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Comparisons of induced pathogenesis-related proteins in resistant and susceptible apple cultivars in response to inoculation of the pathogen *Marssonia coronaria*

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Abstract

Differential induction of the enzyme activity of seven pathogenesis-related proteins: chitinase (CHT), phenylalamine ammonia lyase (PAL), superoxide dismutase (SOD), polyphenol oxidase (PPO), peroxidase (POD), catalase (CAT) and malondialdehyde (MDA) from leaf extracts was characterized in both resistant 'Qinguan' and susceptible 'Fuji' apple cultivars at 0, 4, 8, 16, 24, 48, 72 and 96 h post-inoculation (hpi) with the pathogen *Marssonina coronaria*. Activity of all seven enzymes significantly increased in the resistant and the susceptible cultivars after inoculation with the pathogen ($P < 0.05$). However, activity increment after inoculation (AIAI) was very different among the seven proteins, ranging from 52 to 173% in 'Qinguan' and 34 to 137% in 'Fuji'. AIAI of the seven enzymes in both cultivars had either one or two peaks at 16 hpi (CHT and PPO), 24 hpi (CAT, SOD, POD, PAL-'Fuji' and MDA), 48 hpi (PAL-'Qinguan' and CHT-'Fuji') or 72 hpi (CHT-'Qinguan'). Correlation analysis showed that expression of specific enzymes was significantly correlated ($P < 0.05$) with the other enzymes studied within each apple cultivar. Results from a principal coordinate analysis showed that CHT, SOD, PAL and MDA were associated with one another in 'Qinguan', whereas PPO, POD, SOD and CAT were associated in 'Fuji'. A *t*-test showed that the AIAIs of all seven enzymes were significantly different between the two cultivars ($P < 0.05$). The Mantel test showed that co-correlation coefficients of AIAI of the seven enzymes on the whole was non-significant between 'Qinguan' and 'Fuji' ($R_{xy} = -0.318$, $P = 0.060$). Thus, both AIAI and the association pattern among pathogenesis-related proteins on the whole may be two important indicators that are contributing to resistance in apples in response to inoculation of *M. coronaria*. This study adds to our knowledge of apple responses to *M. coronaria* and provides information to potentially control this disease in the future.

Marssonina coronaria (Ellis and J.J. Davis) is the causative pathogen of Marssonina blotch, a wide-spread fungal disease in apples occurring in North America, Oceania and Asia (Lee et al., 2011; Zhou et al., 2012). This disease causes heavy losses in the apple industry world-wide, especially in Eastern Asian countries (Sharma et al., 2006; Shou and Li, 2009; Lee et al., 2011; Zhou et al., 2012), because the pathogen can lead to severe defoliation from early summer until late autumn (Sharma et al., 2006; Shou and Li, 2009). 'Fuji' and 'Qinguan' are two important apple cultivars grown in China, accounting for 65% and 10% of apple production in the country, respectively. 'Fuji' is very susceptible to *Marssonina coronaria* while 'Qinguan' is resistant to this pathogen (Li et al., 2012).

Plant responses to pathogens are very complex. This interaction involves a number of enzymes and metabolic pathways. Enzymes with direct or indirect roles in antimicrobial activity include phenylalanine ammonia lyase (PAL), chitinase (CHT), superoxide dismutase (SOD), catalase (CAT), peroxidases (POD), and polyphenol oxidases (PPO) (Boller and Meins, 1992; Aglika, 2005; van

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Loon et al., 2006). Accumulation of these enzymes was observed in response to microbial attack in several host-pathogen systems (Boller and Meins, 1992; Scandalios, 1993; Milosevic and Slusarenko, 1996).

There have been few reports that compare patterns of induction of protective enzymes in response to attack by *Marssonina coronaria* in apple (Zhou et al., 2012). A survey of host responses to infection would add to the knowledge of the molecular mechanisms involved with host-pathogen systems, and thus may indicate possible control options for this disease. This research characterized the differential induction of seven protective enzymes in both the resistant cultivar 'Qinguan' and in the susceptible cultivar 'Fuji' in response to inoculation with *Marssonina coronaria*.

