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Fireblight *(Erwinia amylovora)* affects Mal d 1-related allergenicity in apple

Miriam Mayer · Christina Oberhuber · Igor Loncaric · Birgit Heissenberger · Marianne Keck · Otto Scheiner · Karin Hoffmann-Sommergruber

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Abstract Fireblight infection of apple and pear trees is a bacterial disease of serious economic concern. In this study, apple fruits (cultivar "Elstar", "Topaz") from healthy trees and trees naturally infected by the fireblight-causing bacterium *Erwinia amylovora,* as well as leaves from infected seedlings (cultivar "Golden Delicious"), were analysed for their Mal d 1 allergen concentrations. In addition, trees were treated with the two *Aureobasidium pullulans* biocontrol strains CF10 and CF40, active ingredients of Blossom Protect FB. Mal d 1, an 18 kDa intracellular protein is the major apple allergen in Northern and Central Europe and is a member of the family 10 of pathogenesis-related proteins, which is upregulated upon stress and pathogen attack. In young symptomless leaves from infected seedlings Mal d 1.01 transcript levels were significantly increased when compared to controls from healthy plantlets. Quantitative Mal d 1 transcript expression in field grown apple fruits did not show significant differences between samples from infected trees and controls. However, Mal d 1 protein content in apple fruits increased in fruits from infected trees. In fruits from trees with fireblight but treated with *A. pullulans*, Mal d 1 transcript and protein levels were reduced. These data show that an increase of Mal d 1-related allergenicity in fruits may result from fireblight infection. Thus, successful strategies against fireblight are needed to protect the orchards and simultaneously control the allergen content in fruits.

Keywords Apple tree infection · *Erwinia amylovora* · Mal d 1 · *Malus domestica* · Pathogenesis-related protein (PR-10) · Plant food allergen

M. Mayer · C. Oberhuber · O. Scheiner · K. Hoffmann-Sommergruber (✉)
Department of Pathophysiology and Allergy Research,
Center for Pathophysiology Infectiology and Immunology,
Medical University of Vienna,
AKH-EBO 3Q, Waehringer Guertel 18–20,
1090 Vienna, Austria
e-mail: Karin.Hoffmann@meduniwien.ac.at

I. Loncaric · B. Heissenberger
Institute for Apiculture,
Austrian Agency for Health and Food Safety, AGES,
Vienna, Austria

M. Keck
Federal Ministry of Agriculture, Forestry,
Environment and Water Management,
Vienna, Austria

Fireblight is a serious plant disease for Rosaceae, including apple and pear. The infection is caused by the necrogenic gram-negative bacterium *Erwinia amylovora* (Enterobacteriaceae). The bacterium enters the plant through blossoms and wounds and causes black, necrotic lesions, finally leading to the death of the plant (Fig. 1). Fireblight was believed to be endemic for North America. However, it has spread across Central Europe in the recent past. In Austria the disease was first detected in 1993 (Keck et al.

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1997) and is now spreading across the country, representing a serious economic concern. In 2002 eradication costs for three Austrian provinces amounted to 1.72 million EUR, and costs for pruning and eradication of 6,000 infected trees came to 1.8 million EUR (Keck 2004). Until now, the only effective treatment for plants already infected is to remove the affected branches from the orchard. Fungi such as *Aureobasidium pullulans* have been recently tested for their potential to prevent *E. amylovora* blossom infection (Lima et al. 1997; Seibold et al. 2004). In a recent study it was shown that susceptible and resistant apple tissues showed different patterns of expression due to *E. amylovora* infection (Baldo et al. 2010).

