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## Analysis of the apple fruit acid/low-acid trait by SSR markers

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**Abstract** It is necessary to find out the genetic characteristics of malic acid in the course of apple genomic research and breeding. In this study, the SSR marker linked to the acid/low-acid trait in apple fruit was identified from 140 SSR primer pairs, using 91 F<sub>1</sub> population hybrids from the intra-specific cross between apple cultivar ‘Dongguang’ and ‘Fuji’ as the experimental materials. Of 140 SSR primer pairs, only primer SDY085 produced a polymorphic band linked to acid trait, and the linkage distance was 8.89 cM. Also, the titrated acid and malic acid in different developmental stages were determined. The SSR marker analysis, coupled with the change of the total acid and malic acid contents, revealed that the acid/low-acid trait was governed by a major gene and acid trait was completely dominant.

**Keywords** apple, *Malus × domestica* Borkh., acid/low-acid trait, SSR marker

### 1 Introduction

Organic acids are important attributes of fruit flavour and, as such, contribute with soluble sugars and aromas to the overall organoleptic quality of fresh apples (Sweeney et al., 1970). Research on the genetic character of apple fruit acidity will not only help to study the *Ma* gene that can control apple fruit acidity, but also offer the markers for the assistant selection of the high-acid processing apples. Presently, the inheritance of apple fruit acidity was studied mainly by the phenotype value of organic acid content. Some researches have shown that apple fruit acidity was controlled by the additive factors of the major gene (*Malma*) and other minor genes, and the recessive homozygote exhib-

ited the low-acid phenotype (Nybom, 1959; Visser and Verhaegh, 1978; Li et al., 1994). Nevertheless, the dominant degree of *Ma* versus *ma* was not in union. For example, *Ma* was considered to be completely dominant to *ma*, and thus the high or mid acidity was attributed to the other polygenes in the dominant homozygote and heterozygote (Nybom, 1959; Visser and Verhaegh, 1978; Liu et al., 2004). Whereas, the incomplete dominance for *Malma* was raised by Li et al. (1994), and the dominant homozygote (*MaMa*) and the heterozygote (*Malma*) exhibited the high and mid acidity, respectively, the successive changes in the hybrids of the same type of acidity were determined by the other polygenes (Li et al., 1994). At the molecular level, the QTL linked to *Ma* was detected by Maliepaard (1998). In our study, the inheritance of apple fruit acidity was analyzed by the phenotype values of acidity and screened SSR molecular markers linked to *Ma*.

### 2 Materials and methods

#### 2.1 Experimental materials

The 12-year-old hybrids were selected from the cross of ‘Toko’ × ‘Fuji’ (*Malus × domestica* Borkh.) in the experimental orchard of the Liaoning Institute of Fruit Tree Science (Liaoning, China). The titratable acid of ripe apple fruits was determined by alkalimetry in the successive three years. The malic acid content was tested with capillary electrophoresis in each 6 hybrids of the high (>0.7%), mid (0.36%–0.7%) and low (<0.36%) acidity every month. Here, the titratable acid of more than 0.36% is considered to belong to the acidic character, when on the contrary, it belongs to the low-acid character.

#### 2.2 Conditions for capillary electrophoresis (Backman)

The conditions for the detection of organic acids were mainly through uncoated capillary electrophoresis (50 μm × 57 cm); effective length: 50 cm; applied voltage: 10 kV; injection time: 3 s; λ = 200 nm; Temp = 20°C;

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100 mmol·L<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>+0.5 mmol·L<sup>-1</sup> CTAB; pH = 7; and indirect detection at 200 nm.

### 2.3 Preparation of template DNA

Genome DNA from young leaves on autumn shoots was extracted with the SDS method, and then it was digested by RNase A. DNA concentration was adjusted to 10 ng·μL<sup>-1</sup> after spectrophotometric analysis.

### 2.4 Construction of the acid and low-acid gene pools

DNA from eight extremely high- or low-acid apple fruits were mixed equally to construct the acid or low-acid gene pool, according to the theory of Bulk Segregation Analysis (BSA).

