Characterization of defense-related genes in the ‘Qinguan’ apple in response to *Marssonina coronaria*

Q. Zhou, H. Gao, M. Wang, Y. Xu, Y.Z. Guo, Y.Z. Wan, Z.Y. Zhao

**Abstract**

*Marssonina coronaria* is a serious fungal disease causing heavy loss of apple production in East Asia including China and Japan. Suppression subtractive hybridization (SSH) was used to identify differentially expressed genes in the ‘Qinguan’ apple (*Malus × domestica*) in response to challenge with *M. coronaria*. The samples were collected at 3, 6, 12, 24, 48, 72, and 96 hours post-inoculate (hpi). A forward subtractive cDNA library was constructed and 2349 cDNA fragments were screened and sequenced. 175 unique cDNA sequences were obtained from the library. The sizes of the sequenced fragments ranged from ~200 to ~1000 bp. Based on BLAST analysis, functions of these ESTs were classified into ten categories, among which the largest three groups were defense/stress, protein metabolism, and photosynthesis. These ESTs were successfully deposited in GenBank, and their accession numbers (JK263619–JK263793) have been released. Quantitative Real Time-PCR (qRT-PCR) was used to characterize the relative expression of catalase (CAT), hypersensitive-induced response protein (HIR), calmodulin (CaM) and distinctive expression patterns of three genes were found in ‘Qinguan’ and ‘Fuji’ hit by spores of *M. coronaria*. In conclusion, responses to *M. coronaria* were complex in apples, nevertheless, certain common genes were found in apples regarding reactions to fungal infection such as *Venturia inaequalis*.

1. Introduction

Apple (*Malus × domestica*) has become one of the world’s largest fruit crops (Sarowar et al., 2011), and also it has become a model fruit tree for study of commercial traits such as disease and pest resistance (Newcomb, 2006). *Marssonina coronaria* causes apple blots. It is a widely-spread fungal disease in apples in North America, Oceania and Asia (Lee et al., 2011), leading to a heavy loss in the apple industry, especially in Eastern Asian countries including China and Japan (Shou and Li, 2009; Zhang et al., 2007), since it can lead to severe defoliation starting at early summer (Zhao et al., 2009). The ‘Qinguan’ apple was bred by the Shaanxi Pomology Research Institute in China using ‘Golden Delicious’ and ‘Jiguan’ as parents. The crossing was made in 1957 and the cultivar was released in 1976. The ‘Qinguan’ apple is the most important commercial cultivar grown in China, occupying 20% of the total apple production in China. Also, it is highly resistant to most fungal diseases including *M. coronaria*, however, the anti-fungal molecular mechanisms in this cultivar remain unclear (Zhang et al., 2007).

As a first step toward understanding the molecular events of defense responses and speeding the discovery of genes presenting defense resistance in ‘Qinguan’ apple, we took advantage of SSH method to characterize the apple transcriptome in the course of *M. coronaria* infection. SSH includes normalization and subtraction steps, and enables rare differentially expressed transcripts to be enriched 1000–5000 fold. Moreover, it yields cDNA fragments that can be used directly for sequencing and further analyses (Bachem et al., 1996; Diatchenko et al., 1996; Diatchenko et al., 1999; Schena et al., 1995). It has been successfully applied to analysis of transcriptomes in several fruit tree species in response to biotic and abiotic stresses (Bae et al., 2010; Egusa et al., 2009; Norelli et al., 2008; Paris et al., 2008; Zhang et al., 2007; Schena et al., 1995).
2. Materials and methods

2.1. Plant materials and fungal pathogen

Plants of *M. domestica* ‘Qinguan’ and ‘Fuji’ were grown at Apple Experiment Station at the Northwest A&F University, Shannxi, China. Conidia of *M. coronaria* were harvested after propagation for one month on potato dextrose agar (PDA) culture medium. The young leaves of the ‘Qinguan’ and ‘Fuji’ apples were inoculated with *M. coronaria*. The treated leaves were immediately covered with plastic bags to keep moisture and to prevent infection from other pathogens. Leaves were sampled at 3, 6, 12, 24, 48, 72, and 96 hours post-inoculation (hpi) as the tester, and the leaves treated with sterile water were used as control materials (driver). All samples were frozen immediately in liquid nitrogen, and stored at −80 °C.

