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Investigation of the Effects of Apple Trees Infection by *Erwinia amylovora* on the Expression of Pathogenesis-Related Proteins Homologous to Allergens

Nasser Beikzadeh*[1], Abdol-Reza Varasteh[2]

Abstract

Background: Pathogenesis-related (PR) proteins are induced in response to biotic and abiotic stresses. Some plant proteins, including Mal d 1, Mal d 2, and Mal d 3 in apple, are allergens. In this study, the effects of *Erwinia amylovora* infection of two apple cultivars, Red and Golden Delicious, on the expression of PR proteins homologous to Mal d 1, 2, and 3 were investigated.

Methods: In natural conditions trees with or without disease symptoms were sampled. In addition, seeds of the cultivars were grown in a greenhouse and seedlings were examined in three groups: 1) those inoculated by *E. amylovora*, 2) those inoculated by sterilized distilled water, and 3) uninoculated. Real-time PCR was used to determine expression of the Mal d 1, 2, and 3 genes (*Mal d 1*, *2*, and *3*) in infected and uninfected samples. Statistical analyses were performed using SPSS and graphs were produced by Excel. P values < 0.05 were considered significant.

Results: The analysis of variance showed that in natural conditions the effect of infection on the mean relative expression of *Mal d 2* and *3* was significant, and more so in Red than in Golden Delicious. The analysis of variance of the greenhouse samples showed that the effect of infection on the mean relative expression of *Mal d 1*, *2*, and *3* in both cultivars was significant.

Conclusions: Our results suggest that Mal d 2 is more related to plant defense than Mal d 1 or Mal d 3, and is more highly expressed in *E. amylovora*-resistant than in *E. amylovora*-sensitive cultivars.

Keywords: Allergens, *Erwinia amylovora*, Homologous, Pathogenesis-related proteins.

Introduction

Plants defend themselves against biotic and abiotic stress in various ways, one of which is production of pathogenesis-related (PR) proteins (1). To date, many plant allergens with amino acid sequences similar to these proteins have been identified (2). Currently, four important allergens have been reported in apple (3). Three of these allergens, Mal d 1, Mal d 2, and Mal d 3, are homologous to PR proteins (2). The bacterium *Erwinia amylovora* is the causal agent of fire blight disease. One response of plants to this disease is increased expression of PR proteins (4). Infection by *E. amylovora* may affect allergen-homologous PR protein abundance. Studies with in El-star and Topaz apples showed that the Mal d 1 concentration was very high in the *E. amylovora*-infected fruits (4). Because the PR protein concentration is directly related to the degree of infection (5), and differences exist between the strains causing this disease (6), allergen production may differ between areas due to infection by different strains of the bacterium.

In Iran, no studies have yet been reported regarding the effect of biotic agents on the expression of PR proteins homologous to apple allergens. In this

1: Khorasan Razavi Agricultural and Natural Resources Research and Education Center, AREEO, Mashhad, Iran.
2: Varastegan Institute for Medical Sciences, Mashhad, Iran.
***Corresponding author: Nasser Beikzadeh; Tel: +98 51 38717122, Fax: +98 51 8717142; E-mail: beiczadeh@gmail.com.**
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study we analyzed PR protein gene expression in fire blight-infected Golden and Red Delicious apple trees. The reason for choosing these two cultivars was that they have been extensively used in breeding programs (7) and Red and Golden Delicious are reported to be moderately resistant and moderately susceptible, respectively, to this disease.

Materials and methods

Samples of 0.1 g were prepared from the parts of the apple trees with and without infection symptoms (8). To isolate the bacteria from the samples in the CCT, King's B, and Levan media extracts were diluted 1:10 and 1:100 in 10 mM PBS (pH 7.2) and streaked on plates containing the growth media. The plates were incubated at 25 °*C* for 48 to 72 hours. Gram stains, and biochemical (such as sugar oxidation) and pathogenicity tests were used to identify *E. amylovora* (9, 10).

To investigate the effect of the infection in greenhouse-grown plants on the expression of PR proteins homologous to allergens, seedlings of the two cultivars were raised in a greenhouse and 20 seedlings of each cultivar were infected with an *E. amylovora* overnight culture on King's B medium by cutting the upper leaves of the seedlings with a blade containing the bacterium. Twenty uninfected seedlings and 20 seedlings treated with sterile distilled water were included as negative controls (4).

