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Isolation and Expression Analysis of a Defensin Gene from Strawberry (*Fragaria×ananassa* cv. Paros)

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ABSTRACT

Plant defensins are the cysteine-rich peptides that are encoded by small multi-gene families in the plant kingdom. In this study, we designed primers based on conserved regions of defensin genes to clone and identify defensin genes in strawberry (*Fragaria×ananassa* cv. Paros) by reverse transcription PCR technique. Sequence analysis showed that the deduced amino acid had significant similarity to other plant defensins from NCBI database and designated *FaDef1*. The predicted strawberry defensin protein encodes a 54 aa protein of 6.18 kDa, pI 9.22 and eight conserved cysteine residues with desired space conservation with other amino acids. Semi quantitative expressions of *FaDef1* were analyzed in root, stem, leaf, flower, and fruit in three strawberry cultivars, namely, Queenelisa, Camarosa, and Paros. The results showed that the *FaDef1* expression patterns were similar in different tissues of the three cultivars. The higher amount of relative expression of *FaDef1* was in fruit and there was no observable expression in the root. The expression of *FaDef1* increased after wounding and salicylic acid treatment. The expression level was higher in developed fruits compared to that of immature fruits. In fruits infected with the Gray mold agent (*Botrytis cinerea*), the expression of *FaDef1* showed significant increase by development of disease symptom. Taken together, these results suggest that *FaDef1* is both responsive to biotic stress signal compounds and strawberry *B. cinerea* and may be used as a candidate gene for engineering plants against gray mold.

Keywords: Gray mold, Pathogenesis related proteins, Resistance to stress, RT-PCR, strawberry.

INTRODUCTION

Plant defensins are small (45-54 amino acids), highly basic and cysteine-rich peptides that are ubiquitous in the plant kingdom (Thomma *et al.*, 2002). Plant defensins inhibit growth of a broad spectrum of fungi and bacteria (Park *et al.*, 2002). All known plant defensins have eight cysteine residues, which form four structures–stabilizing disulfide bridges. Previous studies showed that three-dimensional structure of plant defensins consist of a triple stranded β-sheet with an alpha-helix in parallel. The conserved three-dimensional structure of defensins in different organisms suggests that defensins are ancient peptides of all eukaryotes and originated before divergence of plants and animals. Consistent with a role for defensins in plant disease resistance the expression of many defensin genes showed increase following pathogen attack. The defensins *PDF1.2* and *PDF2.3* from Arabidopsis were induced upon *Alternaria brassicola* infection (Thomma and Broekaert, 1998), and the radish defensins, *AFP-1* and *AFP-2*, also showed induction in leaves following infection with *A. brassicola* (Terras *et al*., 1995). The pea defensins, *DDR230-a* and *DDR230-b*, were induced in immature pods following inoculation

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with *Fusarium solani* (Chiang and Hadwiger, 1991), and *DDR230-a* and *DDR230-c* were induced in pea leaves after inoculation with *Ascochyta pinodes* (Lai *et al.*, 2002). The expression of eight defensin genes from *Nicotiana benthamiana* showed that members of a defensin gene family will respond to a pathogen differently (Bahramnejad *et al.*, 2009). In addition to induction by pathogens, many plant defensins showed induction by abiotic stresses, such as wounding (Do *et al.*, 2004; Lai *et al.*, 2002; Lee *et al.*, 2001; Meyer *et al.*, 1996), drought (Maitra and Cushman, 1994), cold (Koike *et al.*, 2002) as well as exposure to $ZnCl_2$ (Mirouze *et al.*, 2006) and high NaCl levels (Do *et al.*, 2004; Komori *et al.*, 1997; Yamada *et al.*, 1997). Expression of *PDF1.2* in Arabidopsis was induced by ethylene, while expression of *MsDef1* and *MsDef2.1* in alfalfa was down-regulated (Hanks *et al.*, 2005). Salicylic Acid (SA) is a signaling molecule associated with resistance to biotrophic pathogens and the Hypersensitive Response (HR) (Thomma *et al.*, 1999). It is also associated with Systemic Acquired Resistance (SAR), which is a form of induced resistance that spreads systemically in plants after localized pathogen attack and involves a systemic induction of a number of *PR* genes (Lawton *et al.*, 1995). Induction of SAR by exogenous application of SA resulted in increased expression of *CADEF1* in pepper (Do *et al.*, 2004) but did not affect expression of *PDF1.2* in Arabidopsis (Penninckx *et al.*, 1998).

Plant defensin genes exhibit tissue-specific expression pattern (Lay and Anderson, 2005). The Arabidopsis defensins, *PDF2.2* and *PDF2.3*, were expressed in most tissues, but *PDF1.1* was only expressed in seeds and siliques and *PDF1.2* was not detectable in any healthy tissue (Thomma and Broekaert, 1998). The *Brassica* Stamen-Specific Defensin 1 (BSD1) was expressed in stamens of *Brassica campestris* ssp. *pekinensis*, but not in roots, stems, and leaves (Park *et al.*, 2002). Flower-specific expression was also observed for several defensins from the Solanaceous plants, *Nicotiana tabacum* (Gu *et al.*, 1992), *N. alata* (Lay *et al.*, 2003), and *N. paniculata* (Komori *et al.*, 1997).

Strawberries are flavorful and nutritious fruit enjoyed by millions of people in all climates with an increasing demand. The strawberry fruit is valued for its low-calorie carbohydrate, high fibber contents, a source of natural antioxidants, including carotenoids, vitamins, phenols, flavonoids, dietary glutathionine, and endogenous metabolites (Debnath and Teixeira da Silva, 2007). The evidence concerning induced resistance to diseases and signaling molecules in strawberry is very limited and, to our knowledge, nothing has been published on defensin genes.