2.2. Total RNA isolation and mRNA purification

Total RNA was isolated from the leaves of ‘Qinguan’ and ‘Fuji’ apple using CTAB method (Gasic et al., 2004). Briefly, a 200 mg sample of frozen tissue was ground into fine powder in liquid nitrogen and homogenized at 65 °C 90 μl extraction buffer (2% CTAB, 2% PVPK-30, 10 mM Tris–HCl pH 8.0, 25 mM EDTA, 2.0 M NaCl, and 0.5 g spermidine), the sample was vortexed vigorously, and centrifuged for 20 min to separate the phases. After centrifugation, an equal volume of chloroform/isoamyl alcohol (24:1) was added to the upper aqueous phase, the RNA was dried, dissolved in 30–50 μl RNase-free water, it was visualized in a denaturing formaldehyde 1.2% agarose gel and quantified using a Thermo Scientific NanoDrop 2000 (Thermo Fisher Scientific NanoDrop Products Wilmington, DE, USA). Both tester and driver mRNA were isolated using the Oligotex mRNA Spin-Column from Oligotex mRNA Mini Kit (Qiagen GmbH, Germany).

2.3. SSH library construction

SSH library was constructed using the PCR Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA), following the manufacturer’s instructions. Basically, driver and tester cDNA were synthesized from mRNA of ‘Qinguan’ apple using an oligo(dT) primer that allowed the amplification of the complete mRNA population in each sample. Tester and driver cDNA were separately digested with the restriction enzyme *RsaI* to obtain shorter, blunt-ended molecules. Tester cDNA is then subdivided into two portions, and each is ligated with a different cDNA adaptor, yet driver cDNA has no adaptors. Two rounds of hybridization and PCR amplification were processed to normalize and enrich the differentially expressed cDNAs. The secondary PCR amplification was performed using the nested primers, to further reduce any background PCR products and enrich for differentially expressed sequences.

Then, PCR products were purified by Universal DNA Purification kit (Tiangen Biotech (Beijing) Co., LTD), and cloned into pGEM-T Easy Vector System (Promega, USA) and transformed into *Escherichia coli* strain TOP10 (Tiangen Biotech (Beijing) Co., LTD).

2.4. Fragment sequencing and analysis

White colonies were isolated from selective media, Luria-Bertani ampicillin (LB-Amp) solid medium, and these clones were grown overnight in 100 μl LB-Amp medium at 37 °C, respectively. 2 μl of bacterium culture from each colony was used to amplify the inserts with the M13 primer: M13 forward primer 5′-CGCCAGGGTTTCCCAGT CACGAC-3′; M13 reverse primer 5′-AGCCGATAACATTTTACACAGGA-3′. The cDNA fragments were sequenced by Beijing Genomics Institute. Vector, polyA and redundancy sequences were manually removed. All the inserted sequences were checked for homologies based on the Genome Database for Rosaceae (GDR, http://www.rosaceae.org/projects/apple_genome), while putative physiological functions of SSH-derived ESTs were categorized using the BLAST algorithms based on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Quantitative Real Time-PCR