To extract total RNA, five seedlings from each treatment of each cultivar and 10 leaves from each seedling were chosen. The leaves from each seedling were combined into a single sample and total RNA was extracted (11). Five trees from each infected and uninfected cultivar in natural conditions were also chosen. Skin and flesh of 10 fruits from each tree were combined and considered as a single sample (12, 13). Total RNA was extracted from each sample. The cDNA was synthesized using an Easy^{TM} cDNA Synthesis Kit according to the manufacturer's instructions (Pars Toos Zist Fanavar, Mashhad, Iran) and *Mal d 1, 2* and *3* were amplified by PCR and real-time PCR with specific primers (Tables 1 and 2).

Table 1. Primers used in PCR

Genes	Primers	Sequence (5' – 3')	PCR product size (bp)	Reference
Mal d 1	M1For	TCCACCACCAAGATTGTTCA	420	3
	M1Rev	TGGCCCTTGAGGTAGCTCTC		
Mal d 2	M2For	AAAGCTAGCCGATCAGTGGA	478	3
	M2Rev	GAGGGCACTGCTTCTCAAAG		
Mal d 3	M3For	ATGGCTWGCTCTGCARTGAYYAAG	348	3
	M3Rev	TYACTTCACGGTGGCGCAGTTG		

PCRs were performed using 2X Taq PreMix Kit (Pars Toos Zist Fanavar) as follows: initial denaturation for 2 min at 94 °C followed by 35 cycles of 94 °C for 2 min, annealing at 60 °C for 45 sec, and extension at 72°C for 2 min. A final 20 min extension step was performed at 72 °C (3). The PCR products were separated and visualized using 1.5% agarose gel electrophoresis in TBE buffer. The PCR products were ligated into pGEM-T Easy (Promega, USA) according to the manufacturer's instructions. The purified plasmids were sequenced by Bioneer Company *(*South Korea). The sequences were translated using Expasy and the amino acid sequences were compared with the amino acid sequences in genetic databases.

Real-time PCR was performed using the Mx3000P QPCR System (Agilent Technologies, Stratagene, USA) and SYBR® Green Real Time PCR Master Mix kit (Pars Toos Zist Fanavar). PCR conditions were: initial step at 50 °C for 2 min, activation step at 95 °C for 10 min followed by 35 cycles of 15 sec at 95 °C and annealing and extension for 60 sec at 60 °C (14, 15). The amplification efficiency was estimated using the standard dilution curve and the slope of the regression line using the equation: $E=10^{-1/slope}$, and the data were normalized relative to actin as the reference gene. Then, in comparison with the reference gene, the relative expression ratio of the target genes of an infected sample vs. a healthy

sample was determined (14, 16, 17). Each group contained an infected (test) and uninfected (calibrator) fruit, therefore 10 groups were analyzed. In greenhouse experiments, the relative expression of the target genes in each infected sample versus two healthy samples (one without inoculation and another inoculated by sterile distilled water) was determined in comparison with the reference gene; therefore, each gene has two relative expressions: W for the sample inoculated with sterile distilled water and N for the uninoculated sample. Analysis of variance and comparison of the means ($p < 0.05$) were done using SPSS (version 16), and Excel software was used to produce the graphs.

Table 2. Primers used in real-time PCR

Genes	Primers	Sequence (5' – 3')	PCR product size (bp)	Reference
Mal d 1	M1RTF	CACACCAAGGGTGATGTTGAGA	75	3
	M1RTR	CTTGAACAAACCATGAGCCTTCT		
Mal d 2	M2RTF	GCTTGCCTTGCGTTTGGT	66	3
	M2RTR	ACATGTCTCCGGCGTATCATT		
Mal d 3	M3RTF	GCTGCTTGCAACTGCCTGA	70	3
	M3RTR	AGCGATTCGGCATTGCC		
Actin	ActFor	CTATGTTCCCTGGTATTGCAGACC	82	15
	ActRev	GCCACAACCTTGATTTTCATGC		

Results

Pathogen identification

On the basis of the infection symptoms, which included blossom and leave blight, colony characteristics on media, and biochemical and pathogenicity tests, *E. amylovora* infection was confirmed and the results were consistent with previous studies (8, 18, 19).

