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RESEARCH ARTICLE

Genomic organization and sequence polymorphism of a farnesyl diphosphate synthase gene in apples (*Malus domestica* Borkh.)

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Abstract **Primer pairs were designed to amplify the genomic DNA sequence of the farnesyl diphosphate synthase (*FPPS*) gene by PCR. The PCR products were sequenced, spliced and compared to the cDNA sequence in the GenBank (accession No. AY083165). The genomic sequence and intron-exon organization of *FPPS1* gene in the apple cultivar 'Fuji' were thus obtained. The *FPPS1* genomic sequence has been registered in the GenBank (accession No. HM545312). It has 11 introns and 12 exons. The sizes of 11 introns were 559 bp, 108 bp, 144 bp, 114 bp, 84 bp, 690 bp, 373 bp, 168 bp, 87 bp, 91 bp and 97bp, and their phases were 0, 1, 0, 0, 0, 2, 0, 0, 0, 0 and 0, respectively. The sizes of 12 exons were 111 bp, 25 bp, 116 bp, 87 bp, 117bp, 89 bp, 52 bp, 96 bp, 45 bp, 90 bp, 72 bp and > 12 bp, respectively. Gene sequence comparison results of five apple cultivars indicated that the development of apple superficial scald was not influenced by the mutations in the exon sequence of *FPPS1* gene. A 6-bp repeat unit deletion mutation and many SNP mutations in the introns, mainly in the introns of one allele, were identified in the apple scald-resistant cultivar 'Golden Delicious'. This is the first report on the genomic organization and coding region polymorphism of *FPPS* gene in apples and other fruit trees.**

Keywords apple, farnesyl diphosphate synthase, genomic DNA, polymorphism, superficial scald

Introduction

The genes encoding for 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR), farnesyl diphosphate synthase (FPPS), and alpha-farnesene synthase (AFS) are involved in the formation of alpha-farnesene (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). This indicates that it is possible to control apple superficial scald by controlling the genes involved in the formation of alpha-farnesene. As a first step toward a better understanding of the role of HMGR, FPPS and AFS in apples, we have reported some research results of the *AFS* gene and *FPPS* gene promoters (Yuan et al., 2007; 2009). In this paper, we report our new results on the *FPPS* gene in apples.

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Farnesyl diphosphate synthase is the key enzyme in the synthesis of alpha-farnesene, which catalyzes the conversion of IPP (isopentenyl) and DMAPP (dimethylallyl diphosphate) to FPP (farnesyl diphosphate, the substrate of alpha-farnesene synthesis). Although this reaction is considered to be a limiting step in isoprenoid biosynthesis (Cunillera et al., 1996), compared to many reports on *AFS* gene (Pechous and Whitaker, 2004; Pechous et al., 2005; Li et al., 2006; Green et al., 2007; Yuan et al., 2007; Beuning et al., 2010) and *HMGR* gene (Rupasinghe et al., 2001; Pechous and Whitaker, 2002), there was not much work concerning *FPPS* gene in apples. So far, only the cDNA encoding for farnesyl diphosphate synthase in apples has been cloned (GenBank accession No. AY083165) and a sequence consisting of 528 bp promotor and 142 bp 5′UTR of the *FPPS* gene in apples has already been reported (Yuan et al., 2009). There is no report yet on the genomic organization and coding sequence polymorphism of farnesyl diphosphate synthase gene in apples and other fruit trees. Some reports showed that the difference in sesquiterpenes between two corn cultivars contributed to the allelic variation of two terpene synthase genes (*TPS4*, *TPS5*) (Köllner 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 farnesyl diphosphate synthase gene, and to explore their relationship with the development of apple superficial scald in order to control apple superficial scald using genetic engineering.

Materials and methods

Experimental materials

Young leaves of the apple (*Malus domestica* Borkh.) cultivars 'Fuji', 'Golden Delicious', 'Red Delicious' and 'White Winter Pearmain' from the experiment orchard of Shandong Institute of Pomology were used in this study.

Methods

Extraction of genomic DNA

Genomic DNA was extracted from young leaves of the apple cultivar 'Fuji'. The extraction protocol was based on the methods previously described (Chen et al., 2004; Yuan, 2005). The 2% CTAB extraction buffer consisted of 2% CTAB, 100 mmol/L Tris-HCl (pH 8.0), 20 mmol/L EDTA, and 1.4 mol/L 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 supernatant in the sample tubes was transferred to a new tube after centrifugation at 13000 r/min for 10 min. 0.42 mL 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 r/min for 10 min. The pellet obtained was rinsed twice with 420 µL 70% ethanol (−20°C), air-dried, resuspended in 30 or 50 µL 0.2×TE buffer containing RNase A (50µg/mL) and treated at 37°C for 30–60 min, then kept at either 4°C for immediate use or stored in a freezer.