Three genes, CAT, HIR, CaM, with different transcript abundances and functions were selected for qRT-PCR, and their transcript levels were compared between resistant (‘Qinguan’) and susceptible (‘Fuji’) apples upon inoculation with *M. coronaria*. The *M. domestica* housekeeping gene *EF1-α* was used as an endogenous reference for relative quantification. *EF1-α* forward primer: 5′-CAAGCCCATATGTTGAGAGGA-3′, reverse primer: 5′-CACCCTGGAACTCCCTCTC-3′ (Sanzani et al., 2010). qRT-PCR analysis was prepared with total RNA extracted from ‘Qinguan’ and ‘Fuji’ plants at 3, 6, 12, 24, 48, 72, and 96 hpi. Apple leaves treated with sterile water were used as negative control materials (0 hpi). RNA was reverse-transcribed with oligo(dT) and random primers using PrimeScript RT reagent Kit (Takara Biotechnology, Dalian). qRT-PCR was performed in 96-well plates, using an IQ5 real-time PCR cycler (Bio-Rad Laboratories, USA) and SYBR green master mix (Takara Biotechnology, Dalian) in a reaction volume of 25 μl. Touchdown PCR, a cycling program with varying annealing temperatures, was used. Cycling parameters were 95 °C for 30 s, 2 cycles of 95 °C for 5 s, 62 °C for 30 s and 72 °C for 30 s; 2 cycles of 95 °C for 5 s, 60 °C for 37
30 s and 72 °C for 30 s; 3 cycles of 95 °C for 5 s, 58 °C for 30 s and 72 °C for 30 s; following 28 cycles of 95 °C for 5 s, 55 °C for 30 s, 72 °C for 30 s; then 72 °C for 7 min. To evaluate the quality of the dissociation curves, the following program was added after the 40 PCR cycles: 95 °C for 15 s, followed by a constant increase in temperature from 60 °C to 95 °C. Three experimental replicates were run for each real-time PCR.

The primers were used as following: HIR-F: 5′-TGCTCCCA-CAGCACCTGGCC-3′, HIR-R: 5′-s GCTGGGGCTTGGA-CA- TAGCTCGG-3′; CAT-F: 5′-CCCAACCCCGAACAGCAAC-3′, CAT-R: 5′-GCACCCGACAGCAAGAG-3′; CaM-F: 5′-GGCCGATCAGCTCACAGA-3′, CaM-R: 5′-CATATTGCTCAACATCGTCAACCTGGCC-3′.

3. Results

3.1. Construction of the subtracted cDNA library

A subtracted cDNA library was constructed to obtain a comprehensive set of genes that were induced in M. coronaria-resistant apple cultivar ‘Qinguan’. A total of 2349 white colonies were isolated from LB-Amp medium. Each colony was used to amplify the inserted sequences. Most of the inserted fragments ranged from ~200 to ~1000 bp. From these sequences, we obtained 175 differentially expressed ESTs. Among 175 ESTs, one EST showed no homology to any previously identified genes and the remaining 174 ESTs represented non-redundant sequences, we obtained 175 differentially expressed ESTs.

Among 175 ESTs, we categorized ten functional groups (Fig. 1 and Supplementary Table). Other categories included functions involving protein metabolism (15%), photosynthesis (11%), secondary metabolism (10%), signal transduction (8%), transporters (7%), energy metabolism (7%) and cell growth/division (7%), transcription (6%), and unknown function (1%) in the library. Putative functions of all ESTs were annotated based on the highest scoring matches (e-values lower than 10e-4) by BLAST in NCBI. 175 ESTs deposited in the NCBI dbEST database of GenBank with accession numbers between JK263619–JK263793 (Supplementary Table).

3.2. Quantitative Real Time-PCR for CAT, HIR, CaM

qRT-PCR was used to measure the relative expression of CAT, HIR, CaM, in ‘Qinguan’ and ‘Fuji’ apples at different time points after inoculation with spores of M. coronaria. Each transcript levels of genes were changing as time went on, and distinct expression patterns of these three genes were found in ‘Qinguan’ and ‘Fuji’ apples in response to M. coronaria (Fig. 2).

Expression levels of CAT gene were down-regulated at 3 hpi in ‘Qinguan’ apple, then up-regulated and reached the peak at 72 hpi (Fig. 2, CAT), in contrast, catalase activity increased dramatically at 3 and 72 hpi in ‘Fuji’ apple, but down-regulated at 6 to 48 hpi. Expression of HIR was up-regulated from 24 hpi (Fig. 2 HIR), and reached a peak at 72 hpi in ‘Qinguan’, however, its response postponed at 48 hpi in ‘Fuji’, and lasted less than 24 h. Levels of calmodulin (CaM) gene expression showed insignificant changes in ‘Qinguan’ after inoculation with M. coronaria, even slightly decreased at 3 hpi and 24 hpi, and only slightly increased after 48 hpi (Fig. 2 CaM). In contrast, the activity of the CaM gradually increased after 3 hpi in ‘Fuji’, and peaked at 24 hpi.