Materials and Methods

Plant materials. Both resistant 'Qinguan' and susceptible 'Fuji' apple trees (*Malus*×*domestica*) were obtained from the Apple Experimental Research Orchard, Northwest A&F University, Yangling, Shaanxi, China. The trees were four years old, grafted on 'M.26' rootstock and planted in the orchard at a spacing of 1.5 × 2.5 m.

Fungal pathogen and artificial inoculation. Natural ascospores were collected from ten-year-old 'Fuji' apple trees at the Apple Experimental Research Orchard, Horticultural Farm, Northwest A&F University, Yangling, Shaanxi, China from late August to mid-October, when symptoms of Marssonina blotch peak in Yangling (Li et al., 2012), and purified using monoascoporic methods (Torres et al., 2012). The most virulent strain, which showed the most severe symptoms in leaves in *in vivo* inoculation tests, was chosen for propagation *in vitro*. Conidia were harvested after 10 to 12 weeks of propagation on potato dextrose agar (PDA) culture medium at 22±1°C, a light cycle of 12 h/12 h and a light intensity of 5000 lux in a fluorescent lamp illumination box (MGC250BP2, Shanghai, China) (Yu, 2012). A suspension was prepared using sterile distilled water. The concentration of both conidia and mycelial fragments in the suspension was estimated using a hemocytometer and the concentration adjusted to 10^6 propagules·mL^{-1}. The upper third or fourth young leaves in 'Qinguan' and 'Fuji' apple shoots were inoculated with the mycelial/conidium suspension *in vivo* on warm days during late May or early June. Leaves treated with sterile distilled water were used as controls. Leaves treated with either the mycelial/conidium suspension or with sterile distilled water were immediately covered with plastic bags to maintain moisture and to prevent infection from other pathogens. Leaves were collected at 0, 4, 8, 16, 24, 48, 72 and 96 h post-inoculation (hpi). The post-inoculation times were chosen for collection of samples according to previous observations (Panina et al., 2005; Sarowar et al., 2011; Zhou et al., 2012; Koche and Choudhary, 2012; Torres et al., 2012). All samples were frozen immediately in liquid nitrogen and stored at -80°C.

Enzyme extraction and assays. Leaf tissue (0.5 g) was ground in liquid nitrogen and was then homogenized in a 5.0 mL extraction buffer that was specific for each enzyme.

Chitinase (CHT) activity. CHT activity was measured using colloidal chitin (Promega, USA) as a substrate following Boller et al. (1985). The extraction buffer contained 20 mM sodium acetate buffer (pH 5.0), 0.7 mM β-mercaptoethanol and 0.4% PVP-40. The extraction mixture was centrifuged at 12000 rpm for 15 min at 4°C. The reaction mixture consisted of 0.4 mL enzyme extract, 0.4 mL colloidal chitin (1.0 mg·mL^{-1}), and 0.4 mL 20 mM sodium acetate buffer. The blank solution contained only 0.4 mL colloidal chitin (1.0 mg·mL^{-1}) and 0.4 mL 20 mM sodium acetate buffer. The mixture was incubated at 37°C for 3 h and then at 98°C for 5 min following which 1.0 mL paradime thylaminobenzaldehyde (DMAB) and 1.0 mL acetic acid were added to the reaction mixture. The mixture was incubated at 37°C for 30 min and was then centrifuged at 12000 rpm for 5

min at 4°C. The supernatant was used for the colorimetric determination of N-acetylglucosamine at 585 nm. A standard curve was generated using N-acetyl glucosamine solution at concentrations ranging between 0 and 150 µM.

Phenylalamine ammonia lyase (PAL) activity. PAL activity was assayed using *l*-phenylalanine as a substrate for determining the amount of production of *trans*-cinnamic acid using the modified protocol of Thangavelu et al. (2003). The extraction buffer contained 0.1 M sodium borate (pH 8.8) and 0.4 % g PVP 40. The homogenate was centrifuged at 20000 ×g for 30 min. The assay mixture contained 0.5 mL enzyme extract, 3.0 mL 50 mM extraction buffer (pH 8.8), and 0.5 mL of 20 mM *l*-phenylalanine. The mixture was incubated at 37°C for 60 min and the reaction was terminated by adding 0.1 mL of 6M HCl. The sample absorbance was measured at 290 nm. The blank had the same constituents except for the enzyme extract. A standard curve was generated using transcinnamic acid at concentrations between 0.01 and 0.1 mM (Thangavelu et al., 2003).