Primer design, PCR amplification and sequencing genomic DNA

The cDNA sequence of *FPPS* gene in the GenBank was used to design primers f5, f6, f7 and f9 (18 – 22 bp) (Table 1). F8a, f9h, f10k, f10z, f9v, f9u and f10v in Table 1 are the primers designed to sequence some parts of the genomic DNA that were not obtained at the first stage. F9p, f10p and f10q in Table 1 are allele specific primers. The PCR reactions (50 µL) contained 1 µL (approximately 50 ng) genomic DNA and 49 µL reaction solution mixture. The reaction solution mixture was composed of 1µL dNTPs (10 mmol/L), 5 µL Taq buffer, 1.5 µL forward primer (10 µmol/L), 1.5 µL reverse primer (10 µmol/L), 0.5 µL (1U) Taq polymerase and 39.5 µL water. A total of 35 PCR cycles were performed. Each cycle included denaturation at 94°C for 30 s, followed by annealing for 1 min at the appropriate temperature (50–55°C) for each specific marker and final primer extension at 72°C for 2 min. A mixture of 2 µL PCR products, 8 µL water and 2 µL 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.

Analysis of genomic DNA

The sequenced DNA fragments were spliced to obtain the genomic sequence of *FPPS* gene in the apple cultivar 'Fuji'. The spliced sequence was compared to cDNA sequences in the GenBank (accession No. AY083165) using the Blastn tool, to find out the spliced sites of introns and exons in *FPPS* gene sequence and 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.

Gene sequences of other apple cultivars

The partial *FPPS* gene sequences in the apple scald-resistant

Table 1 PCR products and related primers

PCR product	Forward primer	Reverse primer
f5-6	f5: ACATCTCTCTCCCTCTCTG	f6: ATCTTCAGTTAATTCCCTTC
f7-8a	f7: GGATTGTCTGTTATTGACAG	f8a: ATAGGAAACAGCATACAGG
f9-10z	f9: GTGGAATTTCAAACTGCC	f10z: CCAAAGCAATCCAAATAATC
f9h-10z	f9h: CCAAACAGTCTAAACAGCTTG	f10z: CCAAAGCAATCCAAATAATC
f9h-10z		f10k: AGACCAAGGGAGTTATGTACTG
f9v-10v	f9v: TGAATACACAGAAACAGAATCC	f10v: CGCAAATACACCCTACCAC
f9v-10v	f9u: CCTCCGCACAATGGAACTC	
f9-f10p	f9: GTGGAATTTCAAACTGCC	f10p: GACATCAATAGAGAATCAC
f9-f10q	f9: GTGGAATTTCAAACTGCC	f10q: AAGTAAACATGCTGGTGTG
f9p-10k	f9p: CGATGCTGCAGAAAAGCAC	f10k: AGACCAAGGGAGTTATGTACTG

cultivar 'Golden Delicious' and apple scald-susceptible cultivars 'Red Delicious' and 'White Winter Pearmain' were also obtained and analyzed using the above mentioned method.

Results

Genomic sequence and organization

The size of the obtained genomic sequence of *FPPS* gene in the apple cultivar 'Fuji' was 3493 bp. The genomic organization of *FPPS* gene was determined by comparing its genomic sequence with the cDNA sequence of *FPPS* gene in GenBank (accession No. AY083165). It was found that *FPPS* gene had 11 introns and 12 exons. All 11 introns began with GT and ended with AG, following the GT—AG rule. Their phases were 0, 1, 0, 0, 0, 2, 0, 0, 0, 0 and 0, and their sizes were 559 bp, 108 bp, 144 bp, 114 bp, 84 bp, 690 bp, 373 bp, 168 bp, 87 bp, 91 bp and 97 bp, respectively. The sizes of the 12 exons were 111 bp, 25 bp, 116 bp, 87 bp, 117 bp, 89 bp, 52 bp, 96 bp, 45 bp, 90 bp, 72 bp and > 12 bp, respectively (Fig. 1), while the obtained Exon 12 sequence of *FPPS* gene in the apple cultivar 'Fuji' was only 12 bp and the size of the other Exon 12 sequence was 117 bp based on the the cDNA sequence of *FPPS* gene in GenBank (accession No. AY083165). Hence, the size of Exon 12 was 129 bp (Fig. 1). In addition, 66 bp in the upstream of the start codon was also obtained. The obtained genomic sequence of *FPPS* gene in the apple cultivar 'Fuji' has been registered in GenBank (accession No. HM545312).

