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Genetic characterization of the *Ma* locus with pH and titratable acidity in apple

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Abstract Apple fruit flavor is greatly affected by the level of malic acid, which is the major organic acid in mature apple fruit. To understand the genetic and molecular basis of apple fruit acidity, fruit juice pH and/or titratable acidity (TA) were measured in two half-sib populations GMAL 4595 [Royal Gala × PI (Plant Introduction) 613988] and GMAL 4590 (Royal Gala × PI 613971) of 438 trees in total. The maternal parent Royal Gala is a commercial variety and the paternal parents are two *M. sieversii* (the progenitor species of domestic apple) elite accessions. The low-acid trait segregates recessively and the overall acidity variations in the two populations were primarily controlled by the *Ma* (*malic acid*) locus, a major gene discovered in the 1950s (Nybom in Hereditas 45:332–350, 1959) and later mapped to linkage group 16 (Maliepaard et al. in Theor Appl Genet 97:60–73, 1998). The allele *Ma* has a strong additive effect in increasing fruit acidity and is incompletely dominant over *ma*. QTL (quantitative trait locus) analyses in GMAL 4595 mapped the major QTL *Ma* in both Royal Gala and PI 613988, the effects of which explained 17.0–42.3% of the variation in fruit pH and TA. In addition, two minor QTL, tentatively designated *M2* and *M3*, were also detected for fruit acidity, with *M2* on linkage group 6 of Royal Gala and *M3* on linkage group 1 of PI 613988. By exploring the genome sequences of apple, eight new simple sequence repeat markers tightly linked to *Ma* were developed, leading to construction of a fine genetic map of the *Ma* locus that defines it to a physical region no larger than 150 kb in the Golden Delicious genome.

Keywords Apple · Fruit acidity · *Ma* · pH · QTL · Fine mapping

Introduction

Improvement of fruit quality has been one of the major goals in apple breeding programs around the world because of its importance in the marketplace and in the sustainability of the apple industry. However, fruit quality is complex and comprises many traits, including fruit size, texture, fruit acidity, soluble contents and others. Although much effort has been devoted to genetic studies, our understanding of fruit quality remains incomplete. As a result, genetic improvement of fruit quality continues to be challenging for apple breeders.

Fruit acidity and sugar content greatly affect overall eating quality and flavor. The major organic acid in mature apple fruit is malic acid (Zhang et al. 2010),

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although citric, quinic and other acids are also detectable (Zhang et al. 2010). An appropriate level of fruit acidity and sugar and a proper balance between the two are essential for a successful commercial apple variety. In apple breeding programs, evaluation of fruit titratable acid, °Brix (soluble solids content) and their ratio has become an indispensible measure for advancing selections and for planning new crosses. Studies attempting to understand the relationship between objective and sensory measurements of apple taste and flavor found that titratable acidity is the best predictor of acid taste, yet sweet taste was difficult to predict using °Brix, the ratio °Brix/titratable acidity or the content of individual sugars and acids (Harker et al. 2002; Oraguzie et al. 2009; Guerra et al. 2010). Fruit acidity had the highest heritability estimate among the sensory traits that included firmness, crispness, texture, juiciness, flavor, sugar, acidity and global taste (Kouassi et al. 2009).

Because of its importance in determining fruit flavor and quality, fruit acidity has been a subject of genetic investigations. An early inheritance study conducted on fruit acidity (Nybom 1959), based on pH measurements, reported several important findings: (1) Apple varieties can be categorized into two groups—an acid/sub-acid group with a fruit pH < 3.8 and a sweet group of pH ≥ 4.0; (2) the acid/sub-acid group is much more prevalent than the sweet group in cultivated apples; and (3) the sweet flavor is determined by one recessive gene present in 80–90% of apple varieties studied. These findings were independently confirmed in later studies based on fruit pH (Visser and Verhaegh 1978) as well as on malic acid concentration (Brown and Harvey 1971; Yao et al. 2008). The major gene governing fruit acidity was designated *Ma* with the *Ma* allele representing the dominant high and medium acidity, and the *ma* allele for low acidity (Visser and Verhaegh 1978). As there is a relatively wide acidity spectrum in high- and medium-acid fruits, the dominance of high and medium acidity over low acidity was considered to be quantitative (Brown and Harvey 1971). This additional variation within the dominance class is proposed to be explained by the additive gene action model (Visser et al. 1968; Visser and Verhaegh 1978).

The *Ma* gene has been mapped to the proximal end of linkage group (LG) 16 between the simple sequence repeat (SSR) markers CH02a03 and CH05e04 in a presumably *Mama* × *Mama* cross (Prima × Fiesta) (Maliepaard et al. 1998; Schouten et al. 2011). In these studies, fruit acidity was evaluated with pH indicator paper and the progeny were classified into two categories of three genotypes based on fruit pH, i.e., *MaMa/Mama* (pH < 3.8) and *mama* (pH > 3.8). Using QTL (quantitative trait locus)-based approaches, a major QTL for fruit titratable acidity (TA) was detected in the *Ma* region (Liebhard et al. 2003; Kenis et al. 2008). In the cross Fiesta × Discovery, the major QTL was linked closely to marker CH05e04z in Fiesta and accounted for 36% of fruit acidity variation (Liebhard et al. 2003). In the cross Telamon × Braeburn, it was mapped to an interval between markers CH05e04z and CH05c06 and explained 20–34% of the observed variance (Kenis et al. 2008). In addition to the major QTL in the *Ma* region, another major QTL (explaining 33% of the variance) for TA was reported on LG 8 in Fiesta × Discovery (Liebhard et al. 2003). However, this second major QTL could only be identified as one of the six minor QTL in the Telamon × Braeburn cross. The other five minor QTL were detected on LGs 2, 10, 13, 15 and 17 (Kenis et al. 2008).

Diverse patterns in inheritance of low fruit acid have been documented in other species, ranging from major genes, such as *D* for peach (Boudehri et al. 2009), *acitric* for citrus (Fang et al. 1997) and *SS* for pomegranate (Jalikop 2007), to multiple QTL, such as those in tomato (Fulton et al. 2002). Within the group of major genes, low acidity is dominant over high acidity in peach (Boudehri et al. 2009) but recessive in citrus (Fang et al. 1997) and pomegranate (Jalikop 2007), similar to apple (Nybom 1959). These variations in the mode of gene action and in the number of genes/QTL involved suggest that there are diverse mechanisms in the genetic control of fruit acidity. A fine map of the *D* locus in peach has been constructed and its molecular isolation is underway (Boudehri et al. 2009). Although both peach and apple are climacteric fruits and members of the Rosaceae family, the underlying genes for *D* and *Ma* are not likely to be closely related because (1) for low acidity, *D* is dominant while *ma* is recessive, and (2) they do not reside on chromosomes known to be orthologous. *D* is on chromosome (Ch) 5 in *Prunus*, which is considered to be orthologous to apple Ch 6 and Ch 14 that are two largely paralogous chromosomes (Sargent et al. 2009; Velasco et al. 2010). Chromosome 16, where *Ma* is reported, and its paralogous chromosome (Ch 13) in *Malus* (Velasco et al. 2010) are orthologous

to chromosome (LG) 1 in peach (Dirlewanger et al. 2004).

