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*sp. mali***

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Genome-wide identification and expression analysis of apple *ASR* gene family in response to *Alternaria alternata* f. sp. *mali*

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Draft

Abstract: The ABA/water stress/ripening-induced (*ASR*) gene family exists universally in higher plants, and many *ASR* genes are up-regulated during periods of environmental stresses and fruit ripening. Although a considerable amount of research has been performed investigating *ASR* gene responses to abiotic stresses, relatively little is known about their roles in response to biotic stresses. In this report, we identified five *ASR* genes in apple (*Malus x domestica*) and explored their phylogenetic relationship, duplication events, and selective pressure. Five apple *ASR* genes (Md-*ASR*) were divided into two clades based on phylogenetic analysis. Species-specific duplication was detected in *Malus domestica ASR* genes. Leaves of ‘Golden delicious’ and ‘Starking’ were infected with *Alternaria alternata* f. sp. *mali*, which causes apple blotch disease, and examined for the expression of the *ASR* genes in lesion areas during the first 72 h after inoculation. Md-*ASR* genes showed different expression patterns at different sampling times in ‘Golden delicious’ and ‘Starking’. The activities of stress-related enzymes, peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia lyase (PAL) and polyphenoloxidase (PPO), and the content of malondialdehyde (MDA) were also measured in different stages of disease development in two cultivars. The *ASR* gene expression patterns and these physiological indexes for disease resistance suggested that Md-*ASR* genes are involved in biotic stress responses in apple.

Keywords: apple; *ASR* gene; species-specific duplication; *Alternaria alternata* f. sp. *mali*; gene expression

Introduction

Following the isolation of the first water stress/abscisic acid (ABA) stress/ripening-induced protein (ASR protein) in tomato (Iusem et al. 1993), other members of the *ASR* gene family were subsequently identified in succession (Rossi et al. 1996). The ASR proteins are plant-specific, small hydrophilic proteins whose characteristic domains are located near the C-terminal with about 80 amino acids (Xiong et al. 2002). The numbers of *ASR* genes differ among plant species from zero in *Arabidopsis thaliana* (Carrari et al. 2004; Yang et al. 2005), four in tomato (*Solanum lycopersicum*) (Thompson and Corlett 1995; Frankel et al. 2007) and in loblolly pine (*Pinus taeda* L.) (Puryear et al. 1996), six in rice (*Oryza sativa*) (Wibaux et al. 2010), and nine in maize (*Zea mays*), which is the greatest number of *ASR* genes in a single species described to date (Virlouvet et al. 2011). Comparison of sequences from different species suggested that paralogous members in the family were more closely related to each other than to orthologous members (Carrari et al. 2004; Wibaux et al. 2010). Philipee et al. (2010) suggested that these paralogs originated from recent duplication events and the sub-functionalization of each member might be species-specific. The expression patterns of the *ASR* gene family members varied among different species during abiotic stresses and plant development (Maskin et al. 2001; Zhu 2001; Zhu et al. 2002; Shinozaki et al. 2003). Expression of *ASR* genes was determined in lily pollen treated with abscisic acid (ABA), NaCl or dehydration (Yang et al. 2005), water-stressed loblolly pine (Bartels et al. 1992), common ice plant induced by ABA (Maskin et al. 2001), water-stressed maize (Riccardi et al. 1998), salt-tolerant rice seedlings (Vaidyanathan et al. 1999), grape (Maskin et al. 2001) and non-acidic pummelo fruit (Canel et al. 1995). The findings of these studies suggested that the *ASR* genes are involved in broad physiological and developmental processes.

ASR genes were reported to play a role in abiotic stress, but also in response to fungal pathogens (Chen et al. 2013). For example, the *Mp-ASR* gene was up-regulated in *Musa paradisiaca* (banana) leaves, peels and roots infected with *Fusarium oxysporum* f. sp. *cubense*, which possibly indicated that *Mp-ASR* acted in response to the pathogen or disease development (Dai et al. 2011). In tobacco and tomato, *ASR4*-like proteins were required for the *Cf- 4/Avr4*-dependent HR- resistance and for probably for other biological processes (Xu et al. 2012).

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in plant defenses against pathogens. The hypersensitive reaction (HR) is one of the early defense responses of SAR. Rapid generation of reactive oxygen species (ROS) is a characteristic feature of HR response (Doke et al. 1987). Excess ROS causes lipid peroxidation, membrane damage, and consequently, accumulation of malondialdehyde (MDA) (Antunes et al. 2008; Yang et al. 2011). Therefore, plant cells must be protected against the harmful effects of ROS by a complex antioxidant system, which includes a number of SAR-related enzymes, such as peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) (Cao et al. 2011). Phenylalanine ammonia lyase (PAL) and polyphenoloxidase (PPO) are important SAR related enzymes in plants, and their activities are related to plant resistance to pathogens (Finger 1994; Soares et al. 2005). Because biochemical changes can lead to resistance state, monitoring these changes can help to understand the biochemical/physiological process of plant responses to pathogen infection.