Antioxidant enzyme activities. The same extraction buffer containing 100 mM potassium phosphate, pH 7.8, 1.0 mM EDTA, 1% Triton X-100, 15% glycerol and 0.4% PVP-40 (Ni et al.,1996) was used for extraction of superoxide dismutase (SOD), catalase (CAT), peroxidases (POD) and polyphenol oxidases (PPO).

Total SOD activity was assayed according to Roth and Gilbert (1984). The 1.0 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 100 µM EDTA, 20 µL enzyme extracts and 10 mM pyrogallol. Enzyme activity was calculated by monitoring the reaction mixture for 120 s (at 60 s intervals) at 420 nm.

Total CAT activity was determined by the rate of reduction of H_2O_2 according to the method of Aebi (1984). The 1.5 mL reaction mixture consisted of 0.05 M phosphate buffer (pH 7.0), H_2O_2 (3.0%) and 40 µL enzyme extract. The decrease in H_2O_2 was followed by a decline in A_{240}, and the activity was estimated using a molar absorption coefficient of 40 $mM^{-1} \cdot cm^{-1}$ for H_2O_2.

POD activity was determined according to the method of Lin and Kao (1999). The 3.0 mL reaction mixture contained 50 mM phosphate buffer (pH 5.8), 7.2 mM guaiacol, 11.6 mM H_2O_2 and 0.1 mL enzyme extract. One unit of guaiacol was defined as the amount of enzyme needed to produce 1.0 µM tetraguaiacol$\cdot min^{-1}$.

PPO activity was determined by the rate of production of quinone, as indicated by an increase in absorbance at 400 nm, using a modified procedure described by Fehrmann and Dimond (1967).

Malondialdehyde content. The level of lipid peroxidation was measured based on thiobarbituric acid reactive substances (TBARS), using the method of Heath and Packer (1968) and Aghaleh et al. (2011). The extraction buffer contained 0.1% trichloroacetic acid (TCA) and 0.7 mM β-mercaptoethanol. The 5.0 mL reaction mixture contained 1.0 mL enzyme extracts and 4.0 mL of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 98°C for 20 min and quickly cooled in an ice bath and then centrifuged at 10,000 ×g for 20 min. The absorbance of the supernatant was recorded at both 532 and 600 nm and the absorption value at 600 nm was subtracted from the 532 nm value. The concentration of MDA was estimated using an extinction coefficient of 155 $mM^{-1} \cdot cm^{-1}$.

Statistical analyses. Three separate experiments were performed, with leaves being independently inoculated on different days within the study, where leaves were randomly chosen for inoculation from different shoots and trees. Each experiment had three replicates. For each time point in each experiment, three young leaves randomly collected from three trees were mixed for extraction of enzymes.

Analyses of variance (ANOVA) was performed for activity of seven enzymes between treated and control samples in 'Qinguan' and 'Fuji' apples at eight post-inoculation sam-

pling times (df = 1, 14). Correlation analyses were used to estimate correlation coefficients of the activity increment after inoculation (AIAI = treated values – control values) between pairs of seven enzymes within 'Qinguan' or 'Fuji' apple after inoculation of *M. coronaria* (n = 8 post-inoculation sampling times). t-tests were performed to compare AIAI of each enzyme between 'Qinguan' and 'Fuji' after inoculation with *M. coronaria* (n = 8 post-inoculation sampling times). Correlation analyses were performed to estimate correlation coefficients of AIAI of each enzyme between 'Qinguan' and 'Fuji' after inoculation with *M. coronaria* (n = 8 post-inoculation sampling times). ANOVA, correlation analyses and t-tests were performed using SAS Software (SAS Institute Inc., Cary, NC). Principal coordinate analysis (PCoA) was used to depict potential associated patterns in AIAI of pairs among the seven enzymes (21 pairs) in each of the two cultivars. A Mantel test was used to estimate the correlation coefficients of AIAI of seven enzymes between 'Qinguan' and 'Fuji' overall. Both PCoA and Mantel tests were performed using 'GenAlEx 6' software (Peakall and Smouse, 2006).