Higher plants like Rosaceae protect themselves from various stresses by changing their physiological conditions. The proteins actively induced in accordance with this protective reaction are called pathogenesis-related proteins (PR proteins). PR proteins are upregulated in response to pathogen attack such as fungi (Somssich et al. 1986; Walter et al. 1996), bacteria (Matton and Brisson 1989), viruses, by adverse environmental factors, or by wounding (Warner et al. 1994) and other physical stress (Walter et al. 1996; Stintzi et al. 1993). Pathogenesis-related proteins are classified into 17 unrelated protein families based on their diverse structure, function and biological activity (van Loon and van Strien 1999; Breiteneder 2004). Out of these proteins a considerable number have also been identified as allergens (Hoffmann-Sommergruber 2002). Mal d 1 is the major apple allergen for Northern and Central Europe and a member of the PR-10 protein family. This 18 kDa intracellular protein is encoded by multiple genes, named *Ypr10* genes (Wen et al. 1997). There are four major Mal d 1 isoform clusters identified in apple (Mal d 1.01, Mal d 1.02, Mal d 1.03 and Mal d 1.04) which vary in transcriptional and protein levels (Son et al. 1999; Helsper et al. 2002; Puehringer et al. 2003; Beuning et al. 2004).

Infection by the bacterium *E. amylovora* and the fungus *A. pullulans*, if applied to control fireblight, may affect the level of the pathogenesis-related protein Mal d 1 and, concomitantly, the degree of allergenicity in fruits. In the present work fireblight infection and its effect on Mal d 1 transcript and expression levels in apple plantlets and fruits were investigated. In addition, prevention strategies against fireblight were analysed for their possible impact on Mal d 1-related allergenicity. Plantlets grown in greenhouse under controlled conditions were chosen as a model system to study the effect of *E. amylovora* on the vegetative tissue. For the apple fruit study samples from *E. amylovora* infected orchards were taken, to assess Mal d 1 levels.

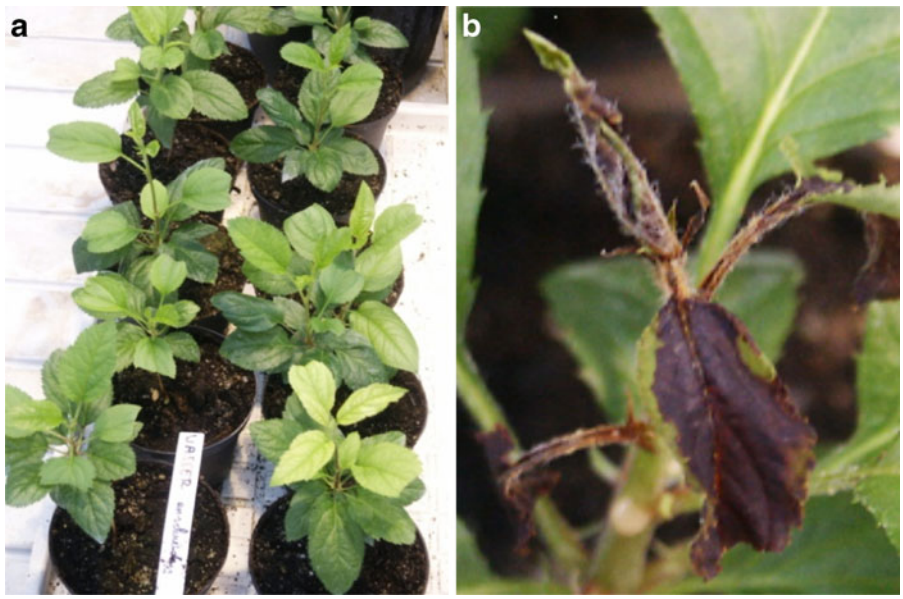

Fig. 1 *Erwinia amylovora* infection of apple plantlets (cv "Golden Delicious"; **b**) and healthy controls (**a**)

For this purpose, apple plantlets ("Golden Delicious" open pollinated) were grown for 6 months in the greenhouse. Twenty plantlets were infected with *E. amylovora* (*E. a.*) strain *295/93* (+FB). In parallel, 40 plantlets were used as controls: 20 plantlets without any treatment (control group 1), 20 plantlets with induced lesions and pure water application (control group 2). For the infection assay a suspension of an overnight culture of strain *E. a. 295/93* on King's medium B (OD 0.1 at 600 nm=ca. 10^8 CFU/ml, measured with Eppendorf BioPhotometer) was used. On day 0 *E. amylovora* was inoculated by cutting the upper leaves with scissors previously dipped into the bacterial suspension. Plantlets of the control group 2 were cut with scissors dipped into water. Infection of plantlets by *E. amylovora* was determined by sample rating according to Hevesi and isolation of the pathogen on King's medium B with ensuing specific PCR and nested PCR (Bellemann and Geider 1992; Llop et al. 2000; Hevesi et al. 2000). Sampling was carried out 18 days after artificial inoculation at a mean rating over 3, i.e., browning of half of the plant and a new sprout close to the necrotic lesion. Growth of the infected plantlets was reduced by 50% as compared to controls (Fig. 1). The young leaves were harvested, shock frozen in liquid nitrogen and stored at −70°C until RNA extraction.