Amino acid sequence and motifs

FPPS gene in the apple cultivar 'Fuji' encoded a protein of 342 amino acids. The sizes of the deduced amino acids of the 12 exons were 37, 9, 38, 29, 39, 30, 17, 32, 15, 30, 24 and 42 (= 4 + 38), respectively. It was reported that there are 5 motifs in the amino acid sequence of FPPS protein (Li and Wang, 2007). Our results indicted that the GKLNR motif was encoded by gene sequence in the joint part of Exon 2 and Exon 3, the first DDxxD motif was encoded by the sequence in Exon 4, GQxxD motif in Exon 6, KT motif in Exon 7, and DDxxD motif in Exon 9 (Fig. 2).

Exon sequence polymorphism

As there is a cDNA sequence of *FPPS* gene in the GenBank (accession No. AY083165, a *FPPS* gene in the apple scald-susceptible cultivar 'Granny Smith'), the obtained genomic sequence of *FPPS* gene in the apple cultivar 'Fuji' was compared to this cDNA sequence. Six single nucleotide polymorphisms were identified (Fig. 3). Among the 6 SNPs, 87 G→T could cause mutations at the amino acid level, but the other SNPs, 105 C→T, 138 A→G, 144 C→T, 306 G→T and 504 C→T, did not induce amino acid mutations.

Figure 3 also shows that both SNPs, 87 G→T and 105 C→T, were in the sequence of Exon 1, the SNP 138 A→G and the SNP 144 C→T were in the sequence of Exon 3, the SNP 306 G→T was in the sequence of Exon 4, the SNP 504 C→T was in the sequence of Exon 6. It needs to be mentioned that the 117 bp sequence of Exon 12 was not obtained; it is not clear whether there was a mutation in this region of Exon 12.

In our further experiments on *FPPS* gene sequences of other apple cultivars, the above mentioned 6 single nucleotide polymorphisms were also identified when the cDNA sequence in the apple scald-susceptible cultivar 'Granny Smith' (GenBank accession No. AY083165) was compared to the obtained exon sequences of *FPPS* gene in the apple

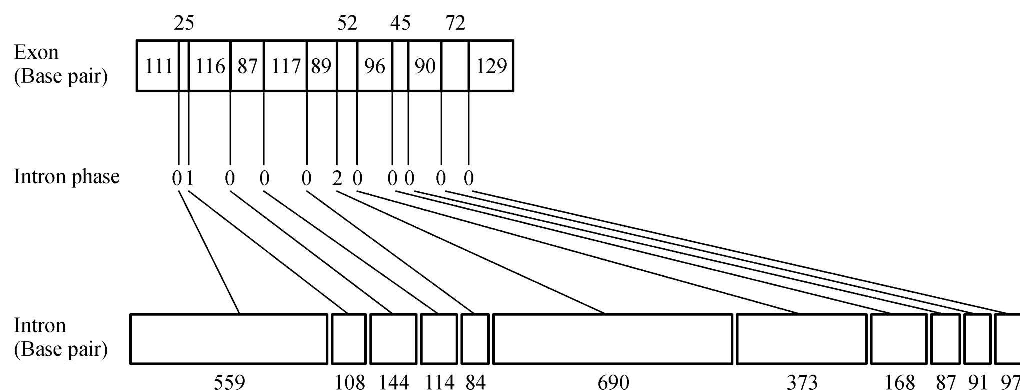

Figure 1 Genomic organization of farnesyl diphosphate synthase gene in the apple cultivar 'Fuji'.

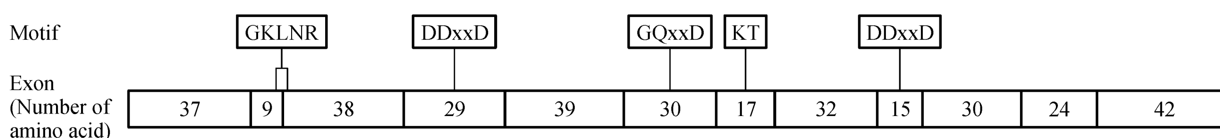

Figure 2 The position of 5 motifs in the deduced amino acid sequence of apple farnesyl diphosphate synthase gene.