The aim of this research was to investigate the expression of defensin gene in three strawberry cultivars following infection by biotic and abiotic stresses. In addition, we aimed to study the gene expression in various tissues, including fruit, at different maturity stages.

MATERIALS AND METHODS

Plant Materials

Strawberry (*Fragaria×ananassa*) cultivars of Queenelisa, Paros, and Camarosa were used in this experiment. Plants were grown under natural sunlight in the greenhouse with temperatures of 23–26°C (day), light intensity of 1300 micromoles per square meter per second, air humidity 70%, and 20–22°C (night). Fresh roots, fresh leaves, stems and full red fruits were collected with random sampling method from all cultivars. All of these samples were washed in freshwater for about 5 minutes to remove the soil particles adhering, then, frozen in liquid nitrogen immediately to keep them under -80°C low temperature, being ready for RNA isolation.

Fruit samples were collected from four individual Paros cultivar plants with random sampling method at different development stages. Between 10 and 18 fruits of the same development stage were harvested every five days and sample collection ended at the over-ripe stage (5th day after the red-ripening stage). These fruit samples coincided with the fruit ripening stages of small green fruit (stage 1), large green fruit (stage 2), green ripe fruit (stage 3), turning red fruit (stage 4), half red fruit (stage 5), red-ripening fruit (stage 6), and full red fruit (stage 7) as shown in (Figure 1). After harvest, all fruits from different ripening stages were washed in water, pooled, and

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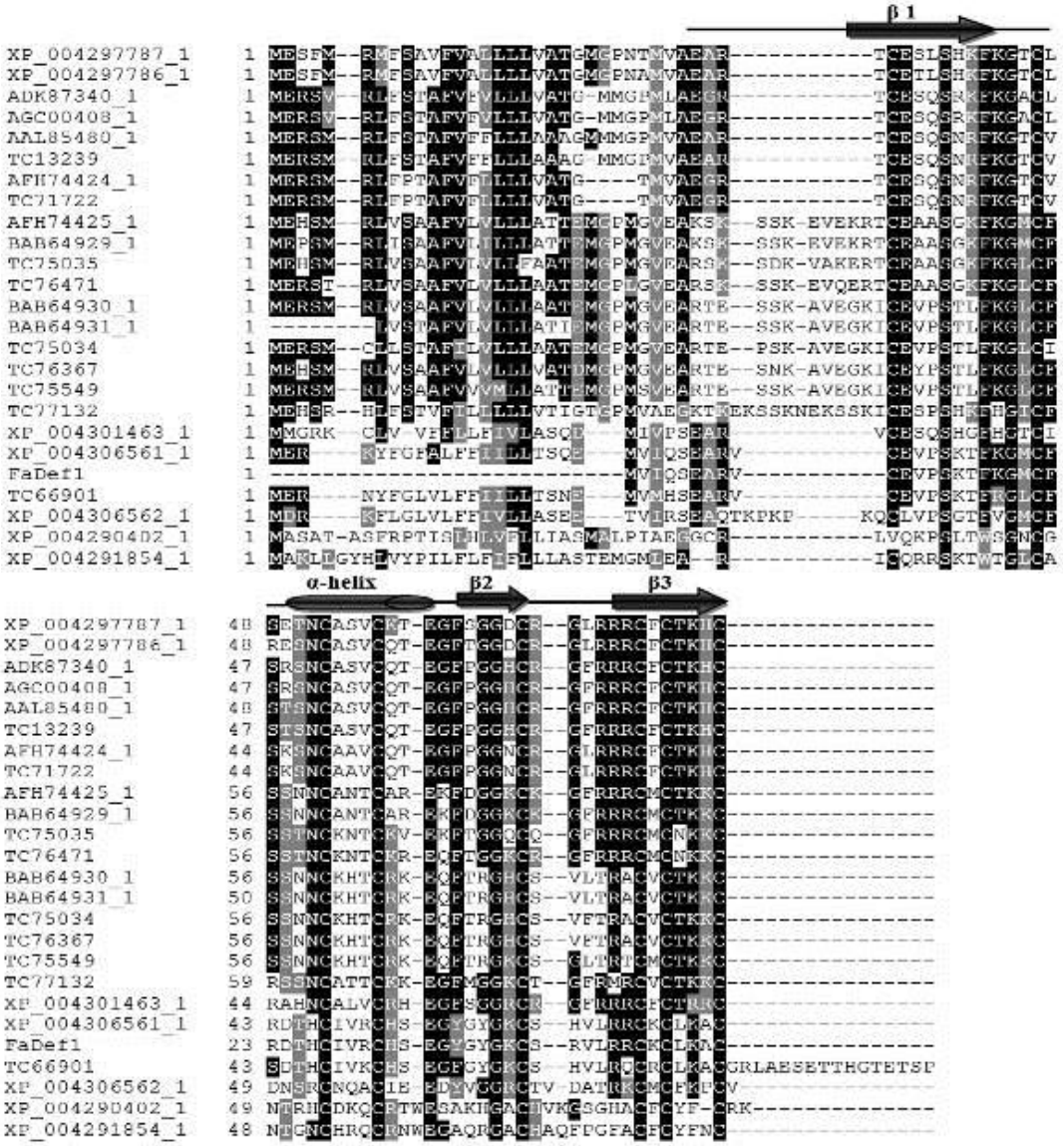

Figure 1. Protein sequence alignment of *FaDef1* (*Fragaria×ananassa* cv. Paros) along with defensins from different Rosaceae plants. Black boxes indicate residues that are strictly identical, and white boxes indicate conservative changes between each defensin sequence.

immediately frozen in liquid nitrogen and kept at –80°C until use for extraction.

Fungal Inoculation

The infection assay was carried out using mycelia (motherboard stock) from *B. cinerea* as described by González *et al.* (2013). The ripe fruits inoculation, taking care of choosing plant material without defects and infection strategy, were based on González *et al.* (2013). Fruit samples were taken daily for up to five days. Collected samples were frozen under liquid nitrogen and kept at -80 °C until processed for transcript analysis.