Results

The activity of seven enzymes significantly increased in both 'Qinguan' and 'Fuji' after inoculation of the pathogen *M. coronaria* ($P < 0.05$; Fig. 1, Table 1). In both cultivars, in most cases, the enzyme activity increased after inoculation to a peak and then declined (Fig. 1). AIAI of each enzyme in the two cultivars had one or two peaks at 16 hpi (CHT and PPO), 24 hpi (CAT, SOD, POD, PAL-'Fuji' and MDA), 48 hpi (PAL-'Qinguan' and CHT-'Fuji') or 72 hpi (CHT-'Qinguan') (Fig. 1). Compared to the controls, the percentage of AIAI of the seven enzymes at the peak ranged from 52 to 173% and from 34 to 137% for 'Qinguan' and 'Fuji', respectively.

The correlation analysis for compari-

Table 1. ANOVA for comparison of enzyme activity between treated and control samples in 'Qinguan' and 'Fuji' apples for seven different enzymes and eight post-inoculation sampling times (df = 1, 14).

F and P	Enzymes						
	CHT	SOD	CAT	POD	PPO	PAL	MDA
F_Q	42.11	27.83	54.98	52.66	40.08	53.65	22.22
P_Q	7.85E-08	4.33E-06	3.73E-09	6.26E-09	1.33E-07	5E-09	2.67E-05
F_F	32.41	22.34	30.55	29.46	27.60	24.97	14.69
P_F	1.1E-06	2.57E-05	1.89E-06	2.63E-06	4.65E-06	1.07E-05	0.000417

F_Q and P_Q are F and P values for 'Qinguan'; F_F and P_F are F and P values for 'Fuji'.

Table 2. Correlation coefficients of AIAI of enzymes between pairs of seven enzymes in 'Qinguan' after inoculation of *M. coronaria* (n = 8 post-inoculation sampling times).

	PAL	POD	CHT	MDA	SOD	CAT
POD	0.846*					
CHT	0.474	0.591				
MDA	0.747*	0.797*	0.154			
SOD	0.859*	0.902**	0.397	0.948		
CAT	0.755*	0.935**	0.386	0.928**	0.937**	
PPO	0.410	0.765*	0.348	0.711	0.654	0.867*

*, $P < 0.05$; **, $P < 0.01$ (Spearman).

