeny (Table 1). This indicates that other genes besides endoPG contribute to genetic variation in mealiness susceptibility. To find them, investigations need to focus only on trees with melting flesh fruit. In a study of a FMF cultivar, fruit that were to remain juicy after cold-storage retained the ability to synthesize in cold storage, or activate after storage, ethylene-regulated enzymes that were impaired in fruit that were to become mealy (Brummell et al., 2004). Following the same principle, practices such as allowing fruit to ripen for a few days before cold storage (“pre-conditioning”), applying ethylene during cold storage, or intermittently raising temperatures during cold storage, can greatly reduce the incidence of mealiness (Lill et al., 1989; Zhou et al., 2001). These studies indicate possible mechanisms for genes contributing to low mealiness in resistant genotypes: such genes could be involved in maintaining a critical level of ethylene production in cold storage, maintaining the expression of particular pectin-metabolizing enzymes in cold storage, or synthesizing/activating the necessary enzymes for normal pectin metabolism after prolonged cold storage.

**Searching for Further Genes Controlling IB**

The non-Normal trait distributions (Fig. 1) indicate that there may be only a few major genes controlling the traits, while the high degree of genetic influence for each of the IB traits (Table 1) improves the prospects for finding these genes. Simple genetic models were constructed and compared to the observed distributions (results not shown). Good matches were achieved with models including as few as 2-3 large-effect genes for each IB trait, with dominant gene action and heterozygosity in ‘Georgia Belle’ being common features. EndoPG accounts for one of these genes for mealiness and probably bleeding also, but where in the genome are the other major genes?

A partial genetic map was constructed for Pop-DG from 80 polymorphic markers (4 morphological, 4 candidate gene, 28 SSR, 21 SRAP, and 25 RAF), of which 70 joined up in 12 linkage groups. Using SSR markers in common with the consensus map of *Prunus* (Aranzana et al., 2003), eight groups were anchored to seven of the eight consensus groups representing the eight chromosomes of *Prunus*. The remaining groups did not include SSR markers and so their consensus position is not yet known. Three polymorphic candidate genes – endoPG, PME (one of eight sequences for this candidate gene), and beta-carotene hydroxylase (BCH) – were placed on the map. EndoPG was located at the F-M locus in group 4, as expected. The PME gene mapped to group 1. BCH, part of the carotenoid biochemical pathway, was mapped to group 2. Another PME gene remained unlinked.

QTL analysis of this partial linkage map identified QTLs (i.e., the approximate locations of influencing genes) for every trait (Table 3). Major QTLs (>25% of phenotypic variation explained) were identified for each of the three IB symptoms, consistent with the outcome from phenotypic analysis that there may be just a few genes with large effects accounting for most of the genotypic variation in these traits, rather than
many genes with small effects. ‘Georgia Belle’ contributed more large-effect alleles associated with high IB in the progeny than did ‘Dr. Davis’. As expected, the \( F-M \) locus (controlled by endoPG) was identified as a major QTL for mealiness and bleeding in every year that data were collected (Table 3). Considering only FMF progeny, another QTL for mealiness was discovered elsewhere in linkage group 4 (Table 3). Independent QTLs were also identified for browning and bleeding (Table 3). No common QTLs were discovered between mealiness and browning to account for their significant positive correlation. Therefore, while some genetic factor appears to determine why fruit that develop mealiness often subsequently develop browning, this is not endoPG at the \( F-M \) locus, or the second mealiness QTL located in group 4. Despite a difference in bleeding observed between white- and yellow-fleshed progeny, no QTL for bleeding was located near the \( Y \) locus of linkage group 1. A bleeding QTL was identified in this linkage group (Table 3), but it was not close to the \( Y \) locus, and originated from ‘Dr. Davis’. Genes controlling bleeding may include (variants of) the loci controlling flesh color around the stone (the \( Cs \) locus of linkage group 3; Dirlewanger et al., 2004) and general red flesh color. The partial Pop-DG map used in the present study did not include the region of the \( Cs \) locus, and the map position of the red flesh color locus has not been reported, so the effect of these loci on cold storage bleeding remains unknown. A QTL for flowering date was found nearby the \( F-M \) locus (results not shown), where flowering QTLs have been reported from multiple \( Prunus \) species (Silva et al., 2005), providing an explanation for the positive correlations between these traits. Other than endoPG, the polymorphic candidate genes did not coincide with QTLs for any of the fruit quality traits, and so can be ruled out as actual candidate genes for these specific traits in our populations.