4. Discussion

4.1. Expression profiling of CAT, HIR, CaM

From our data, we found that two ESTs, CAT and HIR, showed comparatively high abundance among all the inserts. In addition, CaM, a ubiquitous Ca2+-binding protein in eukaryotes, plays a critical role in decoding and transducing stress signals by activating specific targets for cell death and defense responses in plants (Choi et al., 2009). Thus, these three interesting genes were chosen to measure their relative differential expression in resistant (‘Qinguan’) and susceptible (‘Fuji’) apples hit by M. coronaria. Many fungal-induced modifications of the plant cell wall have been reported that could theoretically block the pathogen to enter the cell (Bolwell et al., 2001; Mellersh et al., 2002). One of the earliest occurrences responding to invader is the accumulation of reactive oxygen species (ROS) (Jindrichová et al., 2011; Shetty et al., 2007). However, extra ROS could heavily do harm to nucleic acids, proteins and membranes, therefore, their cellular levels are totally controlled (Jindrichová et al., 2011). In ‘Qinguan’ apple, CAT down-regulated at early stage, this allows H2O2 to accumulate, resulting in antimicrobial activity through strengthening of the plant cell wall, and activation of defense genes, subsequently halt to pathogen infection (Blackman and Hardham, 2008), and then CAT expression up-regulated to maintain ROS homeostasis. In contrast, CAT activity increased dramatically at an early stage in ‘Fuji’ apple, this might give chance for fungus to penetrate, in agreement with that CAT promoted hyphal infection of Magnaporthe oryzae likely through a reduction in the level of H2O2 (Tanabe et al., 2009).
| GenBank acc. | GenBank hit | Annotation | e-Value |
|--------------|-------------|------------|---------|
| JK263634     | AY159555.1  | Putative hypersensitive-induced response protein [Vitis vinifera] | 3.00e-08 |
| JK263665     | AB021789.1  | Calmodulin [Pyrus pyrifolia] | 5.00e-106 |
| JK263701     | AJ496418.1  | Catalase [Prunus persica] | 1.00e-06 |
| JK263619     | AJ243427.1  | Thaumatin-like protein [Malus domestica] | 1.00e-135 |
| JK263620     | U82220.1    | Cysteine protease inhibitor mRNA [Pyrus communis] | 4.00e-10 |
| JK263621     | AB545981.1  | F-box proteins, S ribonuclease [Pyrus pyrifolia] | 8.00e-43 |
| JK263622     | XM_002516763.1 | Transmembrane BAX inhibitor motif-containing protein, putative [Ricinus communis] | 2.00e-43 |
| JK263623     | FN823234.1  | Genomic sequence for BAC clone MC-12 containing a Mal d 1 gene cluster [Malus domestica] | 8.00e-21 |
| JK263624     | EU794447.1  | Clone M18-6Bs Vf apple scab resistance protein HcrVf2-like gene [Malus floribunda] | 3.00e-15 |
| JK263625     | FJ589786.1  | Class IV chitinase mRNA [Pyrus pyrifolia] | 1.00e-23 |
| JK263626     | XM_002265963.1 | GDSL-motif lipase-like (LIP) [Vitis vinifera] | 1.00e-04 |
| JK263627     | AF319165.1  | Dehydration-responsive protein RD22 [Prunus persica] | 8.00e-09 |
| JK263628     | AF577266.2  | Formate dehydrogenase [Quercus robur] | 3.00e-17 |
| JK263629     | AM158274.1  | Asparagine synthetase, type III (sas3 gene) [Glycine max] | 2.00e-25 |
| JK263630     | EU309470.1  | Universal stress protein 1 [Gossypium hirsutum] | 7.00e-32 |
| JK263631     | HM122579.1  | HD domain class transcription factor (HD21) [Malus domestica] | 9.00e-28 |