### 2.5 Screening of SSR markers

PCR was performed in a reaction volume of 15 μL including 10 ng DNA, 10 mmol·L<sup>-1</sup> Tris-HCl, pH 9.0, 0.2 mmol·L<sup>-1</sup> each dNTP, 1.5 mmol·L<sup>-1</sup> MgCl<sub>2</sub>, 50 mmol·L<sup>-1</sup> KCl, 0.2 μmol·L<sup>-1</sup> each of forward and reverse primers, and 1U Taq E. Amplification was carried out with the following cycling parameters: 2 min and 30 s at 94°C; 4 cycles of denaturation at 94°C for 30 s, annealing at 65°C (decrease 1°C every cycle) for 1 min, and extension at 72°C for 1 min; then 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The amplified products were separated by 6% polyacrylamide gel electrophoresis and then displayed by silver staining.

### 2.6 Data analysis

The linkages between SSR markers and target characters were analyzed by Mapmaker/Exp3.0. Recombination rate was transformed to genetic distance (centimorgan, cM) by a Kosambi function.

## 3 Results

### 3.1 Distribution of the titratable acid in the hybrids

In the tested 91 hybrids, the titratable acid ranged from 0.10% to 1.10% and showed a double-peak pattern (Fig. 1). Seventy hybrids showed the acid character while 21 had the low-acid character, which accorded with the distribution of 3:1 and tested by  $\chi^2$  ( $\chi^2 = 0.09$ ,  $\chi^2_{0.05} = 3.84$ ,  $\chi^2 < \chi^2_{0.05}$ ). Therefore, the acid/low-acid trait of the apple fruit corresponded to the single-gene inheritance, i.e., the acid/low-acid trait was controlled by a major gene. In addition, the acid character was dominant to the low-acid one and the parents of the cross were probably heterozygotes (*Mama*).

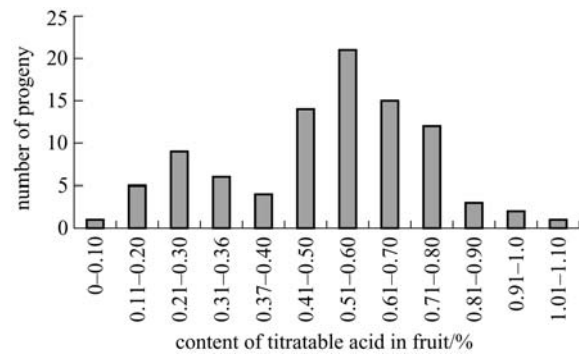


Fig. 1 The distribution of fruit titratable acid in the progeny

### 3.2 Screening of SSR markers linked to the acid/low-acid trait by near isogenic pools

One hundred and forty pairs of SSR primers were used to screen the different PCR bands between the acid and low-acid isogenic pools. Resultantly, 132 pairs of SSR primers could amplify clear bands, of which 117 pairs amplified only one locus including less than two alleles and 15 pairs amplified two different loci including 3–4 different alleles. Five pairs were found to produce the polymorphic bands, whereas only the specific band from the primer pairs of SDY085 (forward primer: 5'-gcc cag aag caa taa gta aac c-3', reverse primer: 5'-att gct cca tgc ata aag gg-3') cosegregated with the acid character.

### 3.3 Cosegregation analysis of the polymorphic bands in the cross population

SDY085 primer pairs amplified the two specific bands of 120–130 bp, designated as band A and B, in both the acid isogenic pool and the acid hybrids, while none in the low-acid pool and the low-acid hybrids. Therefore, it could be concluded that A and B bands together linked to the *Ma* locus. The two bands were also found in both parents 'Toko' and 'Fuji', indicating that both parents had *Ma* locus and their genotypes were *Mama*. The gene recombination was found in 8 of 91 hybrids, with the recombination frequency of 8.79%, which was tested by  $\chi^2$  ( $\chi^2 = 1.94$ ,  $\chi^2_{0.05} = 3.84$ ,  $\chi^2 < \chi^2_{0.05}$ ). The genetic distance between the screened SSR marker and *Ma* locus was 8.89 cM by the Kosambi formula.