In this work, we identified five *ASR* genes in the genome of apple (*Malus x domestica*), the most widely grown fruit tree in the world, and analyzed the phylogenetic relationship, duplication events and selection pressures of the genes. We also determined the expression levels of apple *ASR* genes after infection with *A. alternata* f. sp. *mali*, which causes apple blotch disease, a globally important

early defoliation disease in apple, and associated the expression with the disease symptom development and expression levels changes of the aforementioned defensive enzymes.

Materials and methods

Identification of *ASR* gene family in apple

We used ABA/WDS domain (PF02496) to blast against apple genome using Phytozome website (v10.1) (<http://phytozome.jgi.doe.gov/pz/portal.html>) with the expectation value set to 1. All hits were also submitted to the Pfam analysis (<http://pfam.xfam.org/>) to verify the presence of ABA/WDS domain. The *Malus* EST database at NCBI was used to correct erroneous insertions in the Phytozome gene annotations. The gene structures of *ASR* were drawn by using Fancy GENE (http://bio.eio.eu/fancy_gene).

Identification of *ASR* proteins motif in apple

The Md-*ASR* proteins domain and physico-chemical characteristics were analyzed by using SMART (<http://smart.emblheidelberg.de>), Pfam (<http://pfam.sanger.ac.uk>) and Expasy (<http://web.expasy.org>). The motifs were predicted by using MEME (<http://meme.ncbr.net/meme/intro.html>) with maximum number of motifs at 10 and optimum motif width of 20 to 50 residues.

Sequence alignment and phylogenetic analyses

The peptide sequences of all identified *ASR* genes were aligned using the ClustalW program in MEGA 5.0 (Tamura et al. 2011). A neighbor-joining (NJ) method was then applied to construct the phylogenetic tree of Md-*ASR* genes using MEGA 5.0 with complete deletion, P-distance model and

1,000 bootstraps (Larkin et al. 2007).

A phylogenetic tree of *ASR* genes in apple and other 18 representative species of different plant groups (Table 2) was similarly constructed to gain the evolutionary insight of this group of related genes. The sequences of all *ASR* genes were obtained by search in Phytozome website as were the Md-*ASR* genes except those *ASR* genes from *Lilium longiflorum* and *Ginkgo biloba*, which were retrieved from NCBI (<http://www.ncbi.nlm.nih.gov/>) with Genbank ID AF077629.1 and AY461715.1 (Wang et al. 1998; Shen et al. 2005). There were no reported *ASR* genes in *Physcomitrella patens* and *Chlorophyte* (green algae) including *Chlamydomonas reinhardtii*, *Volvox carteri*, *Coccomyxa subellipsoidea* C-169, *Ostreococcus lucimarinus*, *Micromonas pusilla* CCMP1545 and *Micromonas* sp. RCC299.

Nonsynonymous substitutions and synonymous substitutions

Nonsynonymous substitutions (*Ka*) and synonymous substitutions (*Ks*) were evaluated to detect the duplication time and selection pressure on apple *ASRs*. The CDS of each two members of Md-*ASR* genes were aligned by ClustalW 2.0 based on protein sequences (Larkin et al. 2007). Subsequently, *Ka*, *Ks* and *Ka/Ks* were calculated by using MEGA 5.0 (Tamura et al. 2011).

Preparation of fungi and plant materials with treatments

Alternaria alternata apple pathotype was maintained on potato dextrose agar (PDA) in petri plates for five days with 12 h/12 h day/night photoperiod and 25 °C. Blocks with 4 mm diameter of agar with mycelium were collected on an agar block and used to inoculate apple leaves.

Fully-expanded, entire leaves were collected from two-year old ‘Golden Delicious’ and ‘Starking’ trees in July, 2014. Leaves were disinfected with 75% ethanol and inoculated with a block of agar with the mycelium side in contact with the wound surface (a hole by toothpick). Leaves wounded in a similar manner were inoculated with the same size PDA block, but without fungus, to serve as controls. All leaves were cultured in an incubator with 25 °C, and 12 h/12 h day/night photoperiod. Leaf tissue surrounding the infection sites were collected at 0 , 18 , 36 and 72 h (Li et al., 2011) after inoculation, and photographed with a OLYMPUS SZ61 stereoscopic microscope (Li et al. 2011; Xia et al. 2014). Another set of leaf tissue was collected at 0 h, 18 h, 36 h and 72 h and immediately immersed in liquid nitrogen and stored at –80 °C until RNA extraction. Three independent experiments were replicated, and each treatment contained three leaf samples as biological replicates.

