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RESEARCH ARTICLE

Lesley BEUNING, Sol GREEN, Yar-Khing YAUK

The genomic sequence of AFS-1—an *alpha*-farnesene synthase from the apple cultivar ‘Royal Gala’

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Abstract The genomic sequence encoding *alpha*-farnesene synthase-1 (AFS-1) was amplified from genomic DNA isolated from ‘Royal Gala’ apple (*Malus*×*domestica* Borkh.). The genomic sequence consists of six introns and seven exons, which is consistent with Class III terpene synthases. Four variants of the genomic sequence were amplified. The four variants are based on the presence or absence of a repeat of two sequences, one found in intron 4 (CAGTTATTTAATT) and the other in intron 5 (TA). Although there were small nucleotide differences among the three apple cultivars ‘Royal Gala’, ‘Idared’, and ‘Ralls’, these resulted in only two amino acid changes in the protein sequence, which are unlikely to explain the resistance or susceptibility of an apple cultivar to superficial scald. Given that AFS-1 transcript levels are high in all cultivars, it appears that it is either the reactions downstream of *alpha*-farnesene production that control the accumulation of oxidation products related to superficial scald or that the variation in the level of its substrate, farnesyl diphosphate, may cause differences in the amount of *alpha*-farnesene produced.

Keywords α-farnesene synthase, ‘Royal Gala’, genomic sequence, superficial scald

1 Introduction

(*E*,*E*)-α-Farnesene (3,7,11-trimethyldodeca-1,3*E*,6*E*,10-tetraene) is an acyclic sesquiterpene produced by many plant species and across a range of tissues. Plants produce α-farnesene in response to pathogen attack, as in tobacco (Huang et al., 2003), or in response to wounding by herbivores (Pare and Tumlinson, 1999; Vuorinen et al., 2004). Active synthesis of α-farnesene in response to herbivore attack is thought to attract predators and parasites of the herbivore, hence offering plants a self-control measure over their own consumption (Pare and Tumlinson, 1998).

α-Farnesene is a sesquiterpene; however, phylogenetic analysis demonstrates that the enzyme responsible for α-farnesene biosynthesis in ripe apple (*Malus*×*domestica*) fruits, α-farnesene synthase-1 (AFS-1), clusters with poplar and Kudzu isoprene synthases and, to a lesser extent, with a number of monoterpene synthases, rather than with other sesquiterpene synthases (Martin et al., 2004; Sharkey et al., 2005; Green et al., 2007; Green et al., 2009). Furthermore, AFS-1 also contains the RR(X_8)W motif which is more normally associated with cyclic mono-TPS enzymes (Bohlmann et al., 1998, 1999).

Superficial scald is a physiological disorder of many apple and pear cultivars whereby hypodermal cells of the fruit die during prolonged cold storage. Once the fruits are returned to warmer temperatures, the actual necrosis occurs resulting in browning of the skin (Wang and Dilley, 1999; Tsantili et al., 2007; Pesis et al., 2009). The role that α-farnesene is thought to play in the development of superficial scald is attributed to its autoxidation products. The oxidation products include conjugated trienols (2,6,10-trimethyldodeca-2,7E,9E,11-tetraen-6-ol and 2,6,10-trimethyldodeca-2,7E,9Z,11-tetraen-6-ol), farnesyl hydroperoxide, and 6-methyl-5-hepten-2-one and its alcohol. These oxidation products have been implicated in scald production (Rowan et al., 2001; Fernandez-Trujillo et al., 2003; Whitaker, 2004).

As part of the research into the genes involved in apple flavour being carried out at Plant & Food Research, a putative sesquiterpene synthase (AFS-1), identified from ‘Royal Gala’ ESTs (expressed sequence tags) in the Plant & Food Research EST Database (Newcomb et al., 2006), was shown to encode an α-farnesene synthase (Green et al., 2007). Recently, the genomic sequence (except for the central nucleotides of intron 3) of AFS-1 was reported for another apple cultivar, the superficial scald-susceptible

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Lesley BEUNING (✉), Sol GREEN, Yar-Khing YAUK
The New Zealand Institute for Plant & Food Research Limited, Mt Albert, Auckland 1025, New Zealand
E-mail: lbeuning@hortresearch.co.nz

cultivar ‘Ralls’ (Yuan et al., 2008). This report suggested that an amino acid mutation in the RxR motif of AFS-1 found in the superficial scald-resistant apple cultivar ‘Idared’, but not in ‘Ralls’, may produce the superficial scald resistance of ‘Idared’. The RxR motif is thought to be involved in the separation of the diphosphate ion from the carbon cation upon cleavage of the prenyl diphosphate substrate (Davis and Croteau, 2000). We have amplified the genomic sequence of AFS-1 from ‘Royal Gala’, which has a high resistance to superficial scald (Whitaker, 1998). Here, we present evidence that superficial scald resistance is not caused by a mutation in the RxR motif, as all the sequences we amplified showed no changes in this motif.