Rootstock effects were also assessed through QTL analysis. A common approach in QTL analysis when data sets are available for multiple years is to conduct separate analyses for each year’s trait data. Such an approach gives an idea of the stability of QTLs over seasons, and typically yields both year-specific and general QTLs. As data were available for each rootstock type (own roots and ‘Nemaguard’), QTL analysis was repeated for Pop-DG using the rootstock-specific data sets. No major differences were observed in the sets of QTLs identified for each rootstock type, consistent with the lack of rootstock effects from ANOVA.

Considerable genetic variation for internal breakdown exists, and there is the opportunity to develop new cultivars for both the fresh and canning industries that are free of internal breakdown symptoms. Further mapping and QTL analysis, combined with a continued search for polymorphic candidate genes that may be associated with QTLs, will be conducted, to improve our understanding of the genetic control of internal breakdown in peach, and develop molecular diagnostic tools.

ACKNOWLEDGEMENTS

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Tables

Table 1. Factors affecting internal breakdown symptoms, observed for three years in two peach progeny populations. Significant factors (p<0.01) and their relative magnitude are indicated, as determined by ANOVA.

<table>
<thead>
<tr>
<th>IB trait</th>
<th>Population</th>
<th>Proportion (%) of phenotypic variance&lt;sup&gt;z&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Genotype&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mealiness</td>
<td>Pop-DG</td>
<td>42.6</td>
</tr>
<tr>
<td></td>
<td>Pop-G</td>
<td>51.9</td>
</tr>
<tr>
<td>Mealiness - FMF progeny</td>
<td>Pop-G</td>
<td>31.2</td>
</tr>
<tr>
<td>Browning</td>
<td>Pop-DG</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td>Pop-G</td>
<td>48.5</td>
</tr>
<tr>
<td>Bleeding</td>
<td>Pop-DG</td>
<td>29.2</td>
</tr>
<tr>
<td></td>
<td>Pop-G</td>
<td>47.2</td>
</tr>
</tbody>
</table>

<sup>z</sup> NS = not significant

<sup>y</sup> This proportion of phenotypic variance attributed to Genotype is the broad sense heritability (H).

<sup>x</sup> The Stage effect was determined in one year (2003) only. The corresponding percentages of phenotypic variation attributed to Genotype and Rootstock in this year are shown in parentheses.
Table 2. Phenotypic correlations between internal breakdown symptoms, observed for three years in two peach progeny populations. R-values from Spearman rank correlations on an individual fruit basis (progeny basis in parentheses) are shown, with Pop-DG in the bottom left diagonal and Pop-G in the top right diagonal. Correlations between “Mlns – FMF” and the other traits considered only freestone melting flesh fruit/progeny.

<table>
<thead>
<tr>
<th>IB trait</th>
<th>All progeny</th>
<th>FMF only</th>
<th>Browning</th>
<th>Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mealiness</td>
<td>*</td>
<td>*</td>
<td>0.33 (0.33)</td>
<td>-0.38 (0.70)</td>
</tr>
<tr>
<td>- FMF only</td>
<td>*</td>
<td>*</td>
<td>0.42 (0.63)</td>
<td>-0.06 (0.22)</td>
</tr>
<tr>
<td>Browning</td>
<td>0.34 (0.43)</td>
<td>0.35 (0.37)</td>
<td>*</td>
<td>-0.11 (-0.18)</td>
</tr>
<tr>
<td>Bleeding</td>
<td>-0.18 (-0.63)</td>
<td>-0.10 (-0.22)</td>
<td>-0.05 (-0.19)</td>
<td>*</td>
</tr>
</tbody>
</table>

Table 3. QTLs identified for IB traits in Pop-DG.

| IB trait      | QTL location      | Variance explained | QTL origin
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mealiness</td>
<td>group 4 (F-M)</td>
<td>61%</td>
<td>‘Georgia Belle’</td>
</tr>
<tr>
<td>- FMF only</td>
<td>group 4</td>
<td>24%</td>
<td>‘Georgia Belle’</td>
</tr>
<tr>
<td>Browning</td>
<td>group 5</td>
<td>61%</td>
<td>‘Georgia Belle’</td>
</tr>
<tr>
<td>Bleeding</td>
<td>group 4 (F-M)</td>
<td>43%</td>
<td>‘Georgia Belle’</td>
</tr>
<tr>
<td></td>
<td>group 1</td>
<td>17%</td>
<td>‘Dr. Davis’</td>
</tr>
</tbody>
</table>

* Parental cultivar contributing the allele with high IB effects

Figures

Fig. 1. Distribution of internal breakdown in two peach progeny populations, averaged over three years and two rootstock types for each progeny. Mealiness is shown only for FMF progeny.