A third set of leaves were collected for measuring enzyme activities of POD, SOD, CAT, PAL and PPO, and the content of MDA as physiological indices (Zucker and Milton 1965; Koon et al. 1998b; Zhang 2007; Cang and Zhao 2013). Both infected and control leaf samples were harvested at the same time after inoculation. Three independent biological replicates were collected.

Disease susceptibility/resistance was analyzed using lesion development on 110 inoculated and 110 non-inoculated (control) leaves of both cultivars after 72 h. The severity of infection for each leaf was scored on a grade value of 0, 1, 3, 5, 7 and 9, where 0 = 0% of total leaf area with disease spot, 1 = 0~10%, 3 = 10%~25%, 5 = 25~50%, 7 = 50~75%, and 9 = >75%. The formula for disease index was as follows:

$$\text{Disease index} = \frac{\text{Sum of (Disease class} \times \text{Number of leaves in that class)}}{(\text{Total number of leaves} \times \text{highest disease class})} \times 100$$

The criteria for the classification of disease resistance in vitro was assigned as: high resistance (HR): $0.0 < DI \leq 5.0$; resistance: $5.0 < DI \leq 10.0$; middle resistance (MR): $10.0 < DI \leq 30.0$; susceptible (S): $30.0 < DI \leq 50.0$; high susceptible (HS): $50.0 < DI \leq 100$ (Wang et al. 2007; Ji et al. 2013).

Isolation of total RNA, first strand cDNA synthesis, and qRT-PCR reaction

Total RNA was isolated from leaves using CTAB (cetyltrimethyl ammonium bromide) (Xing et al. 2010), dissolved in 30 μ L of RNase-free water, and stored at -80 °C. RNA was then assessed for quality and quantity using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) with RNase-free water as the zero control.

Single- stranded cDNA was prepared using primescript RT reagent kit with gDNA Eraser (cat#RR047A, Takara, URL:<http://www.takara-bio.com>) as per the manufacturer's protocol with RT Primer Mix. About 2 μ g of total RNA in a single 20 μ L reaction was quantitatively converted to single-stranded cDNA using standard thermal conditions (Deeplanaik et al. 2013).

The 20 μ L reaction mixture for the qRT-PCR experiment contained 10 μ L of SYBR *Premix Ex Taq* II (Tli RNaseH Plus) (2 $\times$) (cat#RR420A, Takara, Japan), 2 μ L of diluted cDNA, 10 μ M of each gene-specific primer and appropriate amount of sterile ddH₂O. PCR reactions were assembled in 0.2 mL, mMultiplate 96-well Unskirted PCR Plates MLP-series (Applied Biosystems, USA) and cDNA amplified in the CFX96 Real-Time PCR System (Applied Biosystems) to monitor DNA synthesis. Individual components of the reaction mixtures were standardized for 20 μ L volumes and carried-out in triplicate. The qRT-PCR thermal cycling program consisted of an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 20 s and extension at 65 °C for 15 s. Melting curve analysis was done for all the reactions and expression

level was calculated by $2^{-\Delta\Delta T}$ method (Schmittgen and Livak). The *18s rDNA* gene of apple was used as a reference for normalizing the expression data to measure the response of Md-*ASR* genes (Yue et al. 2010). To visualize the relative fold difference, all data were normalized based on setting up the relative expression level, the expression level of 0-point treatments for pathogen for two cultivars was set as 1, “above 1” and “below 1” were considered as up-, or down- regulation in *A. alternata* treatment (Wang et al. 2014).

Measurement of enzymatic activities and MDA content

POD activity was measured according to guaiac phenol colorimetric method at 470 nm. The change of 0.01 per minute in optical density value represents one unit of enzyme activity (U /g . min) (Koon et al. 1998a). The measurement of SOD activity was assessed by the NBT (nitroblue tetrazolium) method (Zucker and Milton 1965) and PPO activity was determined according to o-dihydroxybenzene method (Archer and Palmer 1975). CAT activity was measured based on Ultraviolet absorption method at 240 nm (Wang 2011), and the PAL activity was assayed by using the L-phenylalanine test (Zucker and Milton 1965). MDA was measured using a thiobarbituric acid test (Dhindsa and Matowe 1981).

SPSS 2.0 software was used to analyze experimental data. All experiments were carried-out with three biological replicates and three technical replicates per biological replicate. The differences between mean data values were determined using the Student's test ($P < 0.05$).