Regulation of malate metabolism in fruit, which involves complex pathways and a range of enzymes, has been reviewed in detail (Sweetman et al. 2009). In apple, several key enzymes involved in malate synthesis, transport and degradation have been identified and studied. A dedicated investigation into a low-acid variety Usterapfel and its high-acid mutant indicated that the enzymes in malic acid metabolism, PEPC (phosphoenolpyruvate carboxylase), NAD-dependent MDH (malate dehydrogenase) and NADP-dependent malic enzyme (ME), may not play a key role in determining the difference in fruit acidity because there was no difference in the catalytic activity of these enzymes between the two genotypes (Beruter 2004). However, a study on genes *MdPEPC* (EU315246), *MdcyME* (DQ280492) and *MdVHA-A* (EF128033) found that their expression and enzyme activities were different between low and high acid genotypes, suggesting that they may contribute to the variation of fruit acidity (Yao et al. 2009). Another gene *Mal-DDNA* (DQ417661) of unknown function previously appeared to be associated with low acidity in apple fruit (Yao et al. 2007). However, none of these genes are on chromosome 16 where the *Ma* gene resides. In peach, efforts to associate key enzymes involved in organic acid metabolism and storage with the *D* locus have proven to be similarly ineffective (Etienne et al. 2002a, b).

The objectives of this study are: (1) to identify QTL for apple pH and TA in Royal Gala and PI 613988 (*M. sieversii*); (2) to develop DNA markers to saturate the *Ma* region; and (3) to fine-map the *Ma* locus for the map-based isolation of *Ma*.

Materials and methods

Plant materials

Two interspecific half-sib mapping populations of 438 trees in total were used in this study. The first population, GMAL 4595, was derived from a cross Royal Gala (*M.* × *domestica*) × PI 613988 (*M. sieversii*, the progenitor species of domestic apple). This population had 222 individuals, 188 of which were used to construct the genetic maps of its parents (Wang et al. 2011). The second population (GMAL 4590) was of 216 trees developed from a cross Royal Gala × PI 613971 (*M. sieversii*). Both crosses were made in 2002 and the seedlings were planted on their own roots in 2004 in an orchard in Geneva, NY, USA. The maternal parent Royal Gala is a widely grown commercial variety, whereas the paternal parents PI 613988 and PI 613971 were two elite *M. sieversii* clones collected from Kazakhstan (Forsline et al. 2003). PI 613988 and PI 613971 bear fruits of size close to cultivated apples (http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1531529 and http://www.ars-grin.gov/cgi-bin/npgs/acc/display.pl?1498666). Fruiting in these two populations was first recorded on a few trees in 2006 and most of the trees have fruited since 2008.

Evaluation of fruit pH and titratable acidity (TA)

For population GMAL 4595, ten fruits were randomly harvested for each genotype at maturity (initially estimated based on fruit color and aroma) over a six-week period from August 9 through September 20 in 2010. Fruits were stored overnight at 4°C and processed for fruit juice extraction on the day following harvest. To obtain juice from fruits with relatively uniform ripening stages, fruits were cross-sliced into two halves. One half was used for maturity evaluation and the other was processed for fruit juice extraction. The evaluation of fruit maturity was conducted by dipping the cut side of apples in an iodine solution (2.2 g of I_2 plus 8.8 g of KI per liter) for 1 min and then rating from 1 (most immature) to 8 (over-mature) according to the Cornell Starch Index (Blanpied and Silsby 1992). Fruit at stages 4 through 6, a common indicator for mature apples, were selected correspondingly in the second set of halves, resulting in 5–10 fruits (in halves) per genotype for fruit juice extraction. In the case of most fruits which were harvested prematurely, the samples were re-taken at a later time when there were at least five or more fruits matured. The selected fruit halves were pooled and blended using a household food processor (GE Digital Blender, Model 169202, Fairfield, CT, USA), and the fruit juice was obtained by passing through two layers of cheese cloth. The collected juice samples were immediately measured for pH using a pH meter (Accumet AB15, Fisher Scientific, Pittsburgh, PA, USA) and then stored at −20°C. Within 1–2 months of storage, the juice titratable acidity (TA) was determined by titrating samples of 5 ml of juice in a 50 ml dilution

with 0.1 N NaOH to pH 8.2 using an autotitrator (Metrohm 848 Titrino Plus and Metrohm 869 Compact Sample Changer, Herisau, Switzerland). The fruit TA (mg/ml) was calculated based on the formula (Nielsen 2010) below, where the equivalent weight of malic acid is 67.04:

$$\text{Titratable malic acid (mg/ml)} = \frac{(\text{ml base titrant}) \times (\text{N of base in mol/L}) \times \text{equivalent weight of acid}}{\text{sample volume in ml}}$$

For population GMAL 4590, fruit pH values were estimated with pH paper immediately after fruits were picked in the orchard in 2010 (Hydrion Papers, pH 3.0–5.5, Micro Essential Laboratory Inc., Brooklyn, NY, USA). Fruit TA was not determined.

Data analysis

Regression analysis between fruit pH and TA and unpaired means comparisons were carried out with JMP 9.0 software (SAS Institute, Inc., Cary, NC, USA).

Initial QTL analyses of fruit pH and titratable acidity (TA) in population GMAL 4595

The two parental genetic maps of population GMAL 4595, i.e., the Royal Gala map (1,283.4 cM) of 190 SSR markers and the PI 613988 map (1,387.0 cM) of 180 SSR markers, were constructed with 188 of the 222 progeny (Wang et al. 2011). Accordingly, the QTL analyses of fruit pH and TA were conducted using the two single parental maps in the same 188 trees, of which 166 fruited. Fruit pH and TA data were used directly in the QTL analyses. Detection and mapping of QTL were carried out with MapQTL v.4.0 software (Van Ooijen et al. 2002) under three different modes: nonparametric Kruskal–Wallis analysis, interval mapping (IM) and restricted multiple-QTL (rMQM) mapping. In the Kruskal–Wallis analysis, a recommended threshold level of significance at $P = 0.005$ was used to detect a QTL-associated marker (Van Ooijen et al. 2002). For interval mapping, the LOD threshold scores for a significant QTL were obtained with permutation tests of 1,000 at both genome and chromosomal levels (Van Ooijen et al. 2002). However, the chromosome-specific LOD thresholds (Supplementary Table 1) were used to declare the presence of a QTL. QTL positions were defined by the LOD peaks and their surrounding 1- and 2-LOD confidence intervals. For rMQM mapping, co-factors were selected according to the process described previously (Davey et al. 2006). Graphic presentation of QTL mapping results was performed with MapChart (Voorrips 2002).

