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Genomic structure and sequence polymorphism of E,E-alpha-farnesene synthase gene in apples (*Malus domestica* Borkh.)

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Abstract Primer pairs were designed to amplify the genomic DNA sequence of the alpha-farnesene synthase (AFS) gene by PCR. The PCR products were sequenced, spliced and compared to cDNA sequences in the GenBank (accession No. AY182241). The genomic sequence and intron-exon organization of the AFS gene were thus obtained. The AFS genomic sequence has been registered in the GenBank (accession No. DQ901739). It has 6 introns and 7 exons, encoding a protein of 576 amino acids. The sizes of the 6 introns were 108 bp, 113 bp, >1000 bp, 125 bp, 220 bp and 88 bp, and their phases were 0, 1, 2, 2, 0, 0, respectively. The sizes of the deduced amino acids of the 7 exons were 57, 89, 127, 73, 48, 83 and 99, respectively. The AFS protein contained three motifs: the RR(X8)W motif encoded by a sequence in exon 1, and the RxR motif and DDxxD motif encoded by two sequences in exon 4. After comparing the AFS genomic sequence (accession No. DQ901739) to the cDNA sequence (accession No. AY523409) in the GenBank, it was found that there were 6 single-nucleotide polymorphisms between the two sequences, four of which caused mutations at the amino acid level. Interestingly, one amino acid mutation (291R → G) was found in the RxR motif, and further investigation is needed to determine whether the alpha-farnesene synthesis ability and superficial scald susceptibility of apples are influenced by this amino acid mutation and other mutations.

Keywords apple, alpha-farnesene synthase, genomic DNA, polymorphism, superficial scald

1 Introduction

E,E-alpha-farnesene, an acyclic sesquiterpene, is considered to be related to the development of apple superficial

scald (Hu et al., 2002). The genes encoding for HMG-CoA reductase (HMGR), FPP synthase (FPPS), and alpha-farnesene synthase (AFS) are involved in its formation (Rupasinghe et al., 1998). When lovastatin, a competitive inhibitor of HMG-CoA reductase, was used to treat fruits, the formation of alpha-farnesene and the incidence of apple superficial scald were almost completely blocked (Ju and Curry, 2000). Therefore, it is possible to control apple superficial scald by controlling the genes involved in the formation of alpha-farnesene.

Alpha-farnesene synthase is the enzyme in the immediate step in the synthesis of alpha-farnesene. The cDNA encoding for alpha-farnesene synthase has already been cloned (Pechous and Whitaker, 2004; Li et al., 2006). The expression of the alpha-farnesene synthase gene in relation to levels of alpha-farnesene and conjugated trienols in fruit peel tissue has also been studied (Pechous et al., 2005). There is no report yet on the genomic organization and sequence polymorphism of E,E-alpha-farnesene synthase gene in apple cultivars. Some reports showed that the difference in sesquiterpenes between two corn cultivars contributed to the allelic variation of two terpene synthase genes (TPS4, TPS5) (Kollner et al., 2004), and DNA polymorphisms in introns were found to be associated with phenotypic trait (Kuhnlein et al., 1997). Therefore, it is necessary to study the genomic DNA and sequence polymorphisms of alpha-farnesene synthase gene, and to explore their relationship with the development of apple superficial scald in order to control apple superficial scald using genetic engineering.

2 Methods

2.1 Extraction of genomic DNA

Genomic DNA was extracted from young leaves of the apple cultivar 'Ralls'. The extraction protocol was based on methods previously described (Chen et al., 2004; Yuan, 2005). The 2% CTAB extraction buffer consisted of 2% CTAB, 100 mmol·L⁻¹ Tris-HCl (pH 8.0), 20 mmol·L⁻¹

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EDTA, and $1.4 \text{ mol}\cdot\text{L}^{-1}$ NaCl, 2% PVP-40, and 2% β -mercaptoethanol. The extraction buffer was preheated in a 65°C water bath for 5 min, then 0.6 mL extraction buffer was added to each sample. The sample tubes were placed in a 65°C water bath for 30 min, gently shaken 3 times, then 0.6 mL chloroform/isoamyl alcohol (24/1, v/v) was added and sufficiently mixed with the samples. About 0.42 mL of the supernatant in the sample tubes was transferred to a new tube after centrifugation at $13000 \text{ r}\cdot\text{min}^{-1}$ for 10 min. 0.42 mL of cold isopropanol (-20°C) was added into the new tubes, mixed by gentle shaking, then nucleic acids were precipitated with an equal volume of cold isopropanol. The supernatant was decanted after centrifugation at $13000 \text{ r}\cdot\text{min}^{-1}$ for 10 min. The pellet obtained was rinsed twice with $420 \mu\text{L}$ 70% ethanol (-20°C), air-dried, resuspended in 30 or $50 \mu\text{L}$ 0.2X TE buffer, then kept at either 4°C for immediate use or stored in a freezer.