2 Materials and methods

2.1 Amplification of genomic DNA

Genomic DNA was extracted from young leaves of tissue-cultured plantlets of the apple cultivar ‘Royal Gala’ using a Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences). PCR primers (Table 1) were designed from the regions that are in common with other AFS-1 cDNA sequences deposited in GenBank (AY563622, AY523409, and AY182241). The pairings of the primers in PCR amplifications were predicted to amplify at least one intron based on comparing the AFS-1 cDNA sequence with the genomic sequence of other terpene synthases deposited in GenBank. For each intron, the amplification was repeated twice to avoid amplification bias. The PCR amplifications were set up according to the manufacturer’s instructions (Invitrogen). Conditions for the PCR amplifications were denaturation for 4 min at 96°C, followed by 30 cycles of 1 min at 96°C, 1 min at 60°C, and 2 min at 72°C, with the final cycle elongated for 10 min at 72°C.

2.2 Cloning and sequencing of PCR fragments

The PCR products were purified using the Wizard SV Gel and PCR Clean-Up Kit (Promega) and were then either sequenced directly or ligated to the plasmid vector pGEM-T Easy (Promega) and transformed into DH5α (Invitrogen). Plasmids containing inserts were isolated using the QIAprep Spin Miniprep Kit (Qiagen), and for each intron, between 5 and 14 clones were sequenced at the Waikato DNA Sequencing Facility (Hamilton, New Zealand). The genomic sequences were then deposited in the GenBank (AY805408-AY805421).

2.3 mRNA extraction and northern analysis

Fully matured apple fruits of ‘Royal Gala’ (tree-ripened, 150 days after full bloom) (Newcomb et al., 2006) were obtained, and total RNA was extracted from the skin and cortex of these fruits using the method of López-Gómez and Gómez-Lim (1992). Northern analysis was performed using antisense RNA probes (Rueger et al., 1996). Probe templates for AFS-1 were prepared by PCR amplification (Genius thermocycler, Techne, Cambridge, UK) from plasmid DNA using primers 57400NF1 and 57400NR1 (Table 1). Transcription of AFS-1 probes was carried out using T7 RNA polymerase (Invitrogen) according to the manufacturer’s instructions, and the reaction was supplemented with 70 $mmol \cdot L^{-1}$ DIG11UTP (Roche), with unlabelled UTP reduced to 130 $mmol \cdot L^{-1}$. Transcription reactions were incubated at 37°C for one hour and then treated with one unit of RQ1 RNase-free DNase (Promega) in 50-μL total volume for further 15 min at 37°C. Concentration of the RNA probe was estimated by OD_{260} and used at the rate of 100 ng probe per millilitre of hybridisation buffer. RNA loading was measured by rehybridising the blot with an 18S ribosomal RNA PCR product (Table 1). The 343 base pair ribosomal product had a GC content of 40% and hybridised at 42°C with EasyHyb™ according to the manufacturer’s instructions.

Table 1 PCR primers used in amplification of the genomic sequences of α-farnesene synthase-1 from apple

primer name	sequence 5' to 3'
18S-RFT	CTGGCACCTTATGAGAAATC
18S-RTR	CCACCCATAGAATCAAGAAA
57400NF1	GCACATTAGAGAACCACCAT
57400NR1	TAATACGACTCACTATAGG-GATGCTTCCCTTAAGTTTT
57400-5'	TATAGCTTCTTGTATCCCAA
57400-3'	TAAACGACAAACTACAACTT
57400-M1	TTGCAAGAGATAGACTGGTT
57400-M2	AACCAGTCTATCTCTTGCAA
57400-A2	GATGATGAAATGCATCCGTT
57400-A3	AGAGTTCACTTGCAAGCTGA
57400-A5	TTCCATGCATTGTCTATCAT
57400-C1	GCACAAGAGACGATCTCTATG

Note: The PCR primers 57400NF1 and 57400NR1 were also used to generate the probe for northern analysis, and the PCR primers 18S-RFT and 18S-RTR were used to generate the ribosomal probe for testing the RNA loadings of the northern.