### 3.4 Codominance of the screened SSR marker

In the electrophoresis figures, six clear bands of 70–130 bp were found in the dominant homozygotes, whereas four bands below 120 bp did not link to *Ma* for the reason why they existed in some low-acid hybrids while not in some acid hybrids. For the recessive homozygotes, four bands of 140–170 bp were found. From the banding patterns, the genotypes of both parents were heterozygous (*Mama*), while, of 91 hybrids, 25 were dominantly homozygous (*MaMa*), 45 were heterozygous (*Mama*) and 21

were recessively homozygous (*mama*), which corresponded to the segregation of 1:2:1, tested by  $\chi^2$  ( $\chi^2 = 0.84$ ,  $\chi^2_{0.05} = 5.99$ ,  $\chi^2 < \chi^2_{0.05}$ ). In this paper, if the titratable acid of more than 0.7% and 0.36%–0.7% corresponded to the high- and mid-acid genotypes, respectively, only 50% of 22 acid hybrids were dominant homozygotes (Fig. 2(a) and (b)) and 58% of 48 mid-acid hybrids were heterozygotes (Fig. 2(c) and (d)), indicating that genotypes were not consistent with the acidity contents and *Ma* was completely dominant to *ma*.

### 3.5 Position of SDY085 in the linkage map

Liebhart et al. (2002) positioned some SSR primers on the genetic linkage map of the cross 'Fiesta' × 'Discovery'. Here, the screened SSR marker linked to the acid character of apple fruit was positioned on the sixth linkage group of both parents, which would contribute to the precise position on the chromosome of the genes involved in the acid/low-acid trait. In addition, SSR markers are transferable among different species. Therefore, the screened marker also contributes to the construction of the genetic maps of other species.

## 4 Discussion

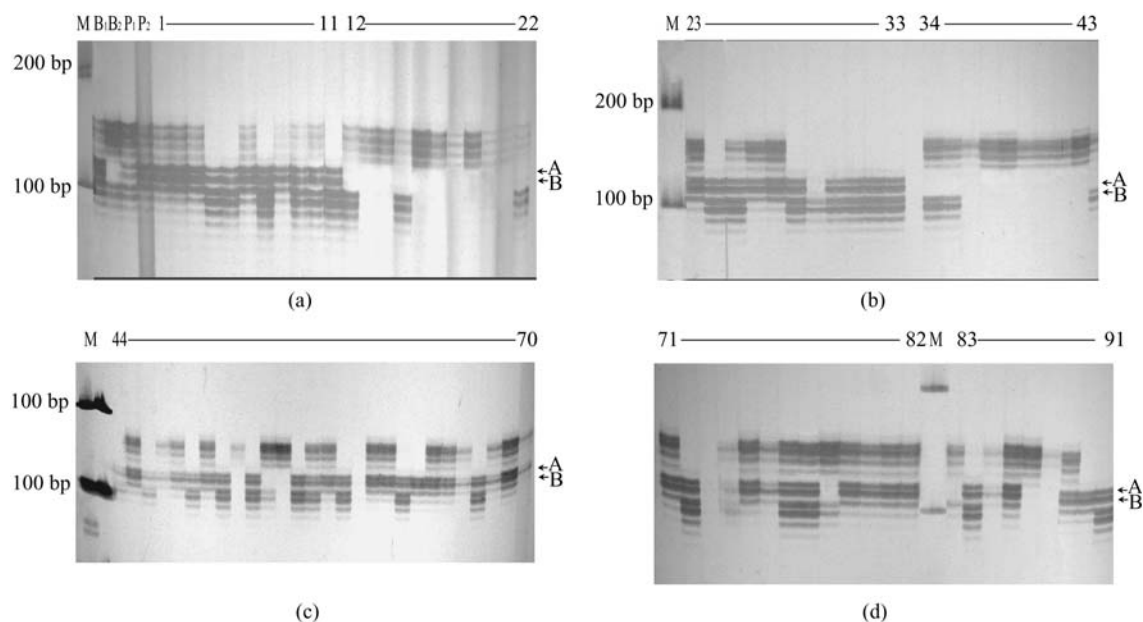
### 4.1 Screening of SSR markers involved in the acid/low-acid trait of apple fruit

SSR markers have some advantages over other molecular markers. They show codominant inheritance and reflect a

large number of alleles per locus. In addition, since the uses of SSRs are based on PCR, this technique is simple and well repeated. It is widely considered that SSRs isolated from a source genome can be transferred to different individuals of the same species or the same genus. However, most of SSRs are located in the untranslated regions, so they can not be used to screened markers at cDNA level. Up to now, only 140 SSRs have been developed and are far from the need for marker screening and constructing genetic linkage maps in apples.

