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Research article

A novel gene, screened by cDNA-AFLP approach, contributes to lowering the acidity of fruit in apple

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Abstract

cDNA-AFLP, coupled with bulked segregant analysis (BSA), was used to screen genes expressed differently between low- and high-acid apple fruits from hybrids of 'Toko' × 'Fuji' (*Malus* × *domestica* Borkh.). Sixty-four combinations of AFLP primers produced 2240 fragments, of which only one showed different expression between low- and high-acid fruits. The specific fragment was cloned and sequenced, and the complete cDNA was achieved by 3′ and 5′ rapid amplification of cDNA ends (RACE). The screened gene, designated as *Mal-DDNA* (GenBank accession no. DQ417661), showed no significant homology to clones in GenBank. The relatedness between fruit acidity and the transcription level of *Mal-DDNA* was identified by RT-PCR analysis on 30 hybrids. RT-PCR analysis indicated that *Mal-DDNA* transcripted in low-acid fruits at both early and ripe stages whereas in high- and mid-acid fruits, it did not transcript at the early stage. RNA gel-blot hybridization indicated that *Mal-DDNA* transcripted only in fruits and had clear difference between low- and high/mid-acid fruits. There was a good indication that *Mal-DDNA* existed as one copy in apple genome by Southern blot. Possible regulation of *Mal-DDNA* in apple fruit acidity is also discussed in the paper.

Keywords: *Malus* × *domestica* Borkh; Fruit acidity; cDNA-AFLP; cDNA cloning; DNA and RNA blot analysis

1. Introduction

Flavor is most complex to analyze, and over 350 volatile compounds have been detected in apple [1]. Organic acids are an important component of fruit flavor and, together with soluble sugars and aromas, contribute to the overall organoleptic quality of fresh apple fruits. Eleven organic acids were identified in apple pulp with an additional five in the whole fruit [2]. Malic acid is the predominant organic acid in apple fruits, and it has been proposed by several authors that malic acid content in fruit is controlled by a major gene [3–5] with homozygous individuals being either too sweet (flat) or too sour, although not always in unison [4]. Also, the identification of low-acid varieties have revealed the effect of a major gene governing acidity in other species including tomato [6], peach [7], grape [8] and pomelo [9]. Genetic studies of the species that accumulate either malic and citric acids (tomato) or only one of the two (malic acid in apple and citric acid in pomelo) have shown that malic and citric acids are each governed by a major gene [6,10,11]. Especially in tomato, malic and citric acids were each governed by a major gene while the inheritance of fruit acidity was shown to be polygenic [12,13]. The low-acid character was found to be recessive in apple [5,11] and citrus [10] but dominant in peach [7]. Therefore, the mechanism determining low acidity should not be identical in different species. Nevertheless, the degree to which the character is dominant or recessive in each species

Abbreviations: cDNA-AFLP, cDNA-amplified fragment length polymorphism; BSA, bulked segregant analysis; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; Mal-DDNA, the name of the gene screened by cDNA-AFLP in the paper; DAB, days after full bloom.

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is not clear so far. In apple, the position of the *Ma* gene was determined by means of a genetic linkage map of the cross 'Prima' × 'Fiesta' [11]. AFLP marker E31M38-0193 and SSR marker CHO5e04z [14] were found to be linked to fruit acidity. Apart from this, almost nothing is known about apple fruit acidity at the molecular level.

Although a gene does not have to be up- or down-regulated to play a key role in a certain process, screening for differentially expressed genes is one of the most straightforward approaches to unravel the molecular basis of a biological system. As a differential screening method, cDNA-amplified fragment length polymorphism (cDNA-AFLP) is more stringent and reproducible than many others and can amplify low-abundance transcripts [15]. In contrast to most hybridization-based techniques, it can distinguish between highly homologous genes from gene families without any prior knowledge of the sequence [16]. A combination of bulked segregant analysis (BSA) [17] and the 'double pseudotest cross' [18] theory, cDNA-AFLP is an effective method to screen differentially expressed genes related to apple fruit acidity via the F1 segregating population and was therefore used in this study.

This work led us to characterize the complete cDNA from the screened gene, which was designated as *Mal-DDNA*. To our knowledge, this is the first report of genes expressed differentially in apple fruits of different acidity.