From field-grown apples (cvs. "Elstar", "Topaz"), Mal d 1 levels were assessed. The apple trees were grown in a 10 ha sized apple orchard in Austria. The apple fruits were grouped into healthy control fruits, apples from trees with naturally occurring fireblight infection (+FB), apples from trees with *A. pullulans* biocontrol strains CF10 and CF40 (AP) treatment without fireblight infection (+F), and fruits from trees with fireblight and AP treatment (+FB+F). During the flowering period of 3–4 weeks the fungus *Aureobasidium pullulans* was applied by four applications into the blossom in a ratio of 1:1; 2.10^{10} blastospores/g; final concentration in water (0.15%). Based on the opening of the florescences (10%, 40%, 70% and 90%) and on the median daily temperature above 15°C the treatment was performed.

The fruits were harvested and stored for 3 months at 4°C under ambient atmosphere.

For PCR analysis, ten apple plantlets (cultivar "Golden Delicious") per treatment were selected. Total RNA was isolated from young leaves according to the method of Chang et al. (1993). Furthermore, total RNA was extracted from fruits (cultivar "Topaz" and "Elstar") as described (Kingston 1995). From each of the three treatments, a total of 10 apple fruits were used for RNA extraction. Reverse transcription was performed and the resulting cDNAs were subjected to PCR amplification by means of appropriate Mal d 1 isoform specific primers (Puehringer et al. 2003) in order to discriminate between the four isoform clusters. RT-PCR and standard PCR were performed as described previously (Willerroider et al. 2003). Mal d 1.01, Mal d 1.02 and Mal d 1.03 were identified, but Mal d 1.04 could not be detected within the limits of the assay. Thus, further experiments were performed with Mal d 1.01, Mal d 1.02 and Mal d 1.03. The cloned and sequenced Mal d 1 nucleotide sequences from plantlets and fruits were compared to nucleotide sequences from EMBL/GenBank nucleotide sequence databases with the accession numbers ***AJ417551*** (Mal d 1.01), ***AF020542*** (Mal d 1.02) and ***AY186248*** (Mal d 1.03) and were in agreement with the respective sequences from the database.

Real Time PCR based on the monitoring of FAM fluorescent labelled probes was applied for relative quantification of Mal d 1 isoform specific transcript expression (Puehringer et al. 2003; Gao et al. 2005). The procedure was carried out using an ABI PRISM 7700 Sequence Detection System Instrument (all reagents from Applied Biosystems, Foster City, CA, USA) under following cycling conditions: 10 min at 95°C, and 40 cycles consisting of denaturation, 15 s at 95°C, and annealing and extension, 1 min at 60°C. In apple plantlets, Mal d 1.01 transcript expression was chosen for quantitative analysis since this isoform is predominantly expressed in vegetative tissues (Atkinson et al. 1996; Puehringer et al. 2003). Plantlets infected by fireblight clearly showed higher Mal d 1.01 transcript levels compared to healthy controls (8.8-fold increase of Mal d 1.01; Fig. 2).

In apple fruits, Mal d 1.01, Mal d 1.02 and Mal d 1.03 transcript levels were found with varying concentrations, with Mal d 1.02 being the most abundant isoform, followed by Mal d 1.01 and only low transcript levels of Mal d 1.03 (Fig. 3). These data are in agreement with previous findings (Puehringer et al. 2003). For future studies it is planned to accomplish the greenhouse studies with cv "Topaz" and "Elstar" plantlets to study cv specific effects.