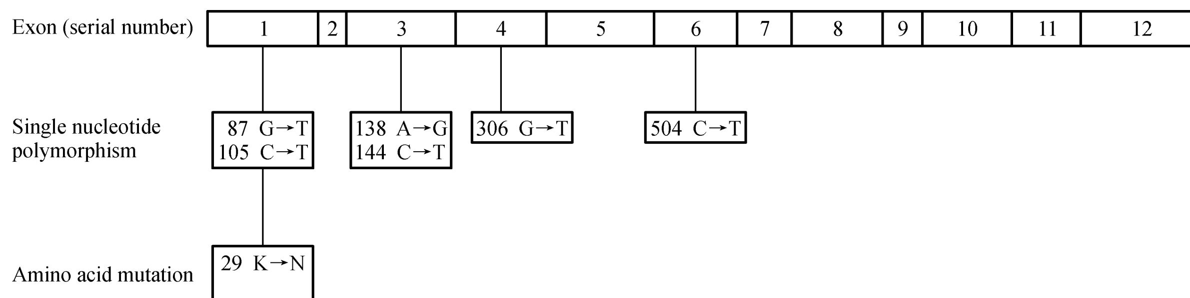

Figure 3 The mutations in the sequences of the *FPPS* gene and its deduced amino acid. Numbers in the boxes indicate the positions in the exon part of *FPPS* gene in the apple cultivar 'Fuji'.

scald-susceptible cultivars 'Red Delicious', 'White Winter Pearmain' and apple scald-resistant cultivar 'Golden Delicious'. These results indicted that the development of apple superficial scald is not influenced by the above mentioned mutations.

Intron sequence analysis and comparison

When the intron sequence of *FPPS* gene in the apple cultivar 'Fuji' was compared to the obtained intron sequence of *FPPS* gene in the apple scald-susceptible cultivar 'Red Delicious' or 'White Winter Pearmain', no mutation was identified.

When the intron sequence of *FPPS* gene in the apple cultivar 'Fuji' was compared to the obtained intron part of *FPPS* gene in the apple scald-resistant cultivar 'Golden Delicious', it was found that there were 25 mutations in the introns of *FPPS* gene, among which three mutations were in Intron 1 (including a 6-bp repeat unit TTACTA deletion), two mutations in Intron 2, two mutations in Intron 3, 25 mutations in Intron 6, one mutation in Intron 7, three mutations in Intron 8 and one mutation in Intron 11 (Table 2). Hence, there were three 6 bp repeat units in the apple scald-resistant cultivar 'Golden Delicious' and four 6 bp repeat units in the apple cultivars 'Fuji', 'Red Delicious' and 'White Winter Pearmain'.

As 24 of 25 mutations in Intron 6 were SNP mutations, 22 SNP sites were polymorphic in the apple cultivar 'Golden Delicious', the obtained sequence of *FPPS* gene in the apple scald-resistant cultivar 'Golden Delicious' was further analyzed using allele specific primers f9p, f10p and f10q, and two alleles were identified. When the Intron 6 sequence of first allele was compared to that of *FPPS* gene in the apple cultivar 'Fuji', only three mutations 11 /→A(insertion), 170 G→A and 285 C→A were found; while the Intron 6 sequence

Table 2 Mutations in the introns of *FPPS* gene

Intron No.	Position in each intron	Nucleotide in 'Fuji'	Nucleotide in GD	Allele no.		Intron No.	Position in each intron	Nucleotide in 'Fuji'	Nucleotide in GD	Allele no.	
				No.1	No.2					No.1	No.2
No.1	230	G	G/A	(G)	(A)	No.6	180	G	G/T	G	T
	246	T	C/T	(T)	(T)		201	C	C/G	C	G
	262	TTACTA	/(deletion)	/	/		285	A	C	C	C
No.2	10	T	T/C	(T)	(C)		288	G	G/A	G	A
	17	G	G/A	(G)	(A)		337	T	T/C	T	C
No.3	87	G	T	T	T		400	T	T/A	T	A
	117	T	C	C	C		402	T	T/C	T	C
No.6	11	/	A(insertion)	A	A		443	T	T/G	T	G
	50	T	T/C	T	C		490	T	T/C	T	C
	58	G	G/A	G	A		589	A	A/G	A	G
	88	T	T/C	T	C		605	A	A/C	A	C
	89	G	G/A	G	A		617	T	T/A	T	A
	99	T	T/C	T	C		619	G	G/A	G	A
	138	T	T/C	T	C	No.7	95	T	C/T	T	C
	139	A	A/G	A	G	No.8	49	T	A/T	(T)	(A)
	141	T	T/A	T	A		66	C	A	A	A
	143	G	G/A	G	A		155	C	T	T	T
	168	G	A/G	G	A	No.11	8	T	C	C	C
	170	A	G	G	G						