Marker development in the *Ma* region

A sequence-based approach was used to develop markers in the *Ma* region by exploiting the draft sequence of the apple genome (Velasco et al. 2010). The strategy was first to establish the connections between markers linked to *Ma* and their corresponding DNA sequences in the apple genome so that the general region of *Ma* could be determined physically, and then to explore the DNA sequences in the region for developing SSR markers closer to *Ma*. In practice, DNA sequences of the SSR markers linked to *Ma* on LG 16, i.e. C5534, C1755, CH02a03, Hi02H08, Hi22f06 and CH05c06 (Silfverberg-Dilworth et al. 2006; Wang et al. 2011), were obtained from the websites of GDR (Genome Database for Rosaseae) (http://www.rosaceae.org/species/apple) and HiDRAS (High-Quality Disease Resistant Apples for a Sustainable Agriculture) (http://www.hidras.unimi.it/) and BLAST searched against the apple genome at GDR. A total of six individual contigs that encompass the six markers, i.e. MDC017634.105 for C5534 and C1755, MDC021909.329 for CH02a03, MDC010932.713 for Hi02H08, MDC002276.243 for Hi22f06, and MDC017428.71 and MDC017158.225 for CH05c06, were identified. All six contigs were confirmed to be of chromosome 16 origin and had the same linear order as the markers on LG 16 (Silfverberg-Dilworth et al. 2006; Wang et al. 2011). The region with these six contigs physically spans a chromosomal segment of around 1.85 Mb. Following these initial steps, DNA sequences of eight representative contigs between the

two markers Hi02H08 and Hi22f06 were downloaded and analyzed for the presence of SSRs (eight or more di-nucleotide repeats, or 6 or more tri-nucleotide repeats) using the web-based program Batch Primer 3 (http://probes.pw.usda.gov/batchprimer3/index.html) (You et al. 2008). The corresponding SSR primers were also designed with this program. Genomic DNA isolation, PCR and SSR analyses were conducted as described previously (Wang et al. 2011).

Fine mapping of the *Ma* locus

Because of the dominance or incomplete dominance effect of the *Ma* allele on fruit acidity and/or pH and the three parents evidently being of the *Mama* genotype, fine mapping of *Ma* could not be routinely conducted. This is because all recombinant plants developed from zygotic combinations between a recombined gamete carrying a crossover event near *Ma* and a non-recombined gamete of the *Ma* allele would bear fruits of high/medium acidity (pH < 3.8), irrespective of the allelotypes of the recombined gamete. Such recombinants would not be informative in mapping of *Ma* as it is often difficult to distinguish the genotype homozygous *MaMa* from heterozygous *Mama* by pH and TA. If a non-recombinant gamete had an allele of *ma, the* paring recombinant would become informative, and its identification would then make it useful for the fine mapping of *Ma*. To select informative recombinants, three markers flanking the *Ma* locus (CH02a03, CH05a09 and CH05c06) were used to screen populations GMAL 4595 and GMAL 4590. Genotypic data of markers CH05a09 and CH05c06 were used to determine the haplotype (*Ma*, *ma* or recombinant) at the *Ma* locus inherited from Royal Gala, whereas the data of markers CH02a03 and CH05c06 were used to type the *Ma* region from PI 613988 or PI 613971.

Results

Segregation of fruit pH and titratable acidity

190 of the 222 trees in population GMAL 4595 bore fruits and were evaluated for fruit pH and TA. The population mean of fruit pH was 3.49 ± 0.46, ranging from 2.76 to 4.65. The average TA showed 7.14 ± 3.74 mg/ml, varying between 1.08 and 18.68. There was a considerable range of variation in both pH and TA in GMAL 4595 (Fig. 1a, b).

There were two peaks in the pH distribution: one in the lower range representing most of the population of high and medium acidity, and the other in the higher pH range for the sweet genotypes, suggesting a bimodal

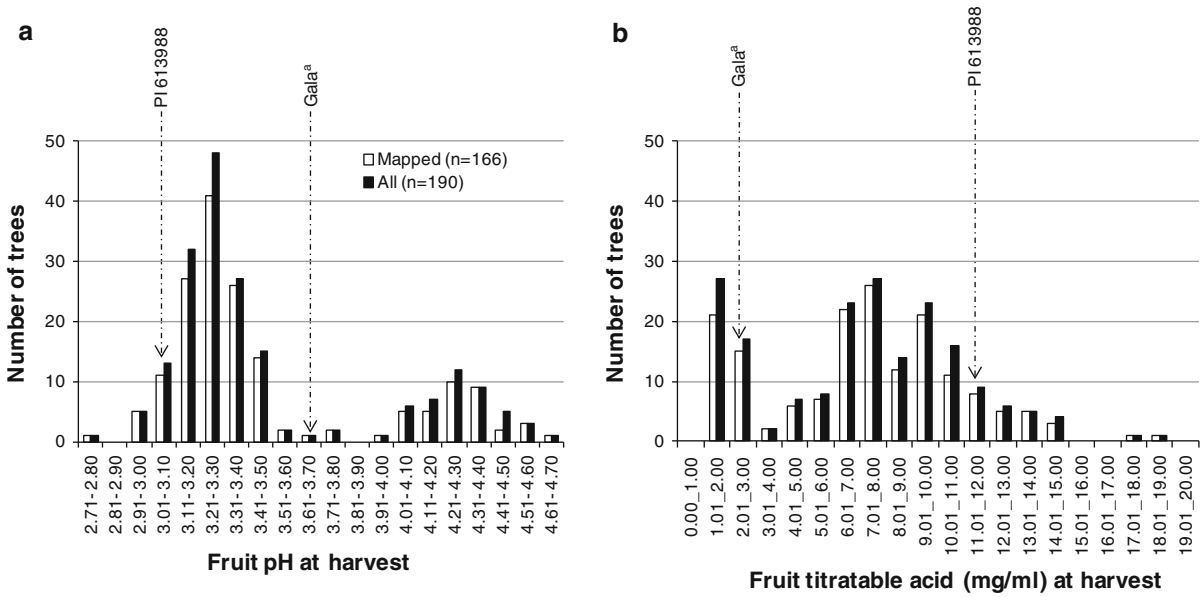

Fig. 1 Distribution of fruit pH (**a**) and titratable acidity (TA) (**b**) at harvest in population GMAL 4595. [a]Fruit pH and TA were not directly measured from the Royal Gala sport, but from its parental variety Gala

distribution for fruit pH. The boundary between the low and high/medium pH seemed to be around pH 3.80 as there were no fruits in the pH 3.81–3.90 range (Fig. 1a). Among the 190 fruiting trees, there are 144 and 46 trees of low and high/medium fruit pH, respectively, a segregation pattern fitting the ratio of 3:1 ($P_{(df=1,X^2>0.344)} = 0.56$). This suggested that there is a completely dominant gene, presumably *Ma* designated previously for controlling fruit pH (Nybom 1959; Maliepaard et al. 1998).