Results

Identification of *ASR* genes in apple genome

Five *ASR* related sequences were obtained from whole-genome sequences of *M. domestica*. They were *MDP0000253074*, *MDP0000136340*, *MDP0000435025*, *MDP0000564469* and *MDP0000128468*. By blasting against *Malus* EST database in NCBI, Md-*ASR1* has an identity of 100% with LOC103444308 (accession number: XM_008383247.1) and Md-*ASR5* has an identity of 100% with LOC103403063 (XM 008341881.1). After deleting erroneous insertion in the Phytozome gene annotations, the two new sequences of Md-*ASR1* and Md-*ASR5* were got and showed in supplementary files. According to the location of chromosome, we named the five genes Md-*ASR1*, Md-*ASR2*, Md-*ASR3*, Md-*ASR4* and Md-*ASR5* with their characteristics listed in Table 1. Each member of *ASR* gene family had at least two exons, ranging from 35 bp to 440 bp in length, and at least one intron from 39 bp to 1586 bp (Fig. 1B).

The length of the five *ASR* proteins ranged from 120 to 202 amino acids (AA). The molecular weights vary from 13, 223.38 kDa to 22, 266.7 kDa. According to the GRAVY and instability index, all were hydrophilic and unstable protein except Md-*ASR5*, which had an instability index of 38.10, lower than those of other four. Their theoretical PIs (isoelectric point) ranged from 5.48 to 6.41. Their secondary structures were mainly alpha helixes. All *ASR* proteins were relatively abundant in Glu, Ala, His, Lys and Gly residues (Table 1).

Every *ASR* protein contained one continuous ABA/WDS domain (Table 1). There were two high similarity sequence regions in these five proteins: one located in the N-terminus about 27 AA and another in the C-terminus about 80 residues (Fig. 2).

The five Md-*ASR* proteins had different motif structures. All Md-*ASR* members had motif 1, 2 and 3. Md-*ASR1* and 5 contained the motif 6 (Fig. 3). Motif 4 was only in Md-*ASR1*, 4 and 5. Only Md-*ASR3* did not contain motif 5.

Phylogenetic tree of the *ASR* genes in apple and in plant kingdom, and *Ks* and *Ka/Ks* values

The phylogenetic tree of five apple *ASR* genes was divided into two clades (Fig. 1A). One clade included Md-*ASR1*, Md-*ASR4* and Md-*ASR5* and the other contained Md-*ASR2* and Md-*ASR3* (Fig. 1A). The *ASR* genes from 18 species (Table 2) and the phylogenetic relationships are shown in Fig. 4. The five *ASR* genes of *Malus domestica* clustered together that formed a natural sub-cluster. The similar species-specific duplications were also detected in *Solanum lycopersicum*, *Populus trichocarpa* and *Selaginella moellendorffii*. The *ASRs* from monocotyledonous Gramineae species, *Zea mays* and *Oryza sativa*, each formed a separate cluster in the tree.

The *Ks* values of nodes were 1.51 (a), 0.05 (b) and 0.74 (c), respectively, in Md-*ASR* gene tree (Fig. 1A), which demonstrated that Md-*ASR2* and Md-*ASR3* were more likely produced by a most recent duplication compared with those in Md-*ASR1*, Md-*ASR4* and Md-*ASR5*. The ratio of *Ka/Ks* of *ASR2/ASR3* genes (2.07) was greater than those of pairs of *ASR1/ASR5* (0.67), *ASR1/ASR4* (0.47) and *ASR4/ASR5* (0.71), which indicated that *ASR2* and *ASR3* were under positive selection compared with the purifying selection on Md-*ASR1*, Md-*ASR4* and Md-*ASR5* (Fig. 1B).

Disease development in two cultivars after *A. alternata* f. sp. *mali* infection

After inoculation with *A. alternata* f. sp. *mali*, leaves developed spots as disease symptoms in both apple cultivars (Fig. 5). Leaves from ‘Golden Delicious’ had a disease index of 33.67, whereas leaves from ‘Starking’ had a disease index of 50.82. In both cultivars, no disease symptoms were observed on leaves inoculated only with agar plugs (controls) (Fig. 5A, B). In leaves, a small necrotic region developed on the upper surface around the inoculation point on ‘Golden Delicious’

leaves after 18 h (Fig. 5C). After 36 h (Fig. 5E), the lesions became clear with scant white fungal mycelium and enlarged after 72 h (Fig. 5F) with the surrounding tissues turning yellow and brown. In ‘Starking’, leaf tissue around inoculation points became soft and appeared water soaked without mycelium after 18 h (Fig. 5D). The spots enlarged and turned necrotic after 36 h (Fig. 5F). Leaf spots with abundant white mycelium, and the surrounding tissues turned necrotic after 72 h (Fig. 5H).

Differential expression of five Md-*ASR* genes in response to infection by *A. alternata* f. sp. *mali*

In ‘Golden Delicious’, *ASR1* gene was shown significant up-regulation at 18 h and not induced at 36 h and 72 h, but the other four genes were down-regulated after 18 h and 36 h compared to the expression level at 0 h. All *ASR* genes were not induced at 72 h relative to the 0 h time point. In ‘Starking’, expression levels of *ASR1*, *ASR2* and *ASR3* genes were significantly down-regulated at all sampling times, as marked in the Figure (Fig. 6B). While *ASR4* and *ASR5* genes were significantly down-regulated ($P < 0.05$) in both 18 h and 72 h, they returned to control levels at the intermediate 36 h time point. These results indicated that Md-*ASR* genes showed different response to *A. alternata* f. sp. *mali* infection in different time points in the two genotypes.