2. Results

2.1. cDNA-AFLP analysis

Six hybrids each of extremely low and extremely high acidity were used for cDNA-AFLP analysis. Their cDNA pools were initially analyzed by 64 pairs of primer combinations to screen the differentially expressed fragments. Sixty-four primer combinations produced a total of 2240 fragments, of which only three were polymorphic between the two cDNA pools, whereas only one 112-bp-sized polymorphic band, amplified with the primer combination *Eco*I-AAC/*Mse*I-CTT, showed consistent difference between each member of the two gene pools (Fig. 1). The fragment was linked to the low-acid trait and existed in all the six extremely low-acid hybrids and in none of the six extremely high-acid hybrids. The specific fragment was then excised, re-amplified and sequenced.

2.2. Molecular cloning and sequence analysis for Mal-DDNA

RACE PCRs (5′ and 3′) were used to amplify the flanking regions of the screened gene with the primers based on the sequence of the fragment gained by cDNA-AFLP. The result indicated a 3728-bp cDNA including an ATG start codon, a TGA stop codon, 11 bp of 5′ untranslated region (UTR), and 326 bp of 3′ UTR. The cDNA open reading frame encoded a 1129-amino-acid protein with a predicted p*I* of 3.99 and an estimated molecular weight of 124,852 Da. The cDNA sequence (GenBank accession no. DQ417661) did not show any significant homology to clones in GenBank. Both TMpred and DAS-TMfilter analysis showed that there were no transmembrane regions for the predicted protein of *Mal-DDNA*. LOCSVMpsi analysis showed the predicted subcellular localization of *Mal-DDNA* in the nucleus and the expected accuracy was 89%, while PSORT analysis indicated *Mal-DDNA* to be localized in the cytoplasm (certainty = 0.650).

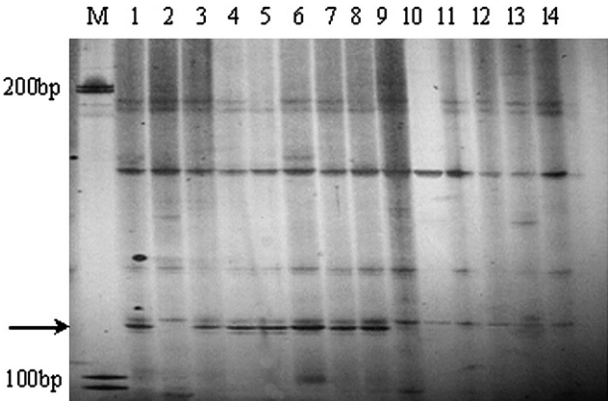

Fig. 1. Silver-stained 6% polyacrylamide gel of cDNA-AFLP products amplified by *Eco*I-AAC/*Mse*I-CTT primer combination. The arrow indicates the band not detected in high-acid hybrids but present in low-acid hybrids. M, DNA marker (100-bp ladder); 1, the low-acid cDNA pool; 2, the high-acid cDNA pool; 3–8, low-acid hybrids; 9–14, high-acid hybrids.

2.3. Genomic complexity of Mal-DDNA

To determine the copy number of *Mal-DDNA*, gel-blot analysis was performed using apple genomic DNA digested with *EcoR*I, *EcoR*V and *Xba*I. There was no cut site within *Mal-DDNA* cDNA for the three enzymes in the probed regions. The blot was probed with part of the coding region of *Mal-DDNA* under high-stringency conditions. The result showed a strong band for each digestion (Fig. 2), strongly suggesting one copy of *Mal-DDNA* in apple genome.

2.4. RT-PCR and Northern blotting of Mal-DDNA

In order to confirm that *Mal-DDNA* correlated to apple fruit acidity, RT-PCR was performed on 30 hybrids, of which 15 were low-acid and others were high- or mid-acid. *Mal-DDNA* was detected in all the low-acid hybrids (Fig. 3A) but not detected in any of the rest except in one high-acid hybrid at 30 DAB (Fig. 3B). Furthermore, *Mal-DDNA* transcripted only in low-acid genotypes at 90 DAB (Fig. 3C). Meanwhile, Northern blot on ripe fruits showed *Mal-DDNA* to have been transcripted in three genotypes (Fig. 3D). The above results suggested the same expression pattern in the mid-acid genotype as in the high-acid one, but different from that in the low-acid genotype.

Expression patterns were determined throughout fruit development, and young leaves, roots and stems were also included in the analysis to find out whether the pattern was specific to the fruit. *Mal-DDNA* was expressed only in the fruit and its expression level was developmentally regulated (Figs. 4 and 5). The expression levels in both the high- and low-acid