The distribution of TA revealed a group of low acidity with TA < 3.00 mg/ml that corresponds to the high-pH genotypes (Figs. 1b, 2). However, there appeared to be two sub-groups in the high/medium acid range with overlapping distributions, as the total distribution showed a clear dip at TA 8.01–9.00 mg/ml. These subgroups showed a perfect 1:1 segregation with 65 trees of a relatively higher TA (9.01–19.00 mg/ml) and another 65 with lower TA (4.01–8.00 mg/ml) if the boundary was placed at TA 8.01–9.00 and the 14 trees falling within the boundary were discounted from the sum (Fig. 1b), thus rejecting the expected 2:1 segregation for *Mama*:*MaMa*. The occurrence of these two subgroups, therefore, must be due to the segregation of an additional, still unidentified fruit acidity modifying gene(s). Regression analysis demonstrated that fruit TA and fruit pH were highly correlated and were predictable with a polynomial function of order 3 ($r^2 = 0.8827$, Fig. 2). Together, these data suggested that fruit pH and TA were under the control of the same major gene *Ma*; but for TA, the dominance of *Ma* over *ma* is incomplete and both additive and dominance effects of the *Ma* allele appeared to be strong (Supplementary Tables 2 and 3, Supplementary Fig. 2, see Discussion).

In population GMAL 4590 of 216 trees, fruit pH was evaluated with pH paper for 150 fruiting trees. There were 116 trees of pH ≤ 3.5 (low pH), 34 of pH ≥ 4.0 (high pH) and 4 of intermediate pH (3.5–4.0) (data not shown). The Chi-squared test confirmed that the segregation of low (116) to high (34) pH also fits the 3:1 ratio ($P_{(df=1,X^2>0.228)} = 0.63$).

Initial QTL analyses of fruit pH and TA in population GMAL 4595

A major QTL, presumably the *Ma* locus, was detected for both fruit pH and TA contents on LG 16 (Fig. 3 and Supplementary Table 1). Interval mapping in Royal Gala found that the *Ma* QTL peaked at or around marker CH05c06 and was supported with LOD scores of 18.34 and 19.82, explaining 41.7 and 42.3% of fruit pH and TA variations, respectively (Fig. 3a). In the Kruskal–Wallis analyses, the *Ma* QTL is supported with highly significant ($P < 0.0001$) values of the *K* statistic, 69.2 for pH and 68.6 for TA (Supplementary Table 1). In *M. sieversii* PI 613988, the *Ma* QTL was also detected near marker CH05c06 on LG 16. However, the peak of the QTL was located in the 8.2-cM interval between markers CH02a03 and CH05c06 (Fig. 3b), and the *Ma* QTL was associated with lower LOD scores (10.35 for pH, 6.13 for TA), a lower percentage of variance explained (28.3% for pH, 17.0% for TA) and lower *K*-statistic values (20.8 for pH and 18.8 for TA) (Supplementary Table 1).

In addition to the *Ma* locus, two minor QTL on LGs 6 and 1, tentatively designated *M2* and *M3*, respectively, were detected for fruit pH and TA based on the Kruskal–Wallis analyses (Supplementary Table 1). *M2* was represented by marker C12360 ($K = 14.1$ and 10.1, $P < 0.0005$ and 0.001, LOD = 1.59 and 2.32, percentage variance explained = 4.3 and 6.2% for pH and TA, respectively) on LG 6 of Royal Gala. *M3* was represented by marker C12063 ($K = 11.1$ and 8.0, $P < 0.001$ and 0.005, LOD = 0.80 and 1.59, percentage variance explained = 2.2 and 4.3% for pH and

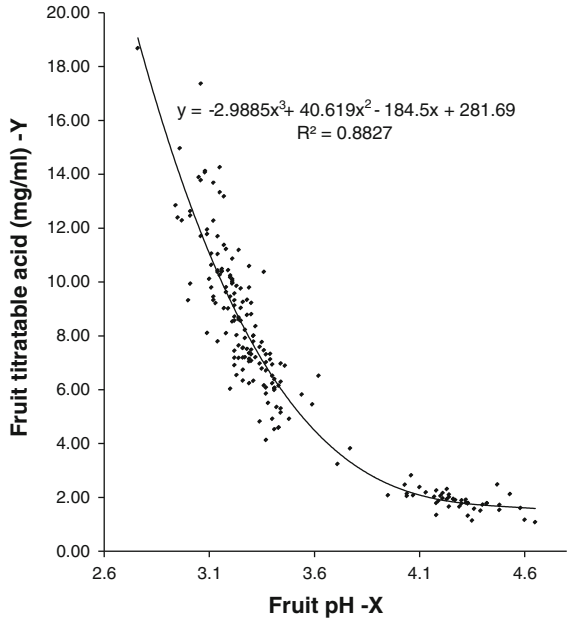

Fig. 2 Relationship between fruit pH and titratable acidity (TA) in population GMAL 4595 ($n = 190$)