3 Results

3.1 The genomic sequence of apple α-farnesene synthase-1

The genomic sequence of apple AFS-1 contained six introns and seven exons (Fig. 1). The size of the genomic sequence obtained for AFS-1 ranged from 3668 base pairs to 3683 base pairs because of the presence of repeated sequences in two of the introns (CAGTTATTTAATT (intron 4) and TA (intron 5)). The sizes of the six introns are 108, 113, 1088, 125-138, 221-223, and 88 base pairs,

respectively. The sizes of the seven exons are ⩾230, 268, 382, 219, 142, 249, and ⩾435 base pairs, respectively.

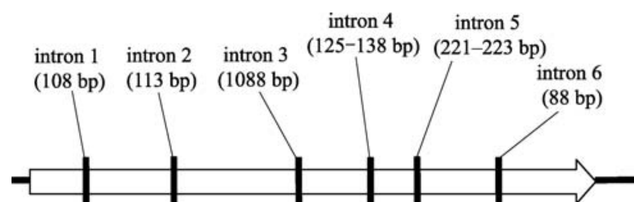

Fig. 1 The genomic organisation of the gene for α-farnesene synthase-1 from 'Royal Gala' apple

Variants of the genomic sequence of AFS-1 were amplified. These variants are based on the presence or absence of the repeat of either of the two repeated sequences found in intron 4 (CAGTTATTTAATT) and intron 5 (TA). All the four possible variants of these repeated sequences were present in 'Royal Gala', i.e., CAGTTATTTAATT alone with TA, CAGTTATTTAATT alone with TA repeated, CAGTTATTTAATT repeated with TA, and CAGTTATTTAATT repeated with TA repeated. The genomic sequence isolated by Yuan et al. (2008) was in correspondence to CAGTTATTTAATT alone with TA.

The genomic sequence of AFS-1 from 'Royal Gala' was compared with that of AFS-1 from the apple cultivar 'Ralls'. There were only two nucleotide differences in the translated sequences, one in exon 4 and the other in exon 6. The nucleotide difference in exon 4 did not alter the protein translation, while that in exon 6 where A in 'Royal Gala' was G in 'Ralls' did alter the protein translation. This nucleotide difference resulted in D (aspartic acid) in the 'Royal Gala' AFS-1 and G (glycine) in the 'Ralls' AFS-1. The amino acid change was also found in 'Idared' at the position 451 of the amino acid sequence of AFS-1. Figure 2 shows an amino acid alignment of AFS-1 for the two scald-resistant cultivars 'Royal Gala' (GenBank AAX19772) and 'Idared' (GenBank AAS01424) and the two scald-susceptible cultivars 'Ralls' (GenBank AAS68019) and 'Laws Rome' (GenBank AAO22848). Comparing the four amino acid sequences shows that any amino acid differences are specific to a single apple cultivar, not to whether the cultivar is resistant or susceptible to superficial scald.

There were a number of differences between the intron sequences of the *AFS-1* gene of 'Royal Gala' and that of 'Ralls'. There existed at least two nucleotide differences in intron 3, one nucleotide difference in intron 4, three nucleotide differences in intron 5 plus an insertion, and two nucleotide differences in intron 6.

3.2 Northern analysis

The level of expression of AFS-1 in 'Royal Gala' apple skin and cortex was examined by northern analysis (Fig. 3). It was found that the level of expression was extremely high in the skin with a much lower level in the cortex.

4 Discussion

We determined that, as found for most of the other sesquiterpene synthases, the genomic sequence of AFS-1 from apples contained six introns and seven exons, and therefore, it was viewed as a Class III terpene synthase (Trapp and Croteau, 2001). In the cultivar 'Royal Gala', there were four variants of the *AFS-1* gene, based on the presence or absence of a repeat of two sequences, CAGTTATTTAATT in intron 4 and TA in intron 5.