| JK263632     | AG374846.1  | Senescence-associated protein SAG102 [Malus domestica] | 3.00e-109 |
| JK263633     | HM042682.1  | Glycine-rich RNA-binding protein 1 (GR-RBP1) [Malus prunifolia] | 3.00e-148 |
| JK263634     | AB021790.1  | Metallothionein-like protein [Pyrus pyrifolia] | 6.00e-33 |
| JK263635     | AF309182.1  | Aldo-keto reductase superfamily member [Fragaria ananassa] | 1.00e-25 |
| JK263636     | AY062129.1  | 1-Aminocyclopropane-1-carboxylate synthase (ACS1) gene [Malus domestica] | 2.00e-06 |
| JK263637     | HM122720.1  | WRKY domain class transcription factor [Malus domestica] | 5.00e-17 |
| JK263639     | AJ000997.1  | Guard cell proline-rich protein [Solanum tuberosum] | 2.00e-20 |
| JK263640     | AY792997.1  | Major allergen and lipid transfer protein Mal d 3 mRNA [Malus domestica] | 1.00e-66 |
| JK263641     | AF362989.1  | Class 4 pathogenesis-related protein mRNA [Prunus persica] | 6.00e-29 |
| JK263642     | XM_002871590.1 | Heavy-metal-associated domain-containing protein, mRNA [Arabidopsis] | 3.00e-13 |
| JK263643     | GU506266.1  | Polyphenol oxidase 5 precursor [Pyrus bretschneideri] | 4.00e-36 |
| JK263644     | AJ078426.1  | Defensin 1 [Prunus persica] | 2.00e-09 |
| JK263645     | AF537127.1  | beta-1,3-Glucosidase-like mRNA [Camellia sinensis] | 4.00e-10 |
| JK263646     | A870964.1   | Thioredoxin M precursor (trxm gene) [Triticum turgidum] | 4.00e-09 |
| JK263647     | EU123921.1  | Expansin 3 (EXPA3) mRNA, partial cds [Eriobotrya japonica] | 2.00e-20 |
| JK263648     | FN812238.1  | Immune mapped protein 1 (imp-1 gene) [Nemata maxima] | 5.00e-59 |
| JK263649     | XM_002879958.1 | DNAJ heat shock N-terminal domain-containing protein, mRNA [Arabidopsis] | 5.00e-04 |
| JK263650     | Z69596.2    | Methionine sulfoxide reductase [Fragaria ananassa] | 8.00e-27 |
| JK263651     | EU794466.1  | Clone M18-6Cs HcrVf4 gene, complete cds [Malus floribunda] | 2.00e-11 |
| JK263667     | DQ907931.1  | Putative mandelonitrile lyase [Lilium longiflorum] | 3.00e-09 |
| JK263668     | DQ252496.1  | Polygalacturonase-like protein [Solanum tuberosum] | 3.00e-22 |
| JK263789     | XM_002320610.1 | Glutaredoxin (PtrGrx9), mRNA [Populus trichocarpa] | 6.00e-13 |
| JK263792     | GU325032.1  | Thioredoxin peroxidase mRNA [Citrus limon] | 3.00e-08 |
| JK263782     | AY347849.1  | Phospholipid glutathione peroxidase [Malus domestica] | 4.00e-06 |
| JK263775     | AF62682.1   | Leaf blotch virus ORF 1, ORF 2 and ORF 3 [Citrus] | 1.00e-16 |
| JK263783     | XM_002528913.1 | Zinc finger protein, putative, mRNA [Ricinus communis] | 1.00e-51 |
| JK263770     | XM_002667692.1 | SPX domain-containing protein, mRNA [Arabidopsis] | 1.00e-06 |
| JK263710     | XM_002511247.1 | Cytochrome P450 [Ricinus communis] | 5.00e-26 |
| JK263763     | AY279312.1  | Cysteine protease-like mRNA, partial sequence [Malus domestica] | 2.00e-06 |
| JK263677     | AB037149.1  | 26S proteasome regulatory particle non-ATPase subunit7, complete cds [Oryza sativa japonica] | 1.00e-18 |
| JK263752     | AF061514.1  | Manganese superoxide dismutase (Mn-SOD) [Gossypium hirsutum] | 1.00e-22 |
| JK263775     | AJ313384.1  | Aspartic proteinase (ap1 gene) [Theobroma cacao] | 3.00e-04 |
| JK263688     | AF397903.1  | AAA-metalloprotease FtsH (FTSH) mRNA [Pisum sativum] | 2.00e-19 |
| JK263785     | XM_002524270.1 | Peroxidase 12 precursor [Ricinus communis] | 1.00e-09 |