Wounding Treatment

Youngest fully developed leaves of Paros cultivar were wounded mechanically by cutting the lamina with a razor blade and allowing the wounded leaf to remain on the

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plant for 0, 24, 48 or 72 hours after treatment. The two wounded leaves were harvested and immediately frozen at -80°C.

Salicylic Acid Treatment

The SAR-inducing chemical, salicylic acid (Merck, Germany), was applied to strawberry cultivar Paros leaves. Plants were sprayed with salicylic acid at final concentration 0.3 mM until run off and kept moist for 24 hours. Control plants were sprayed with distilled water and kept moist in the same condition. The two youngest fully developed leaves were harvested at 0, 24, 48, or 72 hours after treatment and immediately placed at -80°C.

RNA Extraction

All tissue samples were immediately stored at -80°C after harvesting. Total RNA from leaves, flowers, roots, and stems was extracted following the method of Mazzara and James (2000). The RNA was resuspended in 25 µL DEPC-treated with dH_2O and stored at -80°C.

Defensin Sequences and Alignments

A peach (*Prunus persica*) characterized defensin was used as a query for BLASTN against NCBI (http://www.ncbi.nlm.nih.gov) strawberry EST database. An alignment was made of three putative defensin nucleotides sequences from *Fragaria* species (GenBank accession GT151247, GT151426 and EX683843). To amplify defensin genes from *F. annanasa* a forward primer FaDEFf, (5′GAGATGGTGATTCAGAGTGAAGCAA G3′) was designed based on conserved regions of the defensin nucleotides sequences alignment from *Fragaria* species. First strand cDNA for 3′ RACE was synthesized through reverse transcription of RNA from full red fruit of Paros with anchor primer (5′GACCACGCGTATCGATGTCGACTTTT TTTTTTTTTTTTV3′) using first strand VIVA 2-steps RT-PCR kit (Vivantis). The first round PCR reaction was carried out in a total volume of 20 µL including 7 µL H_2O, 10 µL Master Mix, 1 µL first strand cDNA template, 1 µL FaDEFf forward primer, 1 µL PCR anchor primer, GACCACGCGTATCGATGTCGAC. PCR temperature program was 1 cycle of 3 minutes at 94°C, 35 cycles of 30 seconds at 94°C, 30 seconds at 63°C, 30 seconds at 72°C, 1 cycle of 20 minutes at 72°C. The resulting PCR product was separated on 1% agarose gels. All PCRs were done using a BioRad iCycler model (Thermal cycler (BioRad; USA).

Molecular Cloning and DNA Sequencing

PCR products were separated on 1.0 % agarose gels, and the expected fragments were purified from the gels using Nucleic Acid Extraction kit (Vivantis). The concentration of purified DNA was determined by spectrophotometer, then, DNA fragments were ligated into the pTG19-T PCR cloning vector kit (Vivantis) and transformed into competent cells of *Escherichia coli* DH5a strain. Positive clones were identified by colony PCR, and independent sequences per clone were obtained from a commercial sequencing service (Bioneer Inc. Bioneer Corporation).

Bioinformatics Analysis

BLAST program in National Center for Biotechnology Information Server (http://www.ncbi.nlm.nih.gov.) was used to verify the defensin gene homology. Multiple protein sequence alignment was performed using Clustal W program of Jalview 2.3 version. For phylogenetic and sequence alignment analysis, other defensin gene sequences were obtained from the GenBank database (Table 1). The phylogenetic tree of Rosaceae defensin was constructed using MEGA4.0.2 software based on the Neighbor-Joining (NJ) method.

Availability of complete strawberry and wild *Fragaria* species genome sequences has made it possible to identify the putative defensin genes in those plant species. To obtain all the defensins from strawberry and five other wild *Fragaria* species, sequences available in the

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Table 1. Defensin amino acid sequences of Rosaceae family used in this study.

Nucleotide accession number	Amino acid accession	Defensin class[a]	Specis	Name
XM_004297739.1	XP_004297787.1	I	*Fragaria vesca* subsp. vesca-	-
XM_004290354.1	XP_004290402.1	I	*F. vesca* subsp. vesca	-
XM_004297738.1	XP_004297786.1	I	*F. vesca* subsp. vesca	-
XM_004306514.1	XP_004306562.1	I	*F. vesca* subsp. vesca	-
XM_004306513.1	XP_004306561.1	I	*F. vesca* subsp. vesca	-
XM_004301415.1	XP_004301463.1	I	*F. vesca* subsp. vesca	-
XM_004291806.1	XP_004291854.1	I	*F. vesca* subsp. vesca	-
JQ342966.1	AFH74425.1	I	*Malus domestica*	-
JQ342965.1	AFH74424.1	I	*Malus domestica*	-
AY078426.1	AAL85480.1	I	Prunus persica	-
HM044853.1	ADK87340.1	I	Pyrus pyrifolia	-
JX104829.1	AGC00408.1	I	*Pyrus pyrifolia*	-
AB052688.1	BAB64930.1	I	*Pyrus pyrifolia*	-
AB052689.1	BAB64931.1	I	*Pyrus pyrifolia*	-
AB052687.1	BAB64929.1	I	*Pyrus pyrifolia*	-
-	-	I	*Fragaria x ananassa*	*FaDef1*
TC66901	TC66901[b]	I	*Malus domestica*	-
TC71722	TC71722	I	*Malus domestica*	-
TC75034	TC75034	I	*Malus domestica*	-
TC75035	TC75035	I	*Malus domestica*	-
TC75549	TC75549	I	*Malus domestica*	-
TC76367	TC76367	I	*Malus domestica*	-
TC77132	TC77132	I	*Malus domestica*	-
TC76471	TC76471	I	*Malus domestica*	-
TC13239	TC13239	I	*Prunus persica*	-

[a] A *C*-terminal prodomain is absent in Class I defensins but present in Class II defensins. [b] Tentative Consensus (TC) amino acids were obtained using frame finder at the DFCI plant Gene Index (http://compbiodfciharvardedu/tgi/_).