Influence of antioxidant enzymes activities and contents of MDA after *A. alternata* f. sp. *mali* infection

The activities of defense-related enzymes had similar trends in both cultivars (Fig. 7). The maximum activity of these five enzymes occurred in different stages between the two cultivars (Fig. 7). The activity of POD in ‘Golden Delicious’ increased significantly and reached to a maximum of about 180 U/g · min after 18 h, whereas a maximum activity of about 200 U/ g · min was detected in

‘Starking’ 36 h after inoculation (Fig. 7A). The activity of SOD in ‘Golden Delicious’ increased significantly between 0 h and 18 h after inoculation and had a maximum rate of about 250 U/ g at 18 h compared with the activity in of ‘Starking’, which peaked at about 200 U/ g at 18 h after inoculation (Fig. 7B). ‘Golden Delicious’ had a significantly increase in CAT activity at 18 h and 36 h after infection and reached to maximum value of 150 U/ g . min at 36 h. However, the activity in ‘Starking’ reached a maximum of about 140 U/g . min at 36 h after inoculation and was significantly higher than that in ‘Golden Delicious’ ($P<0.05$) (Fig. 7C). The activity of PAL significantly increased in ‘Golden Delicious’ at 18 h after inoculation with the maximum activity of 250 U/ g . min compared to the enzyme activity in ‘Starking’, which peaked at about 150 U/ g . min at 36 h after inoculation (Fig. 7D) and represented significant differences compared to ‘Golden Delicious’ at 18 h and 72 h ($P<0.01$). For PPO , activity in ‘Golden Delicious’ increased after 18 h with maximum value 140 U/ g . min, but ‘Starking’ had its peak level about 150 U/ g . min at 18 h after inoculation (Fig. 7E). We can infer that the five enzymes participated in the response to pathogen of infection in different patterns.

In ‘Golden Delicious’, the MDA contents increased significantly between 0-36 h and reached maximum level of about 20 $\mu\text{mol/g}$ after 36 h, whereas in ‘Starking’ , the peak value was about 17 $\mu\text{mol/g}$ after 18 h (Fig. 7F). After 36 h, the MDA content was a little higher in ‘Golden Delicious’ than that in ‘Starking’, but the difference was not significant ($P<0.05$). The MDA contents were significantly higher in ‘Golden Delicious’ compared to ‘Starking’ 18 h after inoculation. The result suggested that the damage in ‘Golden Delicious’ was heavier than in ‘Starking’.

Discussion

The *ASR* gene family has been studied for their roles in responses to different environmental stresses and during fruit ripening (Negri et al. 2008; Chen et al. 2011), but few studies have explored their functions in response to biotic stresses. In this study, we identified five members of *ASR* genes in apple, a most important fruit crop in the world that is susceptible to many diseases. Each Md-ASR proteins had two highly conserved regions, one with 27 amino acids in N-terminus and another with about 80 amino acids in the C-terminus with a ABA/WDS domain (PF02496), similar to that reported for the rice *ASR* genes (Bartels et al. 1992). All Md-ASRs are highly hydrophilic proteins, rich in His, Lys, Ala and Glu, but lacking in Cys and Thr. The physico-chemical characteristics of apple Md-ASR were also in accord with previous studies that members of the *ASR* family are different from each other in protein molecular weight (70-230), isoelectric point and expression pattern (Iusem et al. 1993).

The main predicted secondary structure of Md-ASR proteins is an alpha helix without a signal peptide, as previously indicated (Dong et al. 2009; Yang et al. 2013). In previous reports, no clear 3-D structure was found in ASR proteins; they are classified as disordered protein (intrinsically disordered protein, IDPs) by bioinformatics analysis (Sun et al. 2013).

The ratios of Ka/Ks , a molecular evolutionary measure of selection pressure (Kimura and Ohta 1974), suggest that three gene pairs of Md-ASRs have been under purifying selection and one gene pair (*ASR2* and *ASR3*) is under positive selection. Therefore, we could infer that *ASR2* and *ASR3* might have evolved faster in response to environmental pressures. Our results on comparison of apple and *ASR* genes from broad plant species suggest that Md-ASRs shared a common evolutionary origin based on conserved structural and sequence characteristics, such as amino acid similarities and conserved motifs. *ASR* genes emerged from *S. moellendorffii* and expansion was primarily

contributed by species-specific duplications (Frankel et al. 2006).