Since fruit trees are perennial and highly heterozygous with a long juvenile phase, it is very difficult to construct the near isogenic lines. Nevertheless, the theory of BSA has been an effective method of screening the molecular markers linked to specific genes. Generally, the DNA pool is composed of 6–10 individuals, whereas the optimum quantity of individuals lies on such factors as the complexity of genome, genetic relationships and types of molecular markers. In this paper, it was found that there were too many polymorphic bands to screen normally between the two pools composed of six hybrids, respectively. On the other hand, the increase of the individual quantity in one pool will raise the gene exchange rate and have some effects on the effective amplification of some bands. Therefore, eight hybrids were selected to compose the gene pool and the polymorphism between two pools was moderate for the next analysis.

Because of the limited quantity of SSR primers, no SSRs, linked closely to *Ma* (<5 cM), were obtained, whereas the SSR marker of 8.89-cM genetic distance to *Ma* was acquired and could be used to analyze the *Ma* genetic character and construct a genetic map and not to



**Fig. 2** Segregation of specific fragment amplified by SDY085 in the progeny

Notes: M, B1, B2, P1 and P2 represent Marker, DNA pool of acid trait, DNA pool of non-acid trait, Dongguang (♀) and Fuji (♂), respectively. 1–11, 23–33, 44–70 and 71–91 are acid individuals and 12–22, 34–43 are non-acid individuals. The specific fragment is arrowed.

assist breeding and perform map-based cloning. At the same time, we carried out cDNA-AFLP to obtain the partial sequence of *Ma* and finally isolate the gene.

#### 4.2 Rules to inheritance of the acid/low-acid trait of apple fruit

So far, there were no unified criteria of compartmentalizing the acid character and low-acid character. Here, the titratable acid content of 0.36% was considered as the borderline between the acid and low-acid characters in the light of its distribution, which was also in accordance with SSR results. The criteria of pH 3.8 and pH 4.2 were also reported by Visser (1978) and Li (1994), respectively. From the above, it is difficult to acquire a uniform criterion to compartmentalize the acid/low-acid traits because the acid content was affected by many factors such as climate, cross population, determination time and method of acidity, and so on.

It is worth mentioning that the content of organic acid is different from the acid trait. The former refers to the summation of all kinds of organic acid, while the latter is raised against the low-acid character, i.e. the content of some organic acid is below its critical value. For example, in tomato, the inheritance of fruit acidity, which had several kinds of organic acids, was shown to be polygenic (Fulton et al., 2000; Saliba-Colombani et al., 2001), whereas its low-acid character was governed by the major gene (Stevens, 1972). The low-acid character was also governed by a major gene in peaches (Yoshida, 1970), grapes (Boubals, 1971) and pummelo (Cameron and Soost, 1977).

The low-acid character was controlled by a dominant major gene in peaches (Monet, 1979), while it was controlled by a recessive one in citrus (Fang et al., 1997), suggesting the different mechanism of inheritance in different species. Malic acid and citric acid were each governed by a major gene in tomato (Maliepaard et al., 1998), which accumulated both of them, and in the pummelo (Saliba-Colombani et al., 2001), which accumulated only citric acid. The acidity of apple fruit is mostly from malic acid. Therefore, the genes controlling the acid/low-acid trait should affect the metabolism of malic acid. Here, the screened SSR marker indicated that the corresponding gene controlled the acid/low-acid trait but did not govern the successive changes of acidity. Therefore, apple fruit acidity was also influenced by the other additive polygenes. It can be speculated that the major and minor genes were each affected by other genes in the regulatory network of acidity. Sequentially the formation of apple fruit

acidity would be determined by so many factors as to the continuous changes.

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