Strawberry Genome and Resource Database ENtry (http://strawberry-garden.kazusa.or.jp/index.html) were used. Strawberry defensin was used as a query sequence for BLAST search. The phylogenetic tree of *Fragaria* defensin was constructed using MEGA4.0.2 software based on the maximum parsimony method.

Relative RT-PCR

Specific primers for the defensin were designed using the primer3 Web version 4.0.0 (http://primer3.ut.ee/). Primers FaDEF-f (5′GAGATGGTGATTCAGAGTGAAGCAAG3′) and FaDEF-r (5′GGATAATGAACAAGACAGATTCGC3′) for amplifying *FaDef1* were used so that the resulting PCR product had approximately the size of 260 bp. Primers GAPDH2-F (5′CAGACTTGAGAAGAAGGCCACCTA3′) and GAPDH2-R (5′GATACCCTTCATCTTTCCCTCAGA3′) for amplifying *GAPDH2* were used so that the resulting PCR product had approximately the size of 200 bp. Single-stranded cDNA was synthesized using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Vivantis) and oligo (dT) primer with total RNA following the manufacturer's instructions. PCR was done in 22 µL reaction final volumes with 1 µL cDNA, 1 µL FaDEF-f primer, 1 µL FaDEF-r primer, 8 µL H_2O, 9 µL Master Mix (Sina gene), 1 µL GAPDH2-F primer and 1 µL GAPDH2-R as housekeeping

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gene. Gene amplification was done with 1 cycle at 94ºC for 3 minutes followed by 28 cycles of 94ºC for 30 seconds, 56ºC for 30 seconds, and 72ºC for 30 seconds, and a final extension period of 5 minutes at 72ºC.

The RT-PCR products were separated in 1.2% TAE agarose gels with ethidium bromide. Gel pictures were taken using UVdoc camera and saved as tiff electronic image files. Pictures were imported in GelQuantNET software for quantification. In each lane, the band intensities were determined for both genes of *GAPDH2* and *FaDef1* and relative expressions were calculated. Experiments were replicated with three independent infections or treatments. For each experiment, RNA was extracted and cDNA was made. We quantified three gels from different experiments for each treatment. To confirm the identity of the RT-PCR products, the bands of the defensin gene were directly sequenced. The purified products were sent for direct sequencing at the commercial sequencing service (Bioneer Inc. Bioneer Corporation).

RESULTS

Cloning and Sequence Analysis of *FaDef1*

A strawberry gene encoding a defensin, designated *FaDef1*, was isolated using a PCR-based procedure. *FaDef1* was 260 bp in length, and it has an Open Reading Frame (ORF) of 168 nucleotides with a 3-nucleotide upstream sequence and a 91-nucleotide downstream sequence. The ORF of *FaDef1* starts at nucleotide position 4 and ends at position 169. It encodes a preproprotein of 65 amino acid residues with a predicted signal peptide of 12 amino acid residues at the N-terminus. The calculated molecular mass of the mature protein is approximately 6.18 kDa with a predicated isoelectric point of 9.22. A GenBank Blastx search revealed that *FaDef1* shares 98% identity with *Fragaria vesca subsp. vesca* (XP_004306561.1). It also shares a high degree of similarity with *Cucumis sativus* (XP-004151187.1) (54% identity), *Vitis vinifera* (XP_002272913.2) (50% identity) and *Triticum urartu* (EMS52277.1) (50% identity). *FaDef1* shares lower degrees of similarity to known proteins of other rose family members. For example, the homology scores are 45% identity for a *Pyrus pyrifolia* (BAB64931.1), and 45% identity for a *Malus domestica* (AFH74425.1). The ORF of *FaDef1* showed 100% identity with two genomic sequences of *F. ananassa* FAN_iscf00311607.1.g00002.1 and FAN_iscf00363510.1.g00001.1 which is available at Strawberry Genome and Resource Database ENtry (http://strawberry-garden.kazusa.or.jp/index.html). The genome of cultivated strawberry (*F. ananassa*) and its wild relatives was dissected using deep sequencing (Hirakawa *et al.*, 2014). Searching strawberry genome database showed that *F. ananassa* has 29 annotated defensins and defensins like genes. In other *Fragaria* species, the number of annotated defensins was 12,14,14,10 and 24 for *F. iinumae*, *F. nipponica*, *F. nubicola*, *F. orientalis* and *F. vesca*, respectively. The phylogenetic analysis of *Fragaria* species defensins showed that this gene family is classified into about 14 sub clusters, in which ortholog gene from different species clustered together (Supplemental Figure 1). In each cluster, usually more than one gene from F. *ananassa* exist, which is due to polploidization.

Multiple Sequence Alignment and Phylogenetic Analysis

A sequence alignment of *FaDef1* and other Rosaceae defensins showed that the eight amino acid residues reported to be crucial to the antifungal activity of the latter three proteins are also conserved in *FaDef1*, as indicated in Figure 1. The positions of the eight cysteine residues are absolutely conserved in *FaDef1* predicted peptide, suggesting *FaDef1* share the same secondary structure with other plant defensins. Multiple amino acid sequence alignments showed that *FaDef1* had high similarity with its counterparts from other plant species. *FaDef1* conserved and semi-conserved regions are shown in black and gray, respectively, indicating that the protein structure and functional manner were strongly conserved.