Alternaria alternata f. sp. *mali* causes Alternaria blotch in the apple host by producing a host-specific AM toxin (Harimoto et al. 2008) and different cultivars display variations of resistance/susceptibility (Li et al. 2011). In our study, ‘Golden Delicious’ showed slight and ‘Starking’ showed high susceptibility, respectively (Fig. 5). Our results for these two cultivars were similarly to those reported by (Chen et al. 2012; Duan 2012; Li et al. 2013), but different from those stated by (Zhang et al. 1994; Yin et al. 2013a; Zhang et al. 2014) in which ‘Golden Delicious’ was resistant to the pathogen. This may be explained by use of a different race of the pathogen and inoculation of leaves at a different stage of maturation (Johnson et al. 2000).

Cakir suggested that ASR dimers might function as part of a transcription-regulating complex involved in sugar and ABA signaling by means of a yeast one-hybrid approach (Cakir et al. 2003). Maskin put forwarded that tomato ASR proteins may act as downstream components of a common signal transduction pathway involved in responses to environmental signals based on a RT-PCR strategy under normal and stress conditions (Maskin et al. 2001). In this article, all five Md-*ASR* genes had different expression patterns in ‘Golden Delicious’ and ‘Starking’, and in a time course study of infection and disease development. The results indicate that some Md-*ASR* genes were more likely to be involved in responding to *A. alternata* f. sp. *mali* infection and disease progression than others, and possibly acted as downstream components of a common signal transduction pathway based on characterization of tomato ASR proteins (Maskin et al. 2001). Because Md-*ASR2* and Md-*ASR3* did not show significantly different response to *A. alternata* f. sp. *mali* infection and control, they may play roles in other physiological functions; for example, Carrari et al (2004) showed an *ASR* gene in *Vitis vinifera* was induced by sucrose and ABA, and that Vv-ASR protein

bound to the promoter of a grape putative monosaccharide transporter VvHT1.

SOD, POD and CAT are important antioxidant enzymes, and PAL and PPO are also the main SAR related enzymes in plant which activities are related to plant resistance, all involved in the defensive reaction against pathogenic agents (Mohammadi and Kazemi 2002). Increased SOD activity could enhance the ability of cells in leaf tissue to convert O^{2-} into H_2O_2 , whereas enhanced CAT, PPO and POD activities contribute to elimination of H_2O_2 (Yin et al. 2013b). Increase activities of these enzymes may account for the lower MDA levels observed in less susceptible cultivar ‘Golden Delicious’ following the *A. alternata* f. sp. *mali* treatment. In our experiments, the less susceptible ‘Golden Delicious’ has higher maximum values of CAT and PAL enzymatic activities than the highly susceptible cultivar ‘Starking’. In this work, the content of MDA in ‘Golden delicious’ was lower than that in susceptible ‘Starking’, suggesting that after *A. alternata* infection, ‘Golden delicious’ suffered less cell membrane damage than ‘Starking’. Lipid peroxidation is an indicator of oxidative stress and is estimated as MDA, the principal product of polyunsaturated fatty acid peroxidation (Sorahinobar et al. 2015). Our present results showed that the MDA content increased first, suggesting that lipid peroxidation might have been induced due to the production of H_2O_2 generated in response to pathogen infection and then decreased, suggesting that antioxidant enzymes (POD, SOD, CAT and PPO) played a role in elimination of H_2O_2 .

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Table 1 Physico-chemical characteristics of five *ASR* genes in *Malus domestica*

Gene	Md- <i>ASR1</i>	Md- <i>ASR2</i>	Md- <i>ASR3</i>	Md- <i>ASR4</i>	Md- <i>ASR5</i>
Phytozome Locus Name	MDP0000 253074	MDP0000 136340	MDP0000 435025	MDP0000 564469	MDP0000 128468
Chromosomal localization	9	10	10	10	16
Number of AA	202	133	120	182	200
PI	5.74	6.41	5.48	6	5.91
Mw(Kda)	22125.4	15081.86	13223.38	19799.16	22266.7
GRAVY	-1.421	-1.162	-1.171	-1.462	-1.433
Instability index	42.24	46.3	63.06	43.84	38.10
Number of domain ABA/WDS	1	1	1	1	1
Number of low complexity	3	0	1	3	1
Domain Length	78	80	80	80	78
Content of Glu (%)	15.3	14.3	15	13.2	14.5
Content of Ala (%)	8.9	12.8	15	8.2	7.0
Content of His (%)	10.4	16.5	10	11.1	12.0
Content of Lys (%)	8.4	13.5	13.3	8.2	9.5
Content of Gly (%)	16.8	6.8	7.5	14.8	13.5

Table 2 *ASR*-like genes among different species.