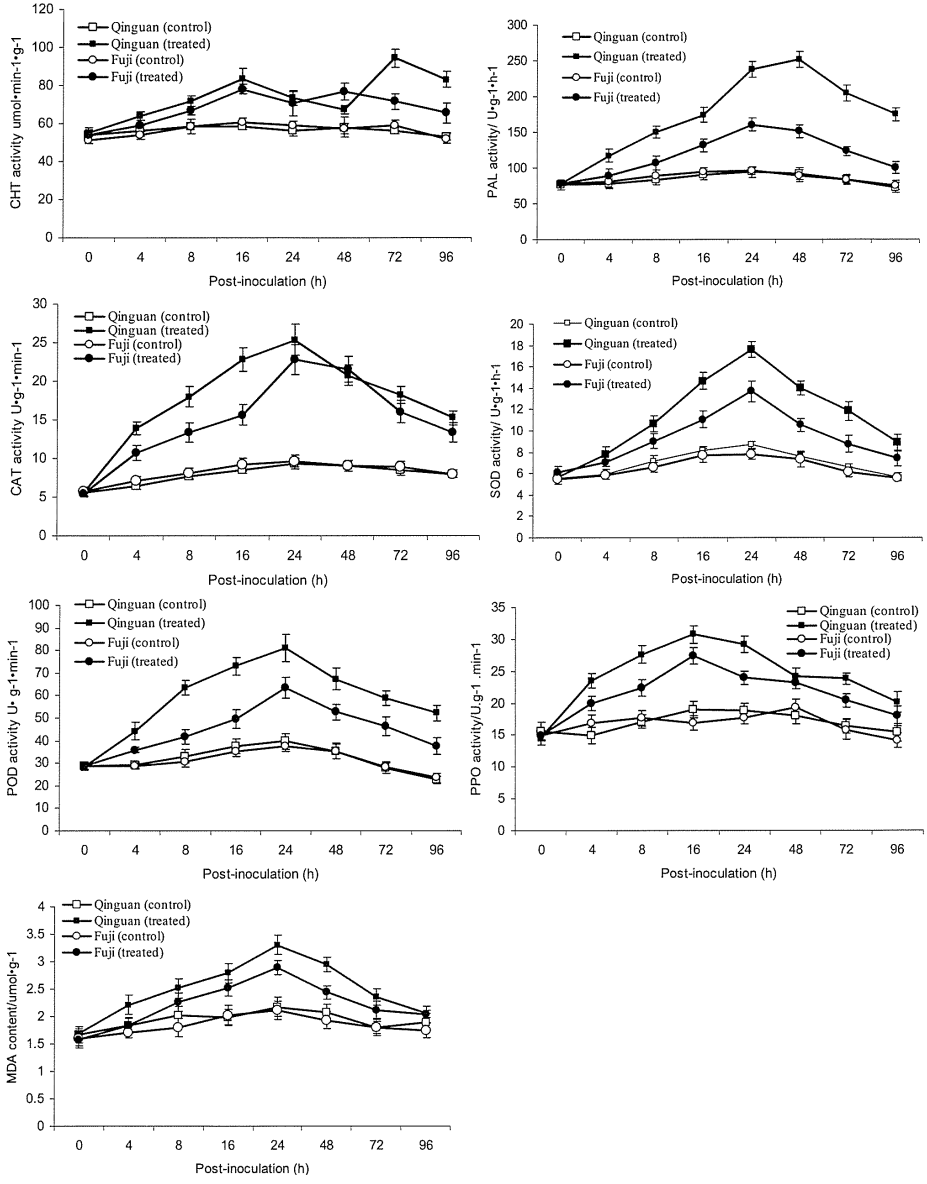


Fig. 1. Activity of seven enzymes in leaf extracts of 'Qinguan' and 'Fuji' collected at different time intervals following inoculation with *M. coronaria* or in non-inoculated (control) plants. Data shown are the mean ± standard deviation from three replicates among three independent experiments.

sons of pairs of AIAI of the seven enzymes throughout the eight sampling times in each cultivar showed that eleven (48%) of twenty one correlation coefficients were significant in both 'Qinguan' and 'Fuji' (Tables 2 and 3), indicating that expression of certain en-

Table 3. Correlation coefficients of AIAI of enzymes between pairs of seven enzymes in 'Fuji' after inoculation of *M. coronaria* (n = 8 post-inoculation sampling times).

	PAL	POD	CHT	MDA	SOD	CAT
POD	0.914**					
CHT	0.788	0.675				
MDA	0.855*	0.896*	0.651			
SOD	0.875*	0.915**	0.538	0.953**		
CAT	0.974*	0.916**	0.723	0.883**	0.881**	
PPO	0.509	0.584	0.635	0.697	0.626	0.464

*, $P < 0.05$; **, $P < 0.01$ (Spearman).