The genomic sequence of AFS-1 was amplified from the scald-susceptible apple cultivar 'Ralls' (Yuan et al., 2008). The comparison of this genomic sequence for AFS-1 and the cDNA sequence of AFS-1 from the scald-resistant apple cultivar 'Idared' indicated that there were 6 single-nucleotide differences between the two sequences, 4 of which altered an amino acid, and one of the amino acid changes occurred in the RxR motif. They proposed that the mutation in the RxR motif may be responsible for the lower levels of both AFS-1 expression and α-farnesene, which was previously reported by Pechous et al. (2005). These lower levels, they suggested, may affect the susceptibility of an apple cultivar to superficial scald. However, when we compare the genomic sequence of AFS-1 from superficial scald-high resistance 'Royal Gala' to that from 'Ralls', we found that there was no mutation in the RxR motif, and that there were only two nucleotide changes in the translated sequence. Only one of these changes altered the amino acid encoded, which was found in exon 6 at the position 451 where G in 'Royal Gala' was changed to D in 'Ralls'. We also examined the level of expression of AFS-1 by northern analysis, revealing that the mRNA for AFS-1 was highly expressed in 'Royal Gala' apple skin. Based on these findings, we conclude that the mutation in the RxR motif found in the cultivar 'Idared' is unlikely to contribute to the resistance of this apple cultivar to superficial scald. Furthermore, the amino acid change in exon 6, where an aspartic acid in 'Royal Gala' is a glycine in 'Ralls', is also unlikely to be involved in superficial scald susceptibility because the equivalent amino acid in the superficial scald-susceptible apple cultivar 'Laws Rome' is an aspartic acid.

A comparison was made of the amino acid sequence of AFS-1 for two superficial scald-resistant cultivars ('Royal Gala' and 'Idared') with the amino acid sequence of AFS-1 for two superficial scald-susceptible cultivars ('Ralls' and 'Laws Rome'). All amino acid differences were specific to a particular cultivar. Therefore, for these four cultivars, either any difference in the amino acid sequence of AFS-1 cannot affect the superficial scald susceptibility or there is no common mechanism at the level of the amino acid

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RGala    MEFRVHLQADNEQKIFQNQMKPEPEASYLINQRRSANYKPNIWKNDFLDQSLISKYDGDE
Idared   MEFRVHLQADNEQKIFQNQMKPEPEASYLINQRRSANYKPNIWKNDFLDQSLISKYDGDE
LRome    MEFRVHLQADNEQKIFQNQMKPEPEASYLINQRRSANYKPNIWKNDFLDQSLISKYDGDE
Ralls    MEFRVHLQADNEQKIFQNQMKPEPEASYLINQRRSANYKPNIWKNDFLDQSLISKYDGDE
         ************************************************************

RGala    YRKLSEKLIEEVKIYISAETMDLVAKLELIDSVRKLGLANLFEKEIKEALDSIAAIESDN
Idared   YRKLSEKLIEEVKIYISAETMDLVAKLELIDSVRKLGLANLFEKKIKEALDSIAAIESDN
LRome    YRKLSEKLIEEVKIYISAETMDLVAKLELIDSVRKLGLANLFEKEIKEALDSIAAIESDN
Ralls    YRKLSEKLIEEVKIYISAETMDLVAKLELIDSVRKLGLANLFEKEIKEALDSIAAIESDN
         ********************************************:***************

RGala    LGTRDDLYGTALHFKILRQHGYKVSQDIFGRFMDEKGTLENHHFAHLKGMLELFEASNLG
Idared   LGTRDDLYGAALHFKILRQHGYKVSQDIFGRFMDEKGTLENHHFAHLKGMLELFEASNLG
LRome    LGTRDDLYGTALHFKILRQHGYKVSQDIFGRFMDEKGTLENHHFAHLKGMLELFEASNLG
Ralls    LGTRDDLYGTALHFKILRQHGYKVSQDIFGRFMDEKGTLENHHFAHLKGMLELFEASNLG
         *********:**************************************************

RGala    FEGEDILDEAKASLTLALRDSGHICYPDSNLSRDVVHSLELPSHRRVQWFDVKWQINAYE
Idared   FEGEDILDEAKASLTLALRDSGHICYPDSNLSRDVVHSLELPSHRRVQWFDVKWQINAYE
LRome    FEGEDILDEAKASLTLALRDSGHICYPDSNLSRDVVHSLELPSHRRVQWFDVKWQINAYE
Ralls    FEGEDILDEAKASLTLALRDSGHICYPDSNLSRDVVHSLELPSHRRVQWFDVKWQINAYE
         ************************************************************