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Sequence alignment revealed that there was high similarity in the defensin domains, including a Cysteine-Stabilized α-helix β-sheet (CSαβ) motif common to plant and invertebrate defensins. Other conserved residues, such as an aromatic residue Tyr11 and Gly13, were also found in the sequence. The eight strictly conserved Cys residues located in defensin domain, the key amino acid residue responsible for the antimicrobial activity, was found in *FaDef1*. To understand the evolutionary relationships among *FaDef1* and other plants, a phylogenetic tree was constructed based on the amino acid sequences of other plants (Figure 2). It was revealed that *FaDef1* grouped into a cluster along with two *Fragaria vesca* defensin like genes XM_004306513.1, XM_004306514.1 and *Malus domestica*, TC66901 belonging to the Rosaceae and paralleling their evolutionary relationships.

Differential Expressions of *FaDef1* in Different Strawberry Organs

The expression of *FaDef1* in different organs of three cultivars Queenelisa, Paros, and Camarosa were examined using relative RT-PCR analysis. From the results shown in Figure 3, it is clear that *FaDef1* was expressed in leaves, stems, flowers, and red fruits. Among these organs, significantly higher levels of *FaDef1* mRNA were observed in red fruits. A moderate level of *FaDef1* mRNA was observed in leaves, flowers, and shoots. In contrast, the expression of *FaDef1* was barely detectable in the root. The expression patterns were similar in the three cultivars.

Expression Patterns of *FaDef1* in Different Developmental Stages of Fruit

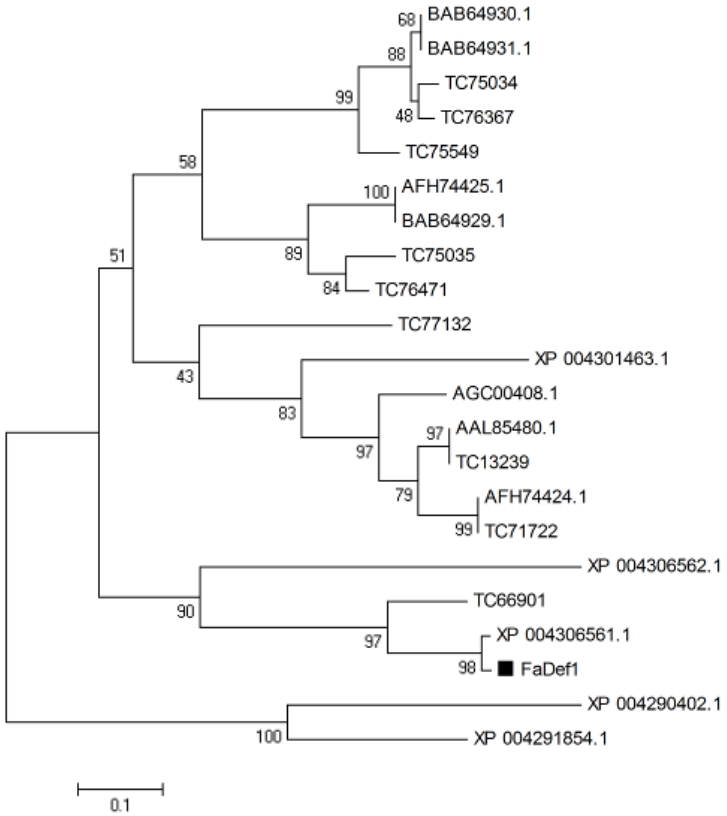

Figure 2. Comparison of rosaceae defensin protein sequence inferred by UPGMA. All defensin proteins analyzed are listed in Table 1. Amino acid sequences were aligned with the program CLUSTALW, and dendrogram was created using distance based phylogeny procedure UPGMA with the program MEGA4.1. The scale bar estimates the genetic distance among defensins.

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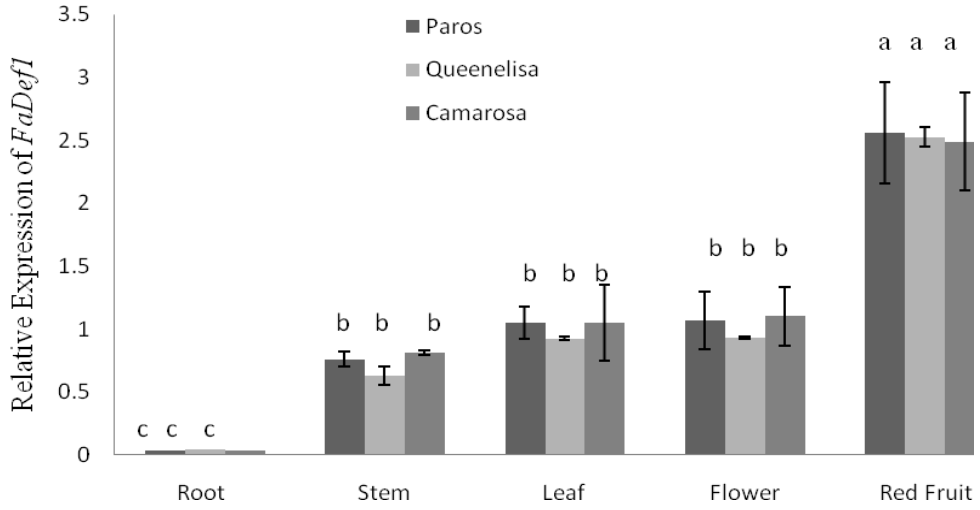

Figure 3. Expression of *FaDef1* in different tissues of three strawberry cultivars Queenelisa, Paros, and Camarosa by relative RT-PCR. The quantity of each defensin mRNA levels was determined relative to the amount (expression) of GAPDH2. Mean values are shown with standard error which is calculated based on three replications.

To further understand whether *FaDef1* was indeed involved in fruit development, we preliminarily explored the expression patterns of *FaDef1* in seven different stages throughout the development of fruit (Figure 4). The results showed that accumulation of *FaDef1* mRNA increased during fruit development and maturation. At the early stages of fruit development (1 to 4), the expression of *FaDef1* was relatively low, while the maximum expression was observed in the completely ripen fruit.