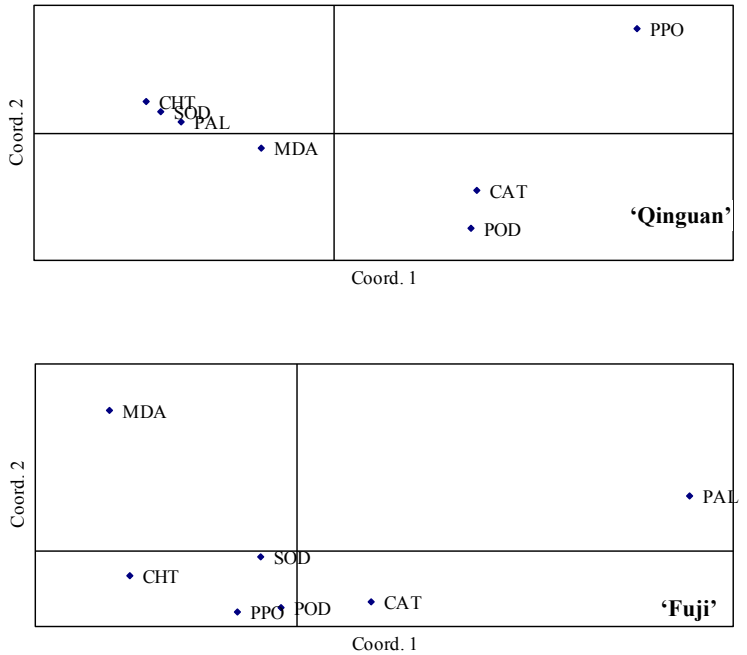


Fig. 2. Potential assocation patterns among seven enzymes in 'Qinguan' and 'Fuji' apple cultivars based on principal coordinate analysis. The cumulated variation of AIAI of seven proteins in the first two coordinates was 79.1% and 73.1% in 'Qinguan' and 'Fuji', respectively.

zymes was significantly correlated with other enzymes in response to inoculation with *M. coronaria*. However, only nine of eleven pairs of comparisons with significant correlation coefficients were the same between the two cultivars (Tables 2 and 3).

PCoA results indicated that CHT, SOD, PAL and MDA were closely associated with one another in 'Qinguan' and that PPO, POD, SOD and CAT were closely associated with one another in 'Fuji' (Fig. 2).

The t-test for comparisons of AIAI for each individual enzyme between 'Qinguan' and 'Fuji' showed that the AIAI for all seven enzymes was significantly different between 'Qinguan' and 'Fuji' (Table 4; $P < 0.05$). The correlation coefficients of AIAI for six enzymes (i.e., excluding CHT) were sig-

nificant between 'Qinguan' and 'Fuji' when single enzymes were used for comparisons (Table 4; $P < 0.05$). However, the Mantel test showed that co-correlation coefficients for AIAI of the seven enzymes overall was non-significant between 'Qinguan' and 'Fuji' (R_{xy} = - 0.318, $P = 0.060$). This indicates that the co-correlation pattern of the seven enzymes overall was highly significantly different between the resistant and the susceptible cultivar.

Discussion

Differential expression of pathogenesis-related (PR) proteins in apples was observed in response to *Marssonina coronaria* in this study as previously reported in other pathogen-host systems. Several (PR) proteins were differentially expressed in the *M. domestica*-resistant cultivar 'Remo' (Gau et al., 2004), and some PR proteins were found in *HcrVf2*-transformed apple plants in response to inoculation with *V. inaequalis* (Paris et al., 2008). In a previous study, several mRNAs that were differentially expressed in the resistant cultivar 'Qinguan', in response to inoculation with *M. coronaria*, were found to be highly homologous with PR proteins (Zhou et al., 2012). In this study, we further compared differential patterns of expression of seven PR proteins between a susceptible and a resistant cultivar.

The AIAI of seven enzymes as revealed by the *t*-test (Table 4) was significantly different between 'Qinguan' and 'Fuji', suggesting that these seven enzymes may play important roles in the responses of apples to inoculation with *M. coronaria*. CHT may act directly by blocking growth of fungal hyphae and so retard the pathogen from invading intercellular spaces, or elicit other defense reactions in the host (Boller et al., 1983; Loon, 1985; Kurosaki and Tashiro, 1988; Barber et al., 1989; Kang et al., 1998; Campbell and Reece, 2005; Mukherjee et al., 2006). PAL plays an important role in regulation of the synthesis of phenolic compounds, such as precursors in lignin biosynthesis, or act as antimicrobial compounds (Retig, 1974; Hammerschmidt and Kuć,1982; Mozzetti et al.,1995; Shiraishi et al.,1995; De Ascensao and Dubery 2000). SOD and POD activities have often been used as important indices to indicate plant responses to stress (Scandalios, 1993; Mehdy, 1994; Milosevic and Slusarenko, 1996). CAT catalyzes H_2O_2 down into H_2O and O_2, and can thus reduce damage to the plant caused by excessive H_2O_2 (Scandalios, 1993; Mehdy, 1994; Milosevic and Slusarenko, 1996). PPO catalyzes lignin into protective quinone compounds that can protect plant cells from further invasion by pathogens (Mayer, 2006). Successive membrane lipid peroxidation can cause membrane permeability to increase that can lead to significant electrolyte leakage; MDA content can be directly related to the degree of damage to cell membranes (Scandalios, 1993; Mehdy, 1994; Milosevic and Slusarenko, 1996).