RGala    KDICRVNATLLELAKLNFNVVQAQLQKNLREASRWWANLGFADNLKFARDRLVECFSCAV
Idared   KDICRVNATLLELAKLNFNVVQAQLQKNLREASRWWANLGFADNLKFARDGLVECFSCAV
LRome    KDICRVNATLLELAKLNFNVVQAQLQKNLREASRWWANLGIADNLKFARDRLVECFACAV
Ralls    KDICRVNATLLELAKLNFNVVQAQLQKNLREASRWWANLGFADNLKFARDRLVECFSCAV
         ****************************************:********* *****:***

RGala    GVAFEPEHSSFRICLTKVINLVLIIDDVYDIYGSEEELKHFTNAVDRWDSRETEQLPECM
Idared   GVAFEPEHSSFRICLTKVINLVLIIDDVYDIYGSEEELKHFTNAVDRWDSRETEQLPECM
LRome    GVAFEPEHSSFRICLTKVINLVLIIDDVYDIYGSEEELKHFTNAVDRWDSRETEQLPECM
Ralls    GVAFEPEHSSFRICLTKVINLVLIIDDVYDIYGSEEELKHFTNAVDRWDSRETEQLPECM
         ************************************************************

RGala    KMCFQVLYNTTCEIAREIEEENGWNQVLPQLTKVWADFCKALLVEAEWYNKSHIPTLEEY
Idared   KMCFQVLYNTTCEIAREIEEENGWNQVLPQLTKVWADFCKALLVEAEWYNKSHIPTLEEY
LRome    KMCFQVLYNTTCEIAREIEEENGWNQVLPQLTKVWADFCKALLVEAEWYNKSHIPTLEEY
Ralls    KMCFQVLYNTTCEIAREIEEENGWNQVLPQLTKVWADFCKALLVEAEWYNKSHIPTLEEY
         ************************************************************

RGala    LRNGCISSSVSVLLVHSFFSITHEGTKEMADFLHKNEDLLYNISLIVRLNNDLGTSAAEQ
Idared   LRNGCISSSVSVLLVHSFFSITHEGTKEMADFLHKNEDLLYNISLIVRLNNDLGTSAAEQ
LRome    LRNGCISSSVSVLLVHSFFSITHEGTKEMADFLHKNEDLLYNISLIVRLNNDLGTSAAEQ
Ralls    LRNGCISSSVSVLLVHSFFSITHEGTKEMAGFLHKNEDLLYNISLIVRLNNDLGTSAAEQ
         ******************************.*****************************

RGala    ERGDSPSSIVCYMREVNASEETARKNIKGMIDNAWKKVNGKCFTTNQVPFLSSFMNNATN
Idared   ERGDSPSSIVCYMREVNASEETARKNIKGMIDNAWKKVNGKCFTTNQVPFLSSFMNNATN
LRome    ERGDSPSSIVCYMREVNASEETARKNIKGMIDNAWKKVNGKCFTTNQVPFLSSFMNNATN
Ralls    ERGDSPSSIVCYMREVNASEETARKNIKGMIDNAWKKVNGKCFTTNQVPFLSSFMNNATN
         ************************************************************

RGala    MARVAHSLYKDGDGFGDQEKGPRTHILSLLFQPLVN
Idared   MARVAHSLYKDGDGFGDQEKGPRTHILSLLFQPLVN
LRome    MARVAHSLYKDGDGFGDQEKGPRTHILSLLFQPLVN
Ralls    MARVAHSLYKDGDGFGDQEKGPRTHILSLLFQPLVN
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Note: Motifs are high-lighted as follows: **RR(X_8)W**, **RxR**, **DDxxD** and amino acid differences **X**

Fig. 2 Amino acid sequence lineup of AFS-1 from superficial scald-resistant apple cultivars 'Royal Gala' and 'Idared' and superficial scald-susceptible apple cultivars 'Laws Rome' and 'Ralls'

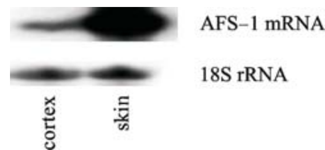

Fig. 3 Northern analysis of total RNA

Note: The total RNA was extracted from mature fruit tissues (tree-ripened 150 days after full bloom) of *Malus×domestica*. Northern analysis was undertaken using a 350 base pair DIG-labelled PCR fragment as a probe, which was amplified from AFS-1 with the primers 57400NF1 (5′-GCACATTAGAGAACCACCAT-3′) and 57400NR1 (5′-GGATGCTTCCCT-3′). Hybridization to 18S rRNA mRNA was used as a RNA loading control.

sequence of AFS-1 that is responsible for an apple cultivar's susceptibility to superficial scald.

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