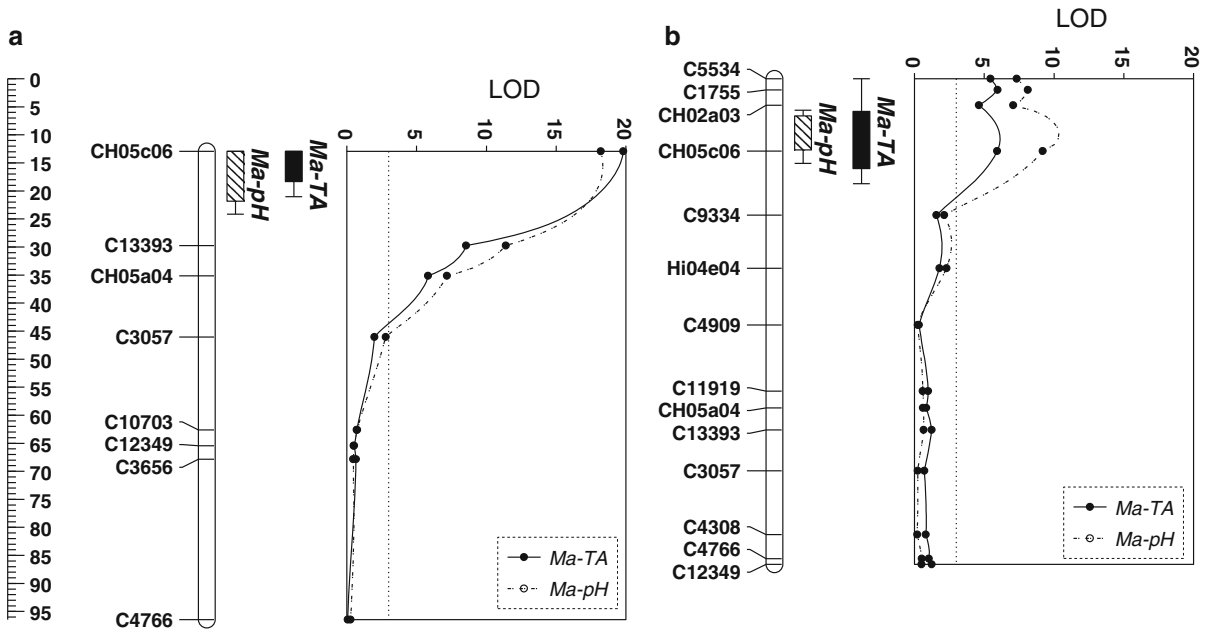

Fig. 3 The *Ma* QTL for fruit pH and TA detected on LG 16 in population GMAL 4595 (Wang et al. 2011). Putative QTL regions are marked with *segments* within 1-LOD (*inner*) and 2-LOD (*outer*) confidence levels from the peak, respectively. The *ruler* measures genetic distance in cM. **a** Royal Gala; **b** *M. sieversii* PI 613988

TA, respectively) on LG 1 of PI 613988. However, there was only one LOD score, LOD 2.32 for *M2* (TA), that was higher than its LG-specific LOD threshold (2.3) obtained from the permutation test, and none exceeded the genome-wide LOD thresholds (3.4–3.8). Moreover, no other significant QTL were detected after an initial round of rMQM mapping was performed for each of the two parental maps. In this round, marker CH05c06 was used as a co-factor as it was found to be effective for controlling the *Ma* QTL effect in both parents. The two markers C12360 and C12063, which represent *M2* and *M3*, respectively, were not used as co-factors in the rMQM analyses as the LODs associated with *M2* and *M3* did not exceed the genome-wide thresholds in the interval mapping (Supplementary Table 1). Overall, the major QTL *Ma* was detected in both Royal Gala and *M. sieversii* PI 613988; but *M2* was only detected in Royal Gala and *M3* was specific to PI 613988.

Fine mapping of the *Ma* locus

To map more genetic markers in the *Ma* region, four published SSR markers (CH05a09, Hi02h08, Hi22f06 and NH026a) that appeared to be linked to *Ma* (Silfverberg-Dilworth et al. 2006) were tested in population GMAL 4595. With the exception of marker NH026a, all other markers were mapped successfully to the *Ma* region in PI 613988 (Fig. 4a, b, Supplementary Fig. 1). The only additional marker that could be mapped in Royal Gala was CH05a09 (Fig. 4c, Supplementary Fig. 1). In population GMAL 4590, when these four markers and CH02a03 and CH05c06 were used, markers CH05a09 and CH05c06 were mapped in Royal Gala while CH02a03, Hi02h08 and CH05c06 were mapped in PI 613971 (Fig. 4c, d; Supplementary Fig. 1).

A total of 36 informative recombinants in the *Ma* region, i.e. the trees arising from zygotic combinations between a recombined gamete carrying a crossover event near *Ma* and a non-recombinant gamete of the *ma* allele, were identified (Supplementary Fig. 1). Out of the 36 recombinants, 15 were of Royal Gala origin and were selected by flanking markers CH05a09 and CH05c06, and of which five were from population GMAL 4595 and ten from GMAL 4590. The other 21 recombinants were from the two pollen parents and were identified by markers CH02a03 and CH05c06, and of which 14 were of the origin of PI 613988 and seven of PI 613971.

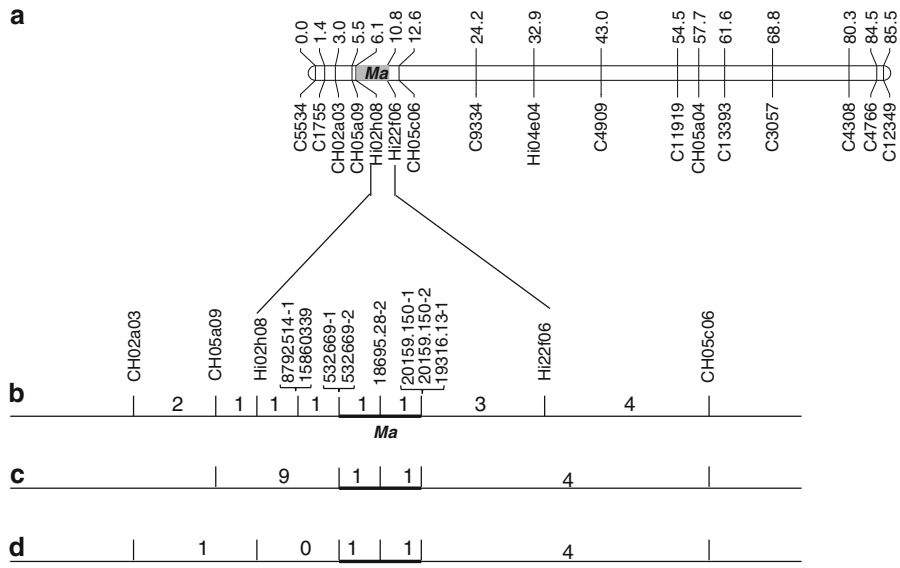

Fig. 4 A fine genetic map of the *Ma* locus. **a** LG 16 of PI 613988. **b** Fine map of the *Ma* locus in PI 613988. The *number* between the markers stands for the number of informative recombinants found in the interval. **c** and **d** The *Ma* region in Royal Gala and PI 613971, respectively

Based on the peak position of the *Ma* QTL in PI 613988, *Ma* was assumed to be in the 8.2-cM interval between markers CH02a03 and CH05c06. A close investigation of the recombinants for the correlation between their markers and pH and TA scores supported the assumption, and suggested a narrowed interval of 4.7 cM harboring the *Ma* locus between markers Hi02h08 and Hi22f06 (Fig. 4a and Supplementary Fig. 1). A BLAST search against the draft sequence of the apple genome (Velasco et al. 2010) identified two contig sequences containing the two markers, MDC010932.713 for Hi02H08 and MDC002276.243 for Hi22f06, which are physically separated by approximately 510 kb in the Golden Delicious genome.