Accumulation of active oxygen and peroxide radicals can be a significant issue in plants when they are attacked by pathogens: such molecules can damage the cells of pathogens, but they can also kill plant cells

Table 4. Correlation coefficients and *t*-test for comparisons of AIAI of each enzyme between 'Qinguan' and 'Fuji' after inoculation of *M. coronaria* ($n = 8$ post-inoculation sampling times).

r and *P*	Enzymes						
	CHT	SOD	CAT	POD	PPO	PAL	MDA
r	0.391	0.825	0.798	0.804	0.774	0.885	0.695
P_r	0.058	6.96E-07	2.89E-06	2.11E-06	9.02E-06	8.6E-09	0.000163
P_t	0.017	2.15E-05	6.04E-05	6.26E-08	7.4E-05	1.63E-08	0.00449

r represents the correlation coefficients for AIAI for each enzyme over eight sampling times between 'Qinguan' and 'Fuji'; P_r is the *P* value for *r*; P_t is the *P* value for the *t*-test for AIAI of each enzyme over eight sampling times between 'Qinguan' and 'Fuji'.

and typically form a necrotic area around fungi that are establishing in the plant to prevent hyphae from spreading further. However, excessive accumulation of active oxygen and peroxide radicals may cause undesirable damage to plant cells. Thus, it is not surprising that the activity of antioxidant enzymes has had a positive correlation with disease resistance in a number of studies (Scandalios, 1993; Mehdy, 1994; Milosevic and Slusarenko, 1996). However, some other reports have shown that there was little correlation or even a negative relationship between the two factors (Mehdy, 1994). In our study, all seven enzymes significantly increased in both 'Qinguan' and 'Fuji', and activity was significantly different between these two cultivars, indicating that the activities of these seven enzymes may reflect resistance of the apple plants to the pathogen *M. coronaria*.

The AIAI of seven enzymes as assessed by the *t*-test (Table 4) and by the overall co-correlation pattern (R_{xy} = - 0.318, P = 0.060; Fig. 2) were significantly different between 'Qinguan' and 'Fuji'. These results suggest that both activity increment and the co-correlation pattern among PR enzymes may be two important indicators of resistance. However, the key enzymes and the related metabolic pathways that are specifically involved in these plant-pathogen interactions need further study. The conclusions from this study also need further confirmation using additional resistant and susceptible apple cultivars to advance the understanding apple responses to infection by *M. coronaria*.

High moisture levels and high temperature conditions facilitate the growth and development of *M. coronaria*. Symptoms in apples caused by *M. coronaria* are epidemic under these conditions (Lee et al., 2011; Li et al., 2012). In this study the responses in apples peaked within one or two days after the plants were inoculated with *M. coronaria* under high moisture conditions with the bagging treatment that was used. This type of response has been observed previously in a number of other studies (Panina et al., 2005; Torres et al., 2012; Sarowar et al., 2011). Our study suggests, therefore, that measures should be taken to control this disease immediately after any significant rain event and before the time which the fungal infection may have fully developed.

Host responses to pathogens are complex and often involve multiple, networked factors. Principal coordinate analysis is increasingly used for population genetics (Peakall and Smouse, 2006) but it does not appear to have been used previously for defining potential associations in networked patterns involving pathogen-related enzymes. The applicability and value of this methodology has been shown in other studies (Peakall and Smouse, 2006). In our study, the cumulated variation of AIAI for the seven enzymes in the two PCA coordinates was 79.1% and 73.1% for 'Qinguan' and 'Fuji', respectively, indicating that the separate patterns in the plots may present basic association features of the seven PR enzymes in each of these two cultivars. The results from this study may indicate useful options for further study of the key enzymes and related pathways involved in plant disease resistance.

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About the Cover:

Freshly harvested green 'Nonpareil' almonds grown by Alan Townsend of Fat Uncle Farms in Wasco, north of Bakersfield in California's San Joaquin Valley. *(Photograph: David Karp)*