2.2 Primer design, PCR amplification and sequencing genomic DNA

The longest cDNA sequence of the AFS gene in the GenBank was used to design primers P1, P2R, P3, P4R, P5 and P6R (18–22 bp) (Table 1). P3f, P4fR and P4gR in Table 1 are the primers designed to sequence some parts of the genomic DNA that were not obtained at the first stage. The PCR reactions ($50 \mu\text{L}$) contained $1 \mu\text{L}$ (approximately 50 ng) genomic DNA and $49 \mu\text{L}$ reaction solution mixture. The reaction solution mixture was composed of $1 \mu\text{L}$ dNTPs ($10 \text{ mmol}\cdot\text{L}^{-1}$), $5 \mu\text{L}$ Taq buffer, $1.5 \mu\text{L}$ forward primer ($10 \mu\text{mol}\cdot\text{L}^{-1}$), $1.5 \mu\text{L}$ reverse primer ($10 \mu\text{mol}\cdot\text{L}^{-1}$), $0.5 \mu\text{L}$ (1U) Taq polymerase and $39.5 \mu\text{L}$ water. A total of 35 PCR cycles were performed. Each cycle included a 30-sec denaturation step at 94°C , followed by a 1-min annealing step at the appropriate temperature (50 – 55°C) for each specific marker, then 2 min of primer extension at 72°C . A mixture of $2 \mu\text{L}$ PCR products, $8 \mu\text{L}$ water and $2 \mu\text{L}$ loading dye was used to check the integrity of the PCR products by electrophoresis at 80 V on a 1.5% agarose gel stained with ethidium bromide. If PCR products were good, the DNA purification and sequencing were conducted by a biotechnology company.

2.3 Analysis of genomic DNA

The sequenced DNA segments were spliced to obtain the genomic sequence of the AFS gene. The spliced sequence was compared to cDNA sequence in the GenBank (accession No. AY182241) using the Blastn tool to find out the spliced sites of introns and exons in the AFS gene sequence. The spliced sequence was also compared to another cDNA sequence in the GenBank (accession No. AY523409, a gene from the apple scald-resistant cultivar 'Idared'), to identify single nucleotide polymorphisms between the two sequences. Sequin software was used to create a GenBank file and to screen for amino acid mutation site(s) between the two apple cultivars.

3 Results

3.1 Genomic sequence and structure

The size of the obtained genomic sequence of the AFS gene in the apple cultivar 'Ralls' was 3009 bp, but a gap sequence in the middle part of the gene could not be obtained. The genomic structure of the AFS gene was determined by comparing its genomic sequence with the cDNA sequence of the AFS1 gene in GenBank. It was found that the AFS gene had 6 introns and 7 exons, encoding a protein of 576 amino acids. The sizes of the 7 exons were 57, 89, 127, 73, 48, 83 and 99, respectively. All 6 introns began with GT and ended with AG, tallying with the GT---AG rule. Their phases were 0, 1, 2, 2, 0 and 0, and their sizes were 108 bp, 113 bp, >1000 bp, 125 bp, 220 bp and 88 bp, respectively (Fig. 1). Furthermore, 5 bp in the upstream of the start codon and 69 bp in the downstream of the end codon were also obtained. The sequence gap was in intron 3. Intron 3 was very big, but only 550 bp could be obtained. Based on the position of the PCR product band on the agarose gel, the size of the gap sequence was about 519 bp. The obtained genomic sequence of the AFS gene in the apple cultivar 'Ralls' has been registered in GenBank (accession No. DQ901739).

Results from the amino acid analysis showed that there were 3 motifs in the amino acid sequence: the RR(X8)W

Table 1 PCR products and related primers

PCR product	forward primer	reverse primer
P1-2R	P1: TATCCCAAACATCTCgAgC	P2R: TAGCgTCAAaggAAgCTTTC
P3-4R	P3: CCTCAAACCTgggTTTCg	P4R: ATTCTCCTCTCAATTTCagc
P5-6R	P5: TgAgCAgCTTCCAgAgTgTATg	P6R: TTCATAATCTTTggCAACAACg
P3f-4fR	P3f: gggCACATTAgAgAACCCAC	P4fR: CgAATgCTACTCCCACAg
P3-4gR	P3: CCTCAAACCTgggTTTCg	P4gR: CATTATgAgggTCgTTgg
P3-4fR	P3: CCTCAAACCTgggTTTCg	P4fR: CgAATgCTACTCCCACAg

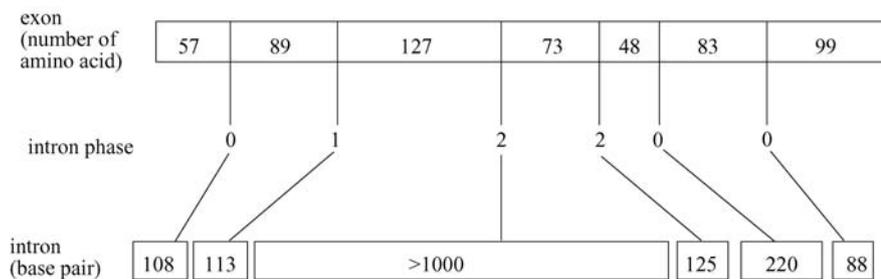


Fig. 1 Genomic organization of apple alpha-farnesene synthase gene

motif, encoded by a sequence in exon 1, and the RxR and DDxxD motifs, were encoded by two sequences in exon 4.