Effects of SA and Mechanical Wounding on Expression of *FaDef1*

To evaluate the different responses of *FaDef1* to abiotic stresses, the mRNA transcript accumulation patterns of *FaDef1* in Paros cultivar leaves treated by wounding and SA were determined. The expression patterns of *FaDef1* at different times after treatments were analyzed (Figure 5-a). Exogenous application of SA appeared to cause a rapid induction of *FaDef1* gene at 24 hours post-treatment and reached a peak at 48h. The expression level then remained unchanged up to 72 hours. The expression pattern of *FaDef1* upon mechanical wounding is shown in Figure 5-b. It appeared that wounding triggered the response of *FaDef1* at a very early stage. A significant induction of *FaDef1* was observed at 24 hours post treatment. The expression level continued to increase until 72 hours time point. Overall, both of the tested abiotic stresses appeared to be able to trigger a significant accumulation of *FaDef1* mRNA within 24 hours post-treatment. Moreover, *FaDef1* was more prominently induced by mechanical wounding.

Expression of *FaDef1* in Infected Cultivar Paros Fruits

Ripe fruits were inoculated with *B. cinerea*, and the accumulation of transcript *FaDef1* was analyzed in different stage of disease development. The expression patterns of *FaDef1* in response to *B. cinerea* are shown in Figure 6. For strawberry plants inoculated with *B. cinerea*, no increase in the expression level of *FaDef1* was observed during the first 48 and 72 hours after infection. However, *FaDef1* expression was significantly increased four and six days after infection compared to that of the control samples. The results showed that with increasing *B. cinerea* infection, *FaDef1* expression level

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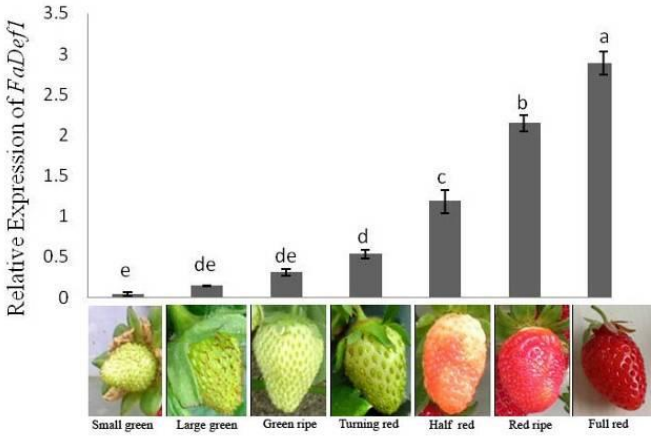

Figure 4. The various stages of *F.×ananassa* cv. Paros fruit development. Relative RT-PCR of *FaDef1* in *F.×ananassa* cv. Paros fruit development stages. The quantity of each defensin mRNA levels was determined relative to the amount (expression) of GAPDH2. Mean values are shown with standard error which is calculated based on three replications.

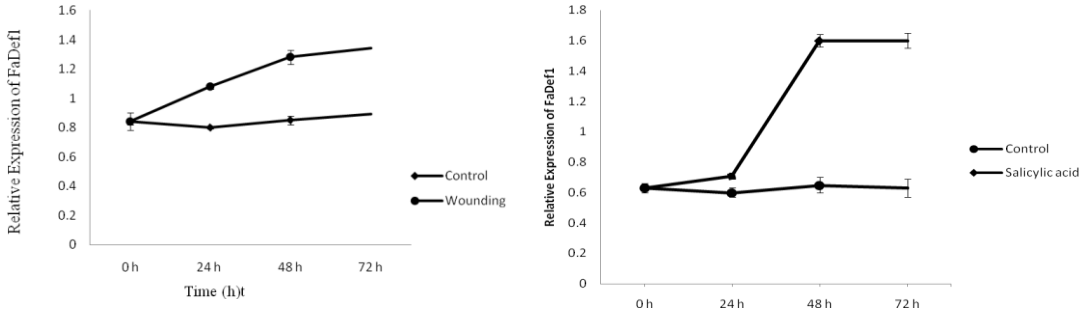

Figure 5. Relative RT-PCR of *FaDef1* in *F.×ananassa* cv. Paros following treatment with 0.3 mM salicylic acid (A) and wounding (B)**.** Control plants were sprayed with distilled water. The quantity of each defensin mRNA levels was determined relative to the amount (expression) of GAPDH2. Mean values are shown with standard error which is calculated based on three replications.

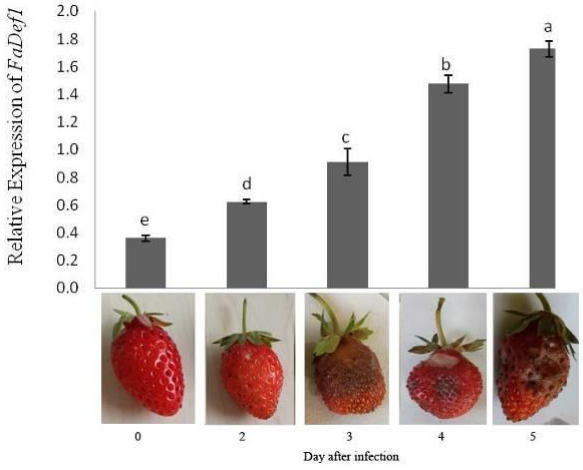

Figure 6. *Botrytis cinerea* infection stages in *F.×ananassa* cv. Paros fruits and relative expression of *FaDef1* in each stage. Ripe fruits were inoculated with *B. cinerea* mycelia, and left at 24°C during the post infection in a growth chamber with 80% humidity. The quantity of each defensin mRNA levels was determined relative to the amount (expression) of GAPDH2. Mean values are shown with standard error which is calculated based on three replications.