Hypersensitive response induces cell death around the infection site to restrict pathogen spread (Heath, 2000; Kombrik and Schmelzer, 2001; Watanabe and Lam, 2006). In this study, expression profiling of HIR was different between resistance and susceptibility cultivars. This indicated that surveillance system in resistance cultivars could be triggered within a few hours following pathogen contact in order to boost a defense response (Greenberg and Yao, 2004). On the other hand, programmed cell death can be retarded by expression of BAX inhibitor 1 (BI-1), a membrane protein that protects cells by an unknown mechanism (Huckelhoven, 2003; Matsumura et al., 2003; Isbat et al., 2009). In this study, we obtained a differentially expressed EST, JK263622 (Table 1), which showed homology to BAX inhibitor motif-containing protein, however, its functions on fungal response were uncertain.

When plant cells are challenged with biotic or abiotic stimuli, Ca²⁺ fluxes are generated to trigger signaling cascades that ultimately lead to proper physiological responses (Lecourieux et al., 2006; Ma et al., 2008). Ca²⁺ signaling is transduced via Ca²⁺ receptors such as CaM, which is a major Ca²⁺ receptor...
CaM isoforms from diverse plant species exhibited differential expression patterns in response to biotic and abiotic stimuli (Ali et al., 2003). They may play a critical role in decoding and transducing stress signals by activating specific targets, hence, calmodulin is considered to be a multifunctional regulatory protein although calmodulin itself usually has no enzymatic or biochemical functions (Chiasson et al., 2005; Choi et al., 2009; Du et al., 2009).

4.2. Defense/stress related ESTs

A great deal of differentially expressed ESTs, which might be related to M. coronaria resistance, was identified in SSH library. The largest set of gene functional category defense/stress (28%) detected in this study was similar to the previous study which categorized disease/defense accounting for 11% in apple under Venturia inaequalis challenge (apple scab) (Paris et al., 2008). Moreover, we also found SSH–ESTs overlap between our work and Degenhardt et al. (2005) in the scab-resistant cultivar ‘Remo’ in response to challenge by V. inaequalis, including chitinase, proline-rich protein, cysteine protease inhibitor, Mal d 1 (Degenhardt et al., 2005). This indicated that there might have been common genes in apples in response to the fungal diseases.

Several pathogenesis-related (PR) proteins are expressed in the apoplast of the M. domestica-resistant cv. ‘Remo’ (Gau et al., 2004), and some of the PR proteins were identified in Her1/2-transformed apple plants in response to V. inaequalis (Paris et al., 2008). PR proteins proved to have anti-fungal activity against phytopathogenic microorganisms and were classified into 17 families (Aglika, 2005; van Loon et al., 2006; Sels et al., 2008; Carvalho and Gomes, 2009). In this study, JK263625 (Table 1), showed homology to chitinase, PR-3 family, which plays a positive role in plant defense by hydrolyzing chitin, the principal constituent of the fungal wall (Jean-Marc, 1999; Veluthakkal and Dasgupta, 2010). Chitinases exhibited antifungal activity in several plant species (Fernandez-Caballero et al., 2009; Kopparapu et al., 2011). JK263619 (Table 1) showed homology to thaumatin-like proteins, which were classified as a class 5-PR protein (PR-5), and proved to function as anti-fungal protein (van Loon et al., 2006). For example, the recombinant thaumatin-like Mal d 2, an important allergen of apple fruits, exhibited antifungal activity against Fusarium oxysporum and Penicillium expansum (Krebitz et al., 2003). Moreover, the transgenic expression of thaumatin-like proteins engendered anti-fungal activity to inhibit mycelial growth (Ho et al., 2007; Kim et al., 2009).