Plant species	Total number	Phytozome locus Name	length (bp)
<i>Malus domestica</i>	5	MDP0000128468	650
		MDP0000136340	1989
		MDP0000564469	1039
		MDP0000435025	1281
		MDP0000253074	1424
<i>Oryza sativa</i>	6	LOC_Os01g73250.1	1780
		LOC_Os01g72910.1	801
		LOC_Os04g34600.1	1234
		LOC_Os02g33820.1	1080
		LOC_Os11g06720.1	991
<i>Zea mays</i>	10	LOC_Os01g72900.1	702
		GRMZM2G009792_T01	1072
		GRMZM2G136910_T01	1381
		GRMZM2G052100_T01	1048
		GRMZM2G168552_T01	978
		GRMZM2G014797_T01	560
		GRMZM2G383699_T01	586
		GRMZM5G854138_T02	1095
		GRMZM2G044132_T02	1451
		GRMZM2G314075_T01	639
<i>Selaginella moellendorffii</i>	3	GRMZM2G057841_T01	626
		29098	321
		25835	719
<i>Fragaria vesca</i>	1	25856	2548
<i>Vitis vinifera</i> Genoscope	1	mrna08120.1-v1.0-hybrid	795
<i>Solanum lycopersicum</i>	5	GSVIVT01034550001	1064
		Solyc04g071610.2.1	1867
		Solyc04g071580.2.1	811
		Solyc04g071600.2.1	1615
		Solyc04g071590.1.1	803
<i>Populus trichocarpa</i>	3	Solyc04g071620.2.1	1683
		Potri.005G193600.1	1454
		Potri.005G193800.1	1130
<i>Lilium longiflorum</i>	1	Potri.005G193900.1	2151
<i>Ginkgo biloba</i>	1	AF077629.1*	1093
<i>Amborella trichopoda</i>	1	AY461715.1*	952
<i>Physcomitrella patens</i>	0	AmTr_v1.0_scaffold00040.48	652
<i>Chlamydomonas</i>	0	-	-

<i>reinhardtii</i>			
<i>Volvox carteri</i>	0	-	-
<i>Coccomyxa subellipsoidea</i>	0	-	-
<i>C-169</i>			
<i>Ostreococcus lucimarinus</i>	0	-	-
<i>Micromonas pusilla</i>	0	-	-
<i>CCMP1545</i>			
<i>Micromonas sp. RCC299</i>	0	-	-

“*” represents the Genbank ID of *ASR* gene from *Lilium longiflorum* and *Ginkgo biloba*.

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Figure 1 (A) Phylogenetic tree of ASR genes in apple genome: the a, b and c represent each of three duplication events with Ks. (B) Gene structures of the five ASR genes. Boxes and lines represent the exons and introns, and the numbers in the boxes and above thin lines indicate the length of exons and introns. The numbers with decimal represent Ka/Ks.

Figure 2 Comparison of amino acid sequences of five Md-ASR proteins. The residues in black box stand for the N-terminus of Md-ASR proteins, the gray box are residues of the C-terminus and the ABA/WDS domain.

Figure 3. Motifs of five Md-ASR proteins. (A) Motif structure of five Md-ASR proteins. Six different marks represented different predicted motifs. (B) Amino acid sequences of the six motifs.

Figure 4 Phylogenetic trees of ASR genes from different plants species. The amino acid sequences used to build up the phylogenetic tree were: *Malus domestica*; *Oryza sativa*; *Lilium longiflorum*; *Ginkgo biloba*; *Selaginella moellendorffii*; *Zea mays*; *Vitis vinifera*; *Fragaria vesca*; *Solanum lycopersicum*; *Populus trichocarpa*; *Amborella trichopoda*.

Figure 5 Leaf spots (red circle) in cultivar 'Golden Delicious' and cultivar 'Starking', infected by *Alternaria alternata* apple pathotype at 0 h (A, B), 18 h (C, D), 36 h (E, F) and 72 h (G, H).

Figure 6 Relative expression levels of Md-ASR genes in cultivar 'Golden Delicious' (A) and cultivar 'Starking' (B) after different infection time by *Alternaria alternata* apple pathotype. The whole experiment was repeated three times. Bars represent standard errors of the mean. Duncan's multiple ranges Test was used to determine differences. The significant statistical differences referred to $P < 0.05$ among the different treatment-times and the controls, and the same word stands for insignificant difference. The letters "a-c" distinguish values that are statistically different as indicated by Duncan's multiple range tests.