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also increased and then remained constant at that level.

DISCUSSION

The deduced peptide encoded by the *FaDef1*gene displays similarities with a wide range of plant defensins. Differences in the primary sequence of defensins may be responsible for their various biological activities. Study of the three-dimensional structure of a number of plant defensins showed that the structure contains a triple-stranded *β*-sheet with an *α*-helix in parallel (Henrik *et al.*, 2009). Three-dimensional structure of *FaDef1* showed the retention (similar) characteristics of a defensin protein. In plant defensins, the conserved sequences are relatively limited, but the eight amino acid cysteine residues, A glycine at position 34 (score by Rs-AFP2) are perfectly preserved. Furthermore, in most cases, the second glycine, serine, glutamic acid and an aromatic residue are conserved (Lay and Anderson, 2005). All of foregoing characteristics existed in *FaDef1* amino acid sequence. *FaDef1* protein had 54 amino acids, of which 13 amino acids are basic, four amino acids are acidic, and predicted isoelectric point of 9.22. Plant defensins are divided into two classes, which differ based on the presence (Class I) or absence (Class II) of a *C*-terminal prodomain. However, defensins with a *C*-terminal prodomain are limited to solanaceous plants (Lay and Anderson, 2005). As members of the Solanaceae, *N. benthamiana* and *N. tabacum* have both classes, while other plants only have Class I (Bahramnejad *et al.*, 2009). *FaDef1* also belonged to Class I.

Sequencing of complete strawberry and wild *Fragaria* species genome has made it possible to identify the putative defensin genes in those plant species (Hirakawa *et al.*, 2014). Defensins are thought to be members of small gene families which contain 15 to 50 members (Silverstein *et al.*, 2005). However, more than 300 defensin-like Cys Cluster Proteins (CCPs) in the legume *Medicago truncatula* (Fedorova *et al.*, 2002) and more than 300 similar unannotated open reading frames of defensin-like sequences in the Arabidopsis have been reported (Silverstein *et al.*, 2005). Numbers of annotated defensins in *Fragaria* species were similar to other plants and ranged from 10 to 29. But, searching genome in more detail and using different bioinformatics approaches may result in more defensin like genes.

The results of relative RT-PCR analysis showed that *FaDef1* expression had similar pattern in three cultivars Paros, Camarosa, and Queenelisa, but was different in different plant organs. Defensin genes showed tissue specific expression pattern in plants. A defensin gene *CADEF1* in *Capsicum annuum* was expressed in stems and roots, but not expressed in leaves or flowers (Do *et al.*, 2004). Defensin gene *PDF2.1* in *A. thaliana* was highly expressed in seeds and roots, but not in healthy leaves. In contrast, *PDF2.2* was expressed in flowers, roots, and healthy leaves, but not in seeds (Thomma and Broekaert, 1998). Each plant tissue expresses at least one defensin gene and some tissues express two or more defensins in Arabidopsis (Henrik *et al.*, 2009). In *A. thaliana* some of defensins show constitutive expression, while the others are up-regulated in leaves following pathogen infection or signaling compound treatment (Lay and Anderson, 2005). Recently, the microarray analysis in two model plants *A. thaliana* and *Medicago truncatula* showed that set of defensin-like genes specifically expressed in seeds or fruits (Tesfaye *et al.*, 2013). Overall, most of plant tissues constitutively express two or more defensin genes, implying that each defensin is expressed under specific conditions or at specific tissues.

The application of salicylic acid increased *FaDef1* expression. A considerable amount of evidence suggests that Salicylic Acid (SA) is involved in the induction of SAR. In both tobacco and Arabidopsis, exogenous SA induced the expression of *PR* (PR-1, PR-2, and PR-5) genes (Antoniw and White, 1980; Uknes *et al.*, 1992; Ward *et al.*, 1991) and increased plant resistance (Uknes *et al.*, 1993; White, 1979). Plant defensins showed variable response to SA. *CADEF1* in *C. annuum* and *NbDef2.2* in *Nicotiana benthamiana* were induced by SA (Bahramnejad *et al.*, 2009; Do *et al.*, 2004). In contrast, other studies showed that *MtDef1.1* and *MtDef2.1* in *M. truncatula* and *MsDef1* and *MsDef2*.1 in *M. sativa* (Hanks

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et al., 2005) and *PDF1.2* in *A. thaliana* (Manners *et al.*, 1998) were not induced by SA.

FaDef1 expression in the wounded leaves of strawberry cultivar Paros showed increase at 24 hours after treatment. Wounding has been shown to induce expression of many plant genes (Reymond *et al.*, 2000). The defensin genes, *CADEF1* and J1 from *C. annuum* (Do *et al.*, 2004; Meyer *et al.*, 1996), *PgD1* from *Picea glauca* (Pervieux *et al.*, 2004), *DRR230-c* from *P. sativum* (Lai *et al.*, 2002) and *NbDef1.1*, *NbDef1.2*, *NbDef1.4* and particularly *NbDef2.2* in *N. benthamiana* (Bahramnejad *et al.*, 2009) were induced upon wounding. Wounding induces signals through an ethylene and/or Jasmonic Acid (JA) dependent pathway (Thaler *et al.*, 2004). The expression of *FaDef1* was significantly induced following both SA and wounding treatment. Induction of a gene by both SA and ethylene has been reported. In Arabidopsis 17 genes, such as 1-Aminocyclopropane-1-carboxylic acid (*ACC*) oxidase, chalcone synthase, lipoxygenase and cellulase were induced by both SA and ethylene (Schenk *et al.*, 2000). Therefore, it is concluded that *FaDef1* may belong to the genes that its expression is induced in both salicylic acid ethylene and/or jasmonic signaling pathway.