To further narrow down the *Ma* region, DNA sequences were downloaded for eight contigs within the region, including MDC008792.514, MDC015860.339, MDC000532.669, MDC018695.28, MDC020140.291, MDC002726.232, MDC020159.150, and MDC019316.13. A total of 17 SSR primer pairs were designed from these contig sequences and analyzed with the recombinants (Supplementary Fig. 1). Eight out of 17 SSRs were successfully mapped in the region in population GMAL 4595 and/or GMAL 4590 (Table 1), leading to the construction of a fine genetic map for the *Ma* locus in the three parents (Fig. 4b–d). Overall, the map showed that marker 18695.28-2 co-segregated with *Ma* in the two populations, and the *Ma* region was delimited by five markers: 532.669-1 and 532.669-2 from contig MDC000532.669, and 20159.150-1 and 20159.150-2 from MDC020159.150 and 19316.13-1 from MDC019316.13, which are genetically supported by the identification of six key recombinants with three (Fig. 5, Supplementary Fig. 1) in each of the two immediate flanking intervals of *Ma* (Fig. 4b–d), respectively. In the Golden Delicious genome, the homologous *Ma* region defined by the five flanking markers is no larger than 150 kb and contained 44 predicted genes (Supplementary Table 4).

Discussion

Evaluation of fruit acidity

Evaluation of fruit acidity is commonly conducted by measuring fruit pH and TA. The latter requires 5–10 ml of juice be extracted. The juice extraction process is time-consuming and labor-intensive because of the juicing and machine cleaning steps. The subsequent titration is also slow, even using an autotitrator. Measuring fruit pH, in contrast, could be quickly conducted in the orchard without extraction of juices using appropriate pH papers graduated in units of 0.2–0.5, such as the Hydrion papers (pH 3.0–5.5, Micro Essential Laboratory Inc., Brooklyn, NY, USA) used here, or the Whatman pH testing strips of range 1.8–3.8 and 3.8–5.5. In this study, TA was highly correlated with fruit pH ($r^2 = 0.8827$) in population GMAL 4595 in which the two measurements were

Table 1 Primer sequences and other relevant information of SSR markers developed in the *Ma* region

Marker name	Primer-F (from 5′ to 3′)	Primer-R (from 5′ to 3′)	IDs of the source sequences	Targeted SSRs	Expected size (bp)	Allele size for *Ma* (bp)[b]	Allele size for *ma* (bp)[c]
8792.514-1	GGAGGGTCTCCATCCAATTTA	TCCCCACACATCTCATATTCC	MDC008792.514	$(GA)_{17}$	181	182 (p3)	Null (p3)
15860.339	GATCATAAATTTGAGTGAGCAAA	TGTCATTCACGAGATTTGAACC	MDC015860.339	$(CA)_9 + (AT)_7$	319	318 (p2, p3)	330 (p2, p3)
532.669-1[a]	GCCGCTTCTGGACTATCACTA	TCTTAACCAACTAAATTGCATGA	MDC000532.669	$(TC)_{22}$	229	210 (p1)	230 (p1)
532.669-2	AAAGCTTCTTCACACCAAGCA	TGGTGATGATGGTGGTAGTCA	MDC000532.669	$(CT)_{22}$	182	184 (p1), 186 (p2, p3)	172 (p1, p2, p3)
18695.28-2	GACGACGACCCTAACATTGAC	GGCAGCCAAACAGAGAAAAA	MDC018695.28	$(CTT)_6$	173	198 (p1), 190 (p2)	165 (p1, p2)
20159.150-1	GGGTTAAAGCTCAACAGAGCA	CAATGCGGTATGTTCCATCTC	MDC020159.150	$(AT)_{13}$	184	168 (p1), 174 (p2, p3)	190 (p1, p2, p3)
20159.150-2	AGGTATTGCCTAAATGTGTGTG	TCACATCATAATGTTTTCCGAAT	MDC020159.150	$(TA)_{11}$	202	210 (p1)	265 (p1)
19316.13-1	GTTGTGTGTTCGAAGGTGGAG	CCCCATATTCCCCCTCCTAT	MDC019316.13	$(AG)_{11}$	175	178 (p1), 222 (p2)	188 (p1), 218 (p2)

[a] The annealing temperature for PCR amplification of marker 532.669-1 was set at 50°C. The annealing temperature for other markers was 55C°

[b] Allele size linked to *Ma* in coupling phase. p1 = Royal Gala; p2 = PI 613988; p3 = PI 613971

[c] Allele size linked to *ma* in coupling phase

made for 190 genotypes (Fig. 2). In addition, the QTL detected by the two measurements were also very close in genomic position and QTL effects (Fig. 3 and Supplementary Table 1). Based on these data, it appeared to be sufficient to simply evaluate fruit pH using pH papers to screen large populations in genetic studies of *Ma* that only need to discriminate the low-acidity genotypes (pH > 3.8) from the high/medium group. Discovery and genetic mapping of the *Ma* locus were largely accomplished by estimation of fruit pH with pH indicators (Nybom 1959; Visser et al. 1968; Visser and Verhaegh 1978; Maliepaard et al. 1998). However, for selection and breeding purposes, TA also should be determined in order to more comprehensively evaluate acid and taste, including its derivative, the fruit sugar to acid ratio.

Segregation of fruit acidity and the genetic effects of *Ma*

There was not a single genotype with fruit pH 3.81–3.90 in population GMAL4595, whereas there are two peaks of distribution at pH ranges 3.21–3.30 and 4.21–4.30, suggesting that classification of fruits with pH values higher than 3.80 into low acidity is rational. Apples with pH > 3.8 are commonly considered to be low acid or sweet of the *mama* genotype (Nybom 1959; Visser and Verhaegh 1978; Maliepaard et al. 1998). Consistent with these findings, the segregation ratio of the high/medium-acid (pH < 3.80) to the low-acid groups fits the 3:1 ratio in population GMAL 4595 and GMAL 4590, suggesting that the parental lines are all of the *Mama* genotype (Fig. 1).

Within the dominance class of high/medium acidity (TA > 3.0 mg/ml), there appeared to be two overlapping classes for TA (Fig. 1). It has been proposed that the variation in this group is under the control of an additive gene action model (Visser and Verhaegh 1978). This theory, however, has received little attention to date because of the dominance effect of the *Ma* allele if measured in pH and the technical difficulties of differentiating the *Mama* genotype from *MaMa* in breeding populations if measured with TA. The genotypic and phenotypic data generated from this study enabled us to estimate the genetic effects of the *Ma* allele in population GMAL 4595.