PCR

Mal d 1, 2 and *3* were amplified and fragments ranging from 400 to 500 bp visualized (Fig. 1). The deduced amino acid sequences were deposited in GenBank under accession numbers APG29328 (for Mal d 1), APG29329 and APG29330 (for Mal d 2), and APG29331 (for Mal d 3). GenBank blastp searches revealed that APG29328 was 100% identical to CAT99609, APG29329 was 100% identical to AAC36740, and APG29331 was 100% identical to AAR22488. The PCR results and comparison of the sequences confirm that the amplified genes are the studied ones.

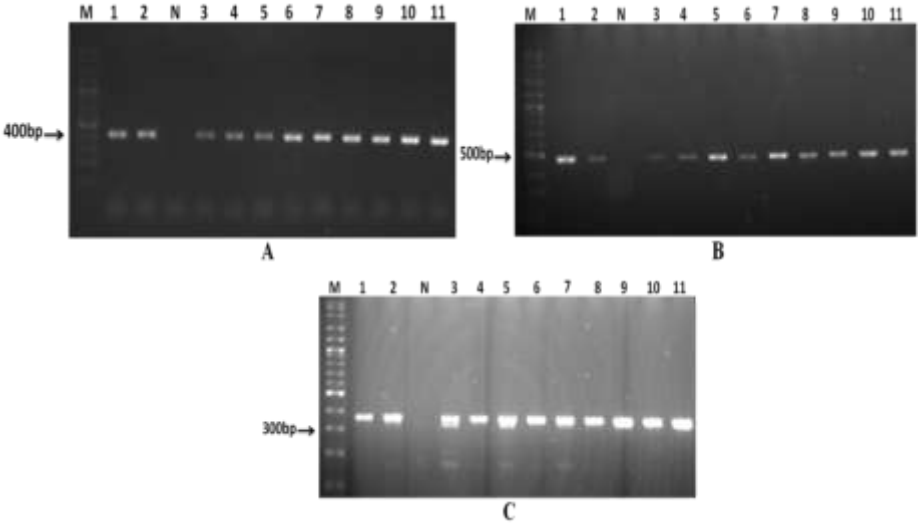

Fig. 1. Agarose gel electrophoresis of the PCR products (A: *Mal d 1*, B: *Mal d 2*, and C: *Mal d 3*): M: molecular marker, N: negative control, lanes 1-3: infected Golden Delicious, lanes 4-6: infected Red Delicious, lanes 7-9: uninfected Golden Delicious, lanes 10 and 11: uninfected Red Delicious.

Real-time PCR test

In natural conditions

After the test and determination of Ct values, the relative expression ratios of the target genes in infected versus uninfected samples were calculated in comparison with the reference gene and graphs were drawn (Fig. 2). Of the three genes studied, *Mal d 2* had the highest expression (Fig. 2A). For relative mean expression, in the Golden Delicious, *Mal d 2* and *3* were highest and lowest expressed, respectively, while in the Red Delicious, *Mal d 2* and *1* were the highest and lowest, respectively (Fig. 2B). Analysis of variance found no significant difference in the sources of cultivar change and interaction effect of infection × cultivar at $p < 0.05$ (Table 3) and that the infection effect is significant only for *Mal d 2* and *3*.