Note: GD represents apple cultivar 'Golden Delicious'. Nucleotides in parentheses were predicted and not verified.

of second allele was compared to that of *FPPS* gene in the apple cultivar 'Fuji', 25 mutations were found. As the nucleotide in each checked polymorphic site of first allele was the same as that of *FPPS* gene in 'Fuji', the nucleotides of first allele in Intron 1, Intron 2 and Intron 8 were predicted to be the same as those of *FPPS* gene in 'Fuji' (Table 2). Based on these results, there were 37 mutations in the introns (25 in Intron 6 and 12 in other introns) of the second allele of Golden Delicious.

Discussion

Although farnesyl diphosphate synthase (FPPS) is a key enzyme in the pathway of alpha-farnesene synthesis, there was not much work concerning the *FPPS* gene in the apples. So far, only one cDNA sequence was found in the GenBank (accession No.AY083165) and a sequence consisting of 528 bp promoter and 142 bp 5′UTR of the *FPPS* gene in apples was reported (Yuan et al., 2009). We did some work on the genomic organization and sequence differences of *FPPS* genes in different apple cultivars. At first, we obtained the genomic sequence of *FPPS* gene in the apple cultivar 'Fuji', and then its sequence was compared to the cDNA sequences in the GenBank (accession No. AY083165, an *FPPS* gene in the ripening fruit peel of apple cultivar 'Granny Smith'), and it was found that there were 6 single-nucleotide polymorphisms between the two sequences, one of which caused mutation at the amino acid level. Because 'Granny Smith' is a highly scald-susceptible apple cultivar, and some research found the percentage of scalded fruit reached 10%–27% among cold-stored 'Fuji' apple (Guan et al., 2004), it could be inferred that the occurrence of apple superficial scald is not influenced by the above mentioned mutations. Further experiments on other apple cultivars confirmed that the development of apple superficial scald is not influenced by the above mentioned mutations.

It was reported that, in rice, the expression of *OsBP-73* gene requires the involvement of its intron (Chen and Wang, 2004). In our present work, three big introns (Intron 1, Intron 6 and Intron 7) were found in the *FPPS* gene of apples. Because the apple cultivar 'Granny Smith' was not available, its intron sequence for *FPPS* gene could not be obtained, so the intron part of *FPPS* gene in 'Fuji' and 'Granny Smith' was not compared. When the intron sequence in the apple cultivar 'Fuji' was compared to the obtained intron sequence of *FPPS* gene in the apple scald-resistant cultivar 'Golden Delicious', a 6-bp repeat unit deletion in Intron 1 and many mutations in Intron 6 were identified in the apple scald-resistant cultivar 'Golden Delicious'. As there were three 6-bp repeat units in the apple scald-resistant cultivar 'Golden Delicious' and four 6-bp repeat units in the 'Fuji' and apple scald-susceptible cultivars 'Red Delicious' and 'White Winter Pearmain', further experiments are needed to investigate whether the expression of *FPPS* gene and the development of apple superficial scald are influenced by this repeat unit deletion as well as the other mutations, especially many mutations in Introns 6. As 25 mutations in Introns 6 and 12 mutations in other introns were found in the second allele of apple scald-resistant cultivar 'Golden Delicious', this allele is very special and interesting. We wonder whether there is a relationship between this allele and the scald-resistance of apple cultivar 'Golden Delicious'.

Because some sequence of another *FPPS* gene (which will be reported when we finish related experiments) was also found in this study, we named the above mentioned *FPPS* gene as *FPPS1* gene.

In general, the genomic sequence and intron-exon organization of *FPPS1* gene with 11 introns and 12 exons in apples were obtained. Gene sequence comparison results of five apple cultivars indicated that the development of apple superficial scald was not influenced by the mutations in the exon sequence of *FPPS1* gene. The genotype of *FPPS1* gene in the apple scald-resistant cultivar 'Golden Delicious' was heterozygous, a very special allele with many mutations in the introns was identified in 'Golden Delicious'. To our knowledge, there is no report on these respects of *FPPS* gene in apples and other fruit trees.

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