Figure 7 The changes of defense related enzymes and MDA contents after infection by *A. alternata* apple pathotype. The line with square represented cultivar 'Golden Delicious' and the line with diamond represented cultivar 'Starking'. A: POD; B: SOD; C: CAT; D: PAL; E: PPO; F: MDA. The error bars indicate standard deviations of triplicate experiments. Student's test was used to determine differences. The significant statistical differences referred to $P < 0.05$ with comparison between two genotypes at the same time point. *: $P < 0.05$;

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A

B

Primers of five *ASR* genes in apple for qRT-PCR

Gene name	Forward primer	Reverse primer
<i>18s</i>	GTTACTTTTAGGACTCCGCC	TTCCTTTAAGTTTCAGCCTTG
Md- <i>ASR1</i>	CCAATGATTATTCTGGTGAAGGACG	AAGTTCAGATTCATGAGTGGTGGTG
Md- <i>ASR2</i>	AGACAAACCCTCAGATACCCTCAG	GATGCTTGTGGTGCTTCTCCTC
Md- <i>ASR3</i>	CTACCCTCAGTCTGGCTA	GATGCTTGTGGTGCTTCT
Md- <i>ASR4</i>	ACCCTCAGTCTGGCTAC	GCTTCTCCTCCTTCTTG
Md- <i>ASR5</i>	GAAGACAAACCCTCAAACCTACCC	CAAATCCACCCGAACCAACT

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>Md-ASR1 (CDS)

ATGTCTGAAGAGAAACACCACCGCGGTCTCTCCACCACCACAAGGATGAAGACAAACCTCAGACTACCC
TCAGTCTGGCTACTCTGATGAAGGACGTCCCGGCGGCCTCGGTGGTGGTTACGGCGACACCAATGATTATTC
TGGTGAAGGACGTACCGGCGGCCTAGGTGGTGGCTATGGCGACACCAATGCTTATTCTGGTGAAGGACGTC
CCGGCGGCTATGGCGGCTATAACGAGACCACTGCTTACTCTGAGGAAAGAGTTGAAAGACCCGGCGGTGG
CCGGTACAGTGAGACTACTGCGGCATATGGCAGCACCACCACTCATGAATCTGAACTTGATTACAAGAAGGA
GGAGAAGCACCACAAGCATCTCGAGCACCTCGGCGAGGCCGGTGTAGCTGCTGCCGGCGCTTTTGCCTTG
CATGAGAAGCACAATGAAAAGAAAGACCCAGAGCATGCCACAGGCACAAGATAGAAGAGGAGATTGCTG
CAGCAGCTGCAGTTGGTTCGGGTGGATTTGCCTTCCATGAGCATCATGAGAAGAAAGAGACAAAGGAAGA
AGAGGAAGAGGCTTATGGAAAGAAGAAGCACCACCACTTCTAG

> Md-ASR1 (202)

MSEEKHHRGLFHHHKDEDKPSDYPQSGYSDEGRPGGLGGGYGDTNDYSGEGRGTGGLGGGYGDTNAYSGEGR
PGGYGGYNETTAYSEERVERPGGGRYSETTAAYGSTTTHESELDYKKEEKHHKLEHLGEAGVAAAGAFALHEK
HNEKKDPEHAHRHKIEEEIAAAAAGVSGGFAPFHEHHEKKETKEEEEEAYGKKKHHHF

> Md-ASR5 (CDS)

ATGTCTGAAGAGAAGCACCATCGCGGTCTCTCCACCACCACAAGGATGAAGACAAACCTCAAACCTACCTC
CAGTCTGGCCACTTTGATGAAGGACATCCTAGTGGCCTCGGTGGTAGTTACGGTGACACCAATGATTATTCT
GGGTGGAGGACGTACCTGCGGCCTAGGTGGTGGCTACGGCGACACCAATGCTTATTCTGGTGAAGGACGT
CCCGGCGGCTATAGTGGCTATAACGAGACCACTACTTACTCTGAGGAAAGAGTTGAAAAACCCGAAGGTGG
CCGGTACAGTGAAACCACGGCGGCATATGGCAGCACCACCACTCATGAATCTGAACTTGATTACAAGAAGG
AGGAGGAGCACCACAAGCATCTCAAGCACCTCGACAAAGTCGGTGTGGCTGCTGCCGGCGCTTTTACCTTG
CATGAGAAGCACAATGAAAAGAAAGACCCAGAGCATGCCACAGGCACAAGATAGAAGATGAGATTGTTG
CAGCAGCTGCAGTTGGTTCGGGTGGATTTGCTTTCCATGAGCATCATGAGAAGAAAGAGACAAAGGAAGA
AGAGGAAGAGGCTCATGGAAAGAAGAAGCATCACCACCTTCTAG

>Md-ASR5 (200)

MSEEKHHRGLFHHHKDEDKPSNYPQSGHFDEGHPSGLGGSYGDTNDYSGRTCGLGGGYGDTNAYSGEGRPG
GYSGYNETTTYSEERVEKPEGGRYSETTAAYGSTTTHESELDYKKEEHHKHLKHLKVGVAAGAFTLHEKHNE
KKDPEHAHRHKIEDEIVAAAAGVSGGFAPFHEHHEKKETKEEEEEAHGKKKHHHF