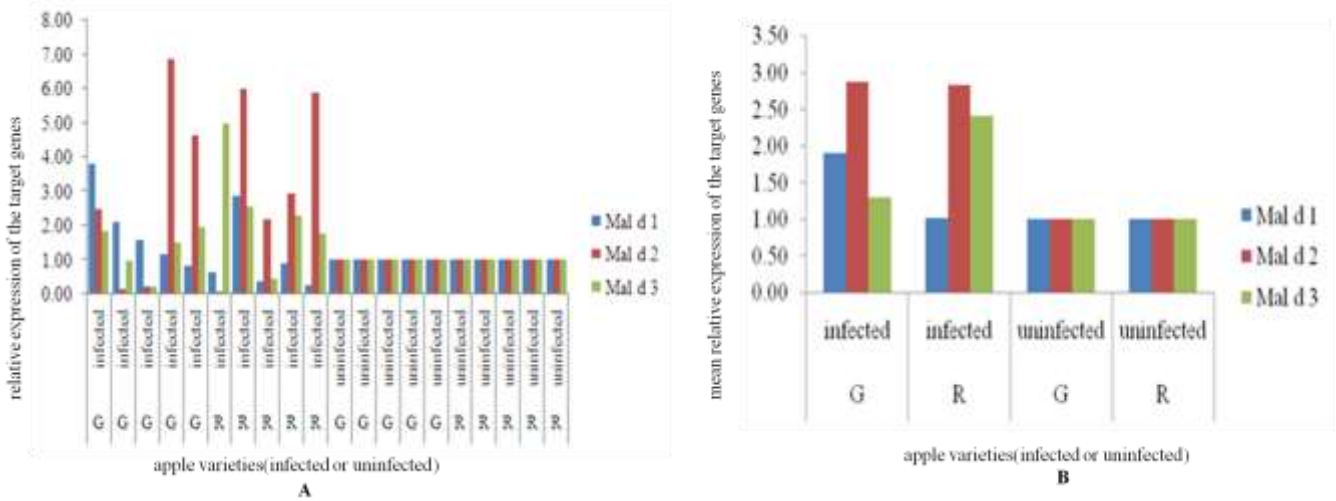

Fig. 2. Relative (A) and mean relative (B) expression of the target genes in *E. amylovora*-infected and uninfected samples in Golden (G) and Red (R) Delicious plants

Table 3. Analysis of variance of relative expression of *Mal d 1*, *2*, and *3* in *E. amylovora*-infected samples in natural conditions at $p < 0.05$, DF: Degree of freedom.

Sources	Variables	DF	Significance level
Cultivar	Mal d 1	1	0.226
	Mal d 2		0.756
	Mal d 3		0.185
Infection	Mal d 1	1	0.216
	Mal d 2		0.024
	Mal d 3		0.049
Infection×Cultivar	Mal d 1	1	0.226
	Mal d 2		0.756
	Mal d 3		0.185

Greenhouse studies

Of the three studied genes, *Mal d 2* was expressed at the highest level in infected plants (Figs. 3 and 4), and was slightly greater in Red than in Golden Delicious. However, the mean relative expression of *Mal d 1* and 3 were decreased in Red in both cultivars and this decrease was greater in Red than in Golden Delicious. Analysis of variance (Table 4) showed no significant difference between the cultivars and the interaction effect of infection × cultivar at the level of $p < 0.05$; however, this analysis shows that infection significantly affects expression of all three target genes. Greenhouse experiments results indicate that the mean expression of *Mal d 2* in the infected samples of the both cultivars is greater than *Mal d 1* and *3*. On the other hand, *Mal d 1* and *3* mean expression is greater in Golden than in Red Delicious and *Mal d 3* mean expression was less in the infected samples of both cultivars than in the uninfected samples. The differences between the mean expressions of the three proteins from infected and uninfected samples is significant.

Discussion

These results indicate that the mean relative expression of *Mal d 2* and *3* in the infected samples of both cultivars increased due to the infection. The effect of this infection on *Mal d 1* expression was less in the Red than in the Golden Delicious. The differences between the mean relative expression for *Mal d 2* and *3* in infected vs. uninfected plants was significant, but not significant for *Mal d 1*. Our *Mal d 1* finding agrees with those of previous studies (4), which showed that transcription levels of the 1.01, 1.02 and 1.03 *Mal d 1* isoforms differ in infected and uninfected fruits in natural conditions, but this difference is not significant. Factors such as pathogen infection, genotype, and ripening stage influence *Mal d 1*, *2*, and *3* expressions (20). No studies have yet addressed the effect of apple trees diseases caused by pathogens, especially *E. amylovora*, on *Mal d 2* and *3* expression; however, studies on the effects of height, stage, shade, and water stress showed that the first three affect *Mal d 1*, *2*, and *3* expressions, while water stress did not significantly affect expression (20). In the current study, the samples were collected from different geographical regions, so environmental factors may affect *Mal d 2* and *3* expression. The Golden Delicious is highly allergenic (4), however factors such as geographical and environmental conditions, possible infection of the Red Delicious samples by other pathogens, and greater resistance to *E. amylovora* by Red than by Golden Delicious (21) may affect the mean relative expression of these genes in Red Delicious.