JK263620 presented homology to cysteine protease inhibitor, which was involved in the protective reactions under stress conditions (Dunaevskii et al., 2005). Protease inhibitors retarded proteases of fungal pathogens and suppressed germination of spores and growth of the fungal mycelium (Dunaevskii et al., 2005; Joshi et al., 1998), however its antifungal mechanism has not been fully elucidated (Wong et al., 2010). JK263644 sequence showed homology to defensin protein 1. Plant defensins constitute a part of the innate immune system and primarily fight against fungal pathogens (Zhu et al., 2007; Stotz et al.,...
2009; Sagaram et al., 2011). A study implicated two MAP kinase signaling cascades in a plant defensin-mediated alteration of fungal growth (Ramamoorthy et al., 2007). JK263640 sequence had homology to lipid-transfer proteins (Table 1). Lipid-transfer proteins (LTP) were classified as a class 14-PR protein, and they were found to be secreted and located in the cell wall (Kader, 1997). Moreover, lipid transfer proteins (LTPs) can retard growth of fungal pathogens and lead to cell death (Borges et al., 2006; Vieira et al., 2010; Wong et al., 2010).

Noteworthily, JK263638 was found homology to WRKY domain class transcription factor. WRKY transcription factors are one of the largest families of transcriptional regulators in plants and constitute integral parts of signaling webs that regulate many plant processes (Mzid et al., 2007; Rushton et al., 2010). WRKY protein functions via interactions with a various array of protein partners, including 14-3-3 proteins (JK263773, Supplementary Table), calmodulin (JK263665), resistance proteins and other WRKY transcription factors (Rushton et al., 2010). WRKY gene expression proved to have dramatic effects on plant defense. For example, in rice, OsWRKY82 was involved in the regulation of defense response to pathogens and tolerance against abiotic stresses (Peng et al., 2011). Transcriptome analysis of populus PtWRKY23 overexpressing revealed a significant concurrence with the Melampsora-infection response (Levee et al., 2009). VvWRKY2 reduces the susceptibility of transgenic tobacco, and suggests its involvement in grape resistance against fungal pathogens (Mzid et al., 2007). Moreover, Fan et al. (2011) proved that pathogen-induced MdWRKY1 enhances disease resistance in ‘Qinguan’ apple (Fan et al., 2011).

5. Conclusions

In conclusion, the interaction between the apple plant host and the *M. coronaria* pathogens is a complex process. This process may be regulated via interactions that involved multiple genes. The pathways and reaction mechanism involved in these reactions are currently unclear. Our primary data provide new hints to the molecular events that were induced by *M. coronaria* infection of apple. In this study, we isolated 175 ESTs from ‘Qinguan’ suffering from *M. coronaria* using a suppressive subtractive hybridization method. Functions of these ESTs were involved in defense/stress, protein metabolism, photosynthesis, secondary metabolism, signal transduction, transporters, energy metabolism and cell growth/division, transcription, and unknown function. The largest set of these genes (28%) was assigned to disease-defense, indicating a general increase in the expression level of defense-related and resistance genes. Many of the identified sequences were implicated in other plant–pathogen interactions and might play similar roles during *Malus–M. coronaria* interaction. Expression profiling by quantitative RT-PCR of 3 genes, CAT, HIR, CaM, showed that they were induced by fungal challenge, and these three genes presented very different expression patterns between ‘Qinguan’ and ‘Fuji’ in response to pathogen infections. These data provide preliminary information on the molecular changes that occur after inoculation, and provide a basis for more detailed future studies. Further work to examine the functions of the differentially expressed genes in response to *M. coronaria* infection would help to understand the molecular mechanisms of the ‘Qinguan’ defense response to fungal infection and to develop new measurements to control this disease, and ultimately to improve apple tolerance by genetic breeding or genetic manipulation.

Supplementary materials related to this article can be found online at doi:10.1016/j.sajb.2012.01.005.

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