3.2 Sequence polymorphism

The obtained genomic sequence of the AFS gene was compared to cDNA sequences in the GenBank (accession No. AY523409, an AFS gene in the apple cultivar ‘Idared’). Six single nucleotide polymorphisms were identified (Fig. 2). Among the 6 SNPs, 426 G→A, 501 A→G, 1747 A→G and 2573 G→A could cause mutations at the amino acid level, but the other two SNPs, 1875 T→C and 2625 C→T, did not induce amino acid mutations. They encoded by 333G (Glycine) and 468R (Arginine), respectively. Interestingly, an amino acid mutation (291 R→G) was found in the RxR motif.

Figure 2 also shows that both SNPs, 426 G→A and 501 A→G, were in the sequence of exon 2, both SNPs 1747 A→G and 1875 T→C were in the sequence of exon 4, and both SNPs 2573 G→A and 2625 C→T were in the sequence of exon 6.

4 Discussion

Alpha-farnesene synthase (AFS) is a key enzyme in the pathway of alpha-farnesene synthesis. One report showed that the maximum levels of the AFS1 transcript, alpha-farnesene, and conjugated trienols were 2.5-, 4-, and 33-fold

greater in ‘Law Rome’ than in ‘Idared’ apples, respectively (Pechous et al., 2005). So far, there has been no report concerning what causes the difference. We did some work on the sequence differences of the AFS gene in different apple cultivars. At first, we obtained the genomic sequence of the AFS gene in the scald-susceptible cultivar ‘Ralls’, and then its sequence was compared to the cDNA sequences in the GenBank (accession No. AY523409, a gene in the scald-resistant cultivar ‘Idared’), and it was found that there were 6 single-nucleotide polymorphisms between the two sequences, four of which caused mutations at the amino acid level. Interestingly, one amino acid mutation (291R→G) was found in the RxR motif, with the amino acid residue being G for scald-resistant cultivar ‘Idared’, and R for scald-susceptible cultivars ‘Ralls’ and ‘Law Rome’. As the residues of the RxR motif and the DDxxD motif are thought to separate the diphosphate ion from the carbon cation upon cleavage of the prenyl diphosphate substrate (Davis and Croteau, 2000), the amino acid mutation (291R→G) in the RxR motif may explain why levels of AFS1 transcript and alpha-farnesene in the apple cultivar ‘Idared’ were lower in Pechous’s experiment (Pechous et al., 2005). Further experiments are needed to verify whether levels of AFS1 transcript and alpha-farnesene of apples are influenced by this amino acid mutation (291R→G) or other mutations.

It was reported that in rice, the expression of the OsBP-73 gene requires involvement of its intron (Chen and Wang, 2004). In our present work, a big intron 3 was

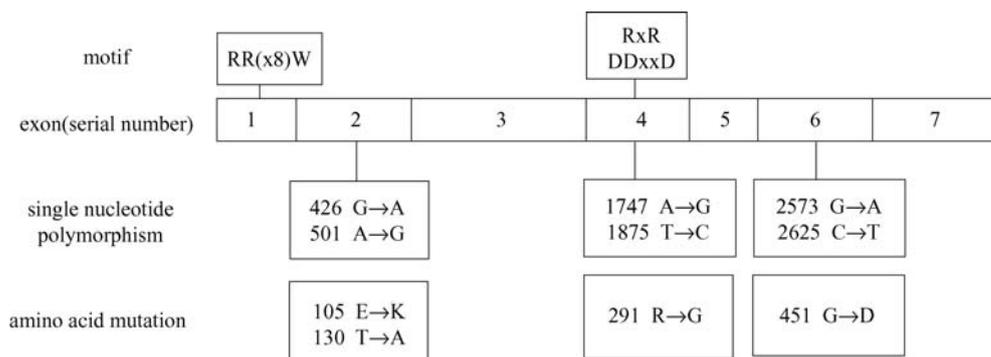


Fig. 2 The mutations in the sequences of the AFS gene and its deduced amino acid

Note: Numbers indicate the positions of the nucleotides in the obtained genomic sequence of AFS gene in the apple cultivar ‘Ralls’.

found in the AFS gene of apples. As the apple cultivar 'Idared' was not available, its intron sequence for the AFS gene could not be obtained, so that only the exon part of the AFS gene in 'Ralls' and 'Idared' was compared. It is not clear whether there is a difference between their intron sequences, and whether such a difference influences the expression of the AFS gene.

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