Various factors including pathogen infection, genotype, and infection severity affect *Mal d 1*, *2*, and *3* expression (20). The Golden is more allergenic than the Red Delicious, and *E. amylovora* infections may drastically increase allergen production in high-allergenic cultivars (4). Because sensitivity to fire blight and severity of systemic infection are greater in Golden than in Red Delicious (21) and, based on Mayer et al. (4) results, we conclude that one reason for the high mean *Mal d 1* and *3* expression in infected Golden Delicious seedlings is its greater *E. amylovora* sensitivity and its high systemic infection severity. Our greenhouse results indicated that the degree of *E. amylovora* resistance has little effect on *Mal d 1* and *3* expression. Further studies are needed to address this finding.

We showed that the mean *Mal d 2* expression in both greenhouse and natural conditions was greater in infected than in uninfected samples, was greater in Red than in Golden Delicious, and greater in greenhouse than in natural conditions. These results agree with those of Venisse et al. (22) who demonstrated that *E. amylovora* infection increased *Mal d 2* expression in *Malus* spp. *E. amylovora* resistance is greater in Red than in Golden Delicious (21); the greater mean Mal d 2 expression in Red than in Golden Delicious may be due to this greater resistance. Our results suggest that plant defense is more related to *Mal d 2* than to the other two genes, and *Mal d 2* is more highly expressed in *E. amylovora*-resistant than in sensitive cultivars.

Table 4. Analysis of variance of relative expression of *Mal d 1*, *2* and *3* expression in *E. amylovora*-infected plants grown in greenhouse conditions

Sources	Variables	DF	Significance level
Cultivar	Mal d 1 W	1	0.577
	Mal d 1 N		0.657
	Mal d 2 W		0.965
	Mal d 2 N		0.951
	Mal d 3 W		0.824
	Mal d 3 N		0.817
Infection	Mal d 1 W	1	0.002
	Mal d 1 N		0.002
	Mal d 2 W		0.023
	Mal d 2 N		0.023
	Mal d 3 W		0.006
	Mal d 3 N		0.007
Infection × Cultivar	Mal d 1 W	1	0.577
	Mal d 1 N		0.657
	Mal d 2 W		0.965
	Mal d 2 N		0.951
	Mal d 3 W		0.824
	Mal d 3 N		0.817