Most of plant defensins are active against a wide range of fungi. In addition to antifungal activity against plant pathogenic fungi (e.g. *Fusarium culmorum* and *Botrytis cinerea*), they showed antifungal activity against the yeast and human pathogenic fungi such (*Candida albicans*) (Henrik *et al.*, 2009). The mechanism by which plant defensins inhibit the growth of the fungus is not well understood (Henrik *et al.*, 2009). Expression of Dahlia defensin, Dm-AMP1, in rice directly inhibits the pathogen, *Magnaporthe oryzae* and *Rhizoctonia solani* by 84% and 72%, respectively (Henrik *et al.*, 2009). Overexpression of a radish defensin RsAFP2 significantly enhanced resistance of tobacco plants to the fungal leaf pathogen *Alternaria longipes* (Terras *et al.*, 1995) and similarly in tomato to *Alternaria solani* (Parashina *et al.*, 2000). In this study, *FaDef1* gene expression was significantly different in infected strawberry fruits compared to the controls fruits. By increasing infection severity, *FaDef1* gene expression was increased. There is not much information on PR proteins in strawberry. Recently, an update on a few recognized components of known families of PR proteins in strawberry cultivars challenged with *Colletotrichum acutatum* are published (Amil-Ruiz *et al.*, 2011). Casado-Díaz *et al.* (2006) analyzed a moderately resistant cultivar (cv.Andana with a very susceptible one (cv. Camarosa) during the process of infection with *Colletotrichum acutatum*. They found that a gene (EST) described as thionin (*Fagthio-1*) was significantly upregulated after 3 days post infection, and this increase gradually diminished from 3 to 7 dpi, while showed a significant repression in Camarosa infected fruit tissue compared with infected crown tissue. The two strawberry pathogenesis related proteins i.e. FcPR5 and FcPR10 showed significant differences in the expression pattern of in *F.* x *ananassa* and *F. chiloensis* infected with *Botrytis cinerea*. (González *et al.*, 2013). In *F. chiloensis*, *FcPR5* showed high transcript level in infected leaves, while *FcPR10* transcripts were high in infected fruits. Authors suggested that expression patterns of these genes in the pathogen response were in a tissue-specific manner. *Phaseolus vulgaris* seed defensin PvD1 caused membrane permeabilization in the filamentous fungi *Fusarium oxysporum*, *Fusarium solani*, and *Fusarium laterithium* and in yeast strains *Candida parapsilosis*, *Pichia membranifaciens*, *Candida tropicalis*, *Candida albicans*, *Kluyveromyces marxiannus*, and *Saccharomyces cerevisiae* (Mello *et al.*, 2011). PvD1 also inhibited glucose-stimulated acidification of the medium by yeast cells and filamentous fungi, as well as to induce the production of reactive oxygen species and nitric oxide in *C. albicans* and *F. oxysporum* cells. Therefore, *FaDef1* high level of expression in the infected fruit may be related to reactive oxygen species and nitric oxide in strawberry fruit.

FaDef1 gene expression was significantly different in developing stages of turning red, half-red, red ripe, and full red. These results demonstrate defensin gene expression increased in advanced stages of development of fruit, which can be related to the interaction

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between signal transduction pathways and multiple function of this gene. The process of development has been poorly understood so far.

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جداسازی و مطالعه بیان یک ژن دفنسین در توت فرنگی (Fragaria×ananassa cv. Paros)

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چکیده

دفنسین‌های گیاهی خانواده کوچک چند ژنی مهمی از پپتیدهای کاتیونی کوتاه در سلسله‌ی گیاهان هستند. این ژن‌ها دارای نواحی حفاظت شده‌ای می‌باشند که در این مطالعه بر اساس توالی نواحی حفاظت شده آغازگر طراحی شد و با استفاده از تکنیک رونوشت برداری معکوس و سپس واکنش زنجیره‌ای پلیمراز، ژن‌های کد کننده پروتئین‌های دفنسین در توت فرنگی (*Fragaria×ananassa* cv. Paros) همسانه سازی و تعیین توالی شدند. مقایسه توالی به دست آمده با توالی‌های موجود در پایگاه داده‌ای مرکز بین‌المللی اطلاعات زیست فناوری صحت ژن دفنسین به دست آمد را نشان داد که نام گذاری شد. تعداد اسیدهای آمینه حاصل از ترجمه‌ی توالی به‌دست آمده با نتایج قبلی *FaDef*

یکسان و برابر با ۵۴ اسیدآمینه و ۸ اسید آمینه سیستئینی حفاظت شده با حفظ فواصل مورد نظر با دیگر اسیدهای آمینه بوده است و نقطه ایزوالکتریک آن۲۲ /۹ بود . بیان نیمه کمی ژن دفنسین در سطح رونوشت در اندام‌های ریشه، ساقه، برگ، گل و میوه در سه رقم مختلف بررسی شد و نتایج نشان داد که در شرایط طبیعی بدون وجود عوامل محرک میزان بیان در اندام‌های مختلف در هر سه رقم برابر است به این صورت که بیشترین میزان بیان نسبی ژن دفنسین در میوه بود، و در ریشه هیچ‌گونه بیانی مشاهده نشد، همچنین بیان ژن دفنسین در اندام برگ با تیمارهای مختلف زخم و اسید سالیسیلیک مطالعه شد و نتایج به‌دست آمده نشان‌دهنده‌ی افزایش بیان ژن در زمان‌های متفاوت بوده است. بررسی بیان ژن دفنسین در مراحل مختلف رشدی میوه نشان دادند که در مراحل پیشرفته رشدی با افزایش بیان ژن همراه بوده است و در آزمایش دیگری که میوه‌ها با قارچ عامل کپک خاکستری آلوده شده بودند بررسی بیان ژن دفنسین در شدت‌های مختلف آلودگی نشان داد که با افزایش شدت بیماری میزان بیان ژن نیز افزایش یافته است.

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