Based on the *Ma*-flanking markers CH02a03, CH05a09 and CH05c06, 38, 72 and 32 trees were

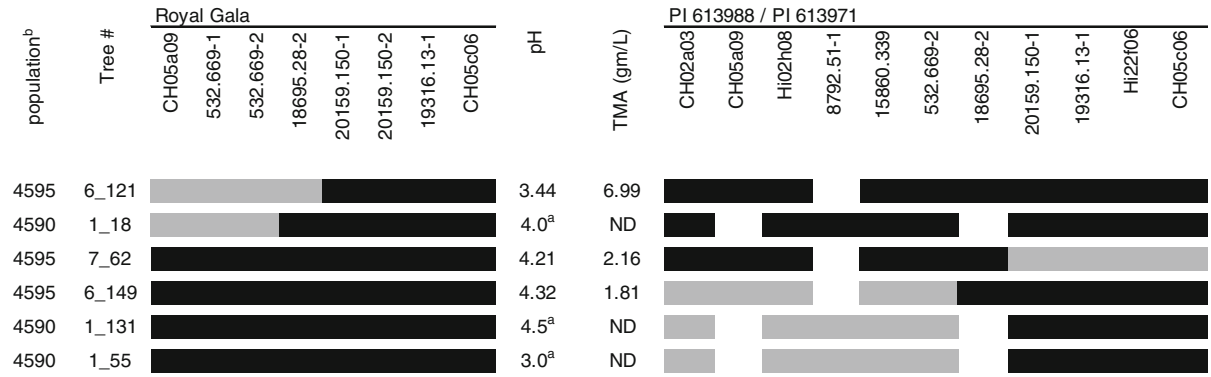

Fig. 5 Key recombinants generically defining the *Ma* locus. *TA* titratable acidity. *ND* not determined. [a]measured by pH paper. [b] *4595* = GMAL 4595; *4590* = GMAL 4590. *Gray* linked to the low-pH or high-acidity allele *Ma*; *Black* linked to the high-pH or low-acidity allele *ma*

determined to be of the *MaMa*, *Mama* and *mama* genotypes, respectively. The mean pH values for genotypes *MaMa*, *Mama* and *mama* were 3.157 ± 0.133, 3.274 ± 0.129, and 4.242 ± 0.215, respectively; and the mean TA contents for the three genotypes were 10.383 ± 2.973, 8.446 ± 2.325, and 2.063 ± 1.106 mg/ml, respectively (Supplementary Table 2). Unpaired means comparisons indicated that the *Mama* genotype differed significantly not only from *mama* ($P < 0.0001$) but also *MaMa* [$P = 0.0002$ (pH) and $P < 0.0001$ (TA)] (Supplementary Table 3). The genotypic values of *MaMa*, $|a_{pH}| = 0.54$ and $|a_{TA}| = 4.16$, which were measured from the midpoint between the two homozygous genotypes, were greater than those of *Mama*, $|d_{pH}| = 0.42$ and $|a_{TA}| = 2.22$, respectively, suggesting that *Ma* was incompletely dominant over the *ma* allele. The degree of dominance was estimated to be 77.8% for pH and 53.4% for TA, largely explaining the distribution and segregation of fruit pH and TA (Fig. 1a, b). The estimated additive effect of the *Ma* allele was significant, i.e. −0.27 units in pH and 1.94 mg/ml in TA. These estimates suggested that an additive-dominant gene action model in controlling the variation of fruit acidity is more appropriate in population GMAL 4595.

Effect of *M2* and *M3* on fruit acidity in genotypes *MaMa*, *Mama* and *mama*

There were 65 trees with a relatively higher TA (9.01–19.00 mg/ml), and another 65 lower (4.01–8.00 mg/ml) in the high/medium-TA range if the boundary was placed at TA 8.01–9.00 and the 14 trees falling within the boundary were excluded from the tally (Fig. 1b). This indicated that the segregation of the high/medium-TA subgroups fitted a perfect 1:1 ratio rather than the expected 2:1 for *Mama*:*MaMa*. TA plotting in the high/medium range showed that the *Mama* progeny had a peak at TA 6.01–7.00 mg/ml versus *MaMa* at 9.01–10.00, and there was a wide overlap in TA between *MaMa* and *Mama* (Supplementary Fig. 2a). Therefore, although there was a significant difference in TA between *MaMa* and *Mama*, there were other factors, including the two minor QTL *M2* and *M3*, that may have caused the overlapping distribution, and thereby the segregation distortion of TA.

To understand if and how *M2* and *M3* might be responsible for the overlapping TA between *MaMa* and *Mama*, the effect of *M2* and *M3* on fruit acidity in genotypes *MaMa*, *Mama* and *mama* were examined with their associated markers C12360 and C12063, respectively. It showed that the segregation of an *M2* allele represented by $C12360_{280bp}$ that was inherited from Royal Gala significantly increased fruit acidity within each group of *MaMa*, *Mama* and *mama*. One consequence of $C12360_{280bp}$ segregation was to abolish the difference in pH and TA between the *MaMa* plants without $C12360_{280bp}$ ($n = 18$) and the *Mama* plants with $C12360_{280bp}$ ($n = 33$) (Supplementary Fig. 3a, b). The segregation of the *M3* (C12063) alleles also had a significant effect on fruit acidity, but limited to genotype *Mama* (Supplementary Fig. 3c, d). The *Mama* plants with two $C12063_{700bp}$ ($n = 34$) alleles had significantly lower pH and higher TA than those of alleles $C12063_{700bp}$ and $C12063_{650bp}$

($n = 30$), leading to the former group being insignificantly different from the *MaMa* plants ($n = 16$) with alleles $C12063_{700bp}$ and $C12063_{650bp}$ (Supplementary Fig. 3c, d). Consequently, the independent allelic segregation of *M2* in genotypes *MaMa* and *Mama*, and that of *M3* in *Mama*, would contribute to the overlapping distribution of TA between *MaMa* and *Mama*, and thus the segregation distortion in the high/medium-TA range.

The occurrence of two sub-groups was not observed for pH (Fig. 1a), despite the presence of a strong correlation between pH and TA (Fig. 2) and the similar effect of *M2* and *M3* on pH (Supplementary Fig. 3a, c). A simple explanation for this discrepancy is the differences in the scale of assessment: pH shows a much smaller range then TA. Moreover, the scores for the lower part of the scale became more compressed, due to which small differences in pH become obscured (Fig. 1a, Supplementary Fig. 2b).

To further investigate whether there are any other factors that may play roles in fruit acidity contributing to the TA segregation distortion, a QTL analysis was performed on the progeny of TA > 4.01 mg/ml using both single parental maps. The results suggested that no other QTL apart from *Ma*, *M1* and *M2* were detectable in population GMAL 4595.

Marker-assisted breeding for fruit acidity

With the QTL and markers identified/developed in this study and elsewhere, it might be possible to screen apple breeding populations at the seedling stage to remove most, if not all, of the *MaMa* and *mama* genotypes, which make up one half of the population in most crosses. The *Mama* genotype may have a selection advantage as most apple varieties are heterozygous (Nybom 1959; Brown and Harvey 1971; Visser and Verhaegh 1978). Fruits with pH < 3.1 or >10 mg/ml in TA are considered to be too high for desert apple varieties, whereas pH > 3.8 or <3.0 mg/ml in TA are too low (Nybom 1959; Brown and Harvey 1971; Visser and Verhaegh 1978). In this study, the *MaMa* genotype has a mean pH of 3.157 ± 0.133 and TA of 10.383 ± 2.973 mg/ml, and the *mama* trees have an average pH of 4.242 ± 0.215 and TA of 2.063 ± 1.106, which are close to or exceed these proposed limits. Removing the *mama* plants, which have been described as "the useless sweet type" in Brown and Harvey (1971), could be done based on the *Ma* tightly linked markers (Table 1). However, to discard the *MaMa* plants, it would be better to consider the effect of *M2* on fruit acidity (Supplementary Fig. 3a, b). Among the *MaMa* plants in population GMAL 4595, there are 19 of an allele of $C12360_{280bp}$, of which 13 are of TA 10.01–19.00 mg/ml, three of TA 9.01–10.00 and three of TA 5.01–9.00 (Supplementary Table 5). To reduce the risk of ridding the *MaMa* genotype of medium acidity, e.g. TA < 10.0 mg/ml (Supplementary Fig. 2, Supplementary Table 5), we propose to first remove one-half of the *MaMa* seedlings that carry the *M2* acid-increasing allele $C12360_{280bp}$. Doing so would discard approx. 37.5% (25% *mama* plus 12.5% *MaMa*) homozygous plants, a fraction close to one-third of the total seedlings of undesirable low- plus high-acid observed in many *Mama* × *Mama* crosses studied (Visser and Verhaegh 1978). However, more studies, especially using real breeding populations to test the selection strategy based on QTL *Ma* and *M2* and their associated markers, are needed to confidently discard the *MaMa* seedlings.

QTL analyses of fruit acidity

QTL analyses of fruit acidity measured with both pH and TA in this study identified a major QTL, the *Ma* locus on LG 16, and two minor QTL on LGs 6 (Royal Gala) and 1 (PI 613988). Detection of the major QTL of *Ma* appears consistent with previous studies (Liebhard et al. 2003; Kenis et al. 2008). The peak of the major QTL of *Ma* was initially located between markers CH02a03 and CH05c06 in PI 613988, and later was confirmed with the fine map of the *Ma* locus in Royal Gala, PI 613988 and PI 613971. The CH02a03–CH05c06 interval was best compared with the interval between markers CH05e04z and CH05c06 in Telamon where a major fruit acidity QTL was detected, although the same interval was inverted in Braeburn (Kenis et al. 2008; Schouten et al. 2011). Except for the *Ma* locus, there were no common QTL detected for fruit acidity among the crosses studied to date. Notably, another major QTL for fruit acidity on LG 8 (Liebhard et al. 2003), was not detected in Telamon × Braebur' (Kenis et al. 2008), nor in population GMAL 4595.

This QTL study was conducted with only one year of fruit pH and TA data. Although a similar previous study was conducted for fruit acidity (Liebhard et al. 2003), the variability of fruit acidity between years

and between individual fruits of the same genotype could still be a concern. Nevertheless, a study addressing such variations between years concluded that the relative acidity trend in 17 cultivars evaluated remained "much the same" between years while fruit malic acid contents varied slightly (Brown and Harvey 1971). The same study also found that the variation in individual fruits harvested at different positions for a given cultivar was negligible compared with those observed between different cultivars, and suggested that "sampling of mixing the juice from a few fruits can be relied upon to give a reasonably accurate figure for the cultivar". This method of bulking several juice samples was also used in Kenis et al. (2008).

Fine mapping of the *Ma* locus

In the fine map, marker 18695.28-2 co-segregates with *Ma* and the *Ma* region is defined by two flanking markers: 532.669-2 on one side, and 20159.150-1 on the other (Table 1, Fig. 4b–d). Successful development of these new SSR markers was due to the availability of the apple (Golden Delicious) genome sequences (Velasco et al. 2010). Genetic evidence supportive of the *Ma* region came from the six key recombinants (Figs. 4, 5 and Supplementary Fig. 1). Together with the new markers, the six key recombinants delimit the *Ma* region within a genomic segment of approx. 150 kb in the Golden Delicious genome. Although a few sequence gaps are present within the 150 kb region (Velasco et al. 2010), there are 44 genes predicted in it (Supplementary Table 4). Based on the current version of apple genome annotation (Velasco et al. 2010) and our own BLAST search against GenBank, the 44 predicted genes are largely hypothetical and yet quite diverse, including 19 of hypothetical proteins, four of ribosomal S3Ae family proteins, three of no significant similarities, three of Med25_VWA (mediator complex subunit 25 von Willebrand factor type A)-like proteins, two of serine/threonine protein phosphatase 2a regulatory subunit A, and several others. It is likely that the candidate genes of *Ma* are among the 19 hypothetical protein-encoding genes. To reveal the identity of *Ma*, one approach may begin with a gene expression experiment to inspect whether the expression pattern of any of these 44 predicted genes is correlated with the fruit acidity variation in some 20 representative apple varieties of genotypes *mama* and *Mama* or *MaMa*. If this experiment succeeds in identifying genes that show positive correlations as expected, they will be considered to be strong candidate genes for *Ma*. To functionally prove these candidate genes, both over- and under-expression approaches will be taken to genetically and/or transiently transform apple plants and/or fruit cell cultures, respectively.

This fine map of the *Ma* locus was constructed using 438 F_1 trees in the two mapping populations, a low number compared with fine mapping of important plant genes/QTL. For example, to define a genomic region of 350 kb for the locus of *Vf*, an apple scab resistance gene, 2,071 plants in seven populations were required (Patocchi et al. 1999; Vinatzer et al. 2001). More than 4,000 F_2 plants were used in the case of the *Sub1* QTL conferring submergence tolerance in rice, which was mapped to a 150-kb region (Xu et al. 2000, 2006). Our ability to find a sufficient number of informative recombinant plants, particularly the six key recombinants, is attributed to the fact that the *Ma* region had a much higher recombination frequency in the two mapping populations: the ratio of genetic/physical distances in the *Ma* region of 150 kb was calculated to be 1 cM per 110 kb, much greater than the genome-wide average of 1 cM per 500–600 kb.

In conclusion, the *Ma* locus has been shown to be the primary genetic factor determining fruit titratable acid and/or pH in both Royal Gala and the two *M. sieversii* accessions PI 613988 and PI 613971. In addition, there are two minor QTL detected for fruit TA and pH, with *M2* specific to Royal Gala and *M3* to PI 613988. The variations in fruit acidity in population GMAL 4595 are better explained by the additive-dominance gene action of allele *Ma*, as it has a strong additive effect in increasing fruit acidity and is incompletely dominant over *ma* although *M2* and *M3* modify acidity in the high/medium-acid range. The eight new SSR markers developed here would be useful in marker-assisted breeding in apple. Construction of the fine map of the *Ma* locus represents an important step forward in isolating the *Ma* gene(s).

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