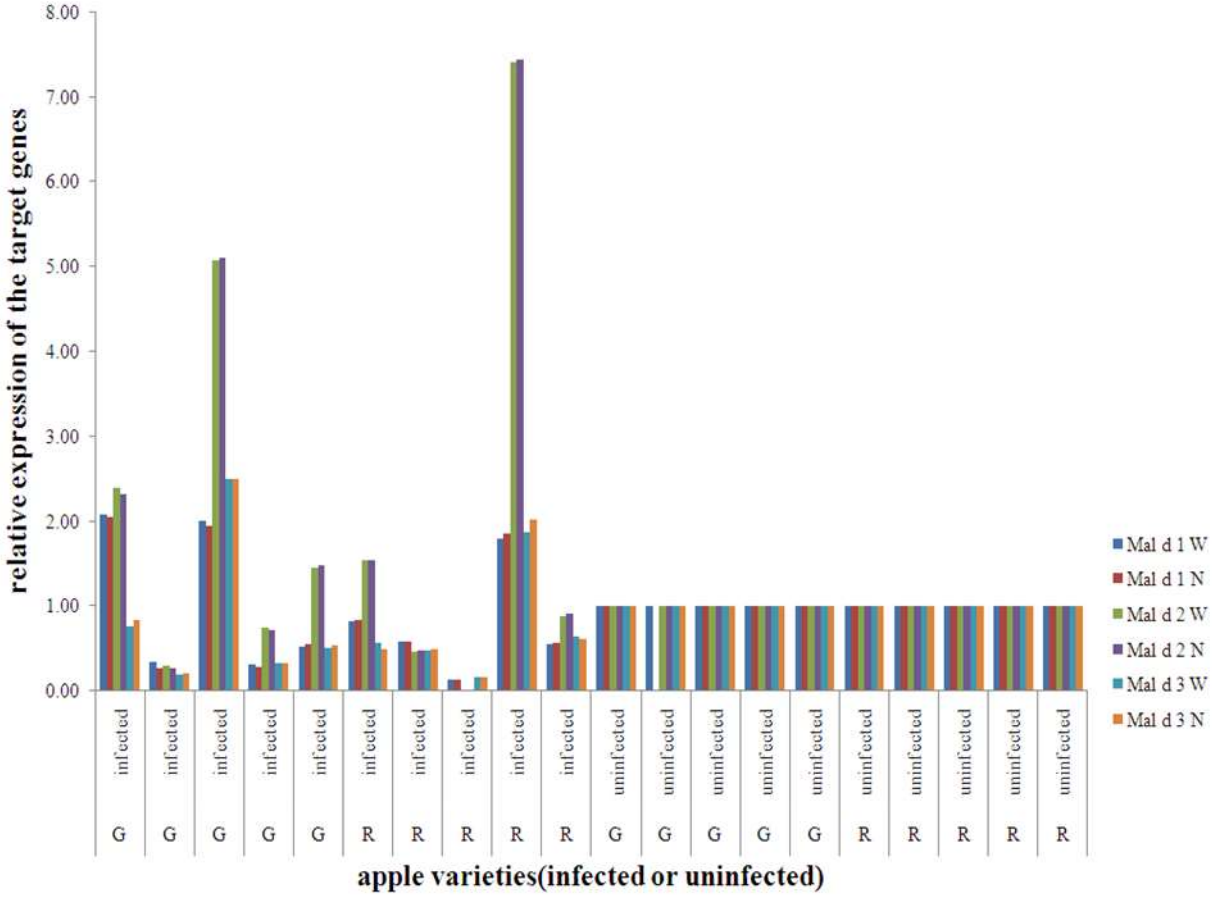

Fig. 3. Relative expression of the target genes in *E. amylovora*-infected and -uninfected Golden (G) and Red (R) Delicious.

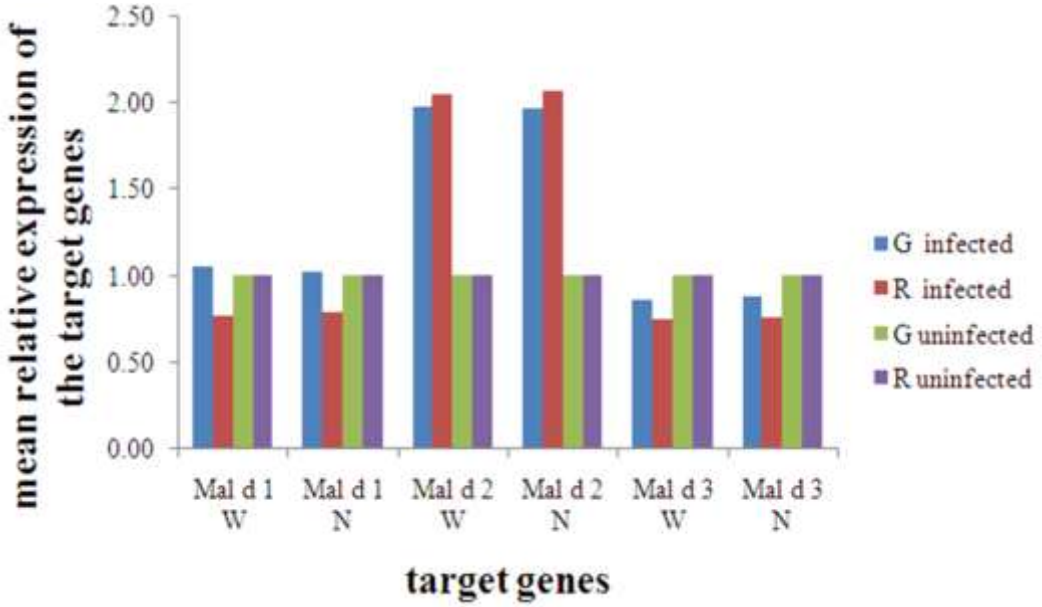

Fig. 4. Mean relative expression of the target genes in *E. amylovora*-infected and -uninfected Golden (G) and Red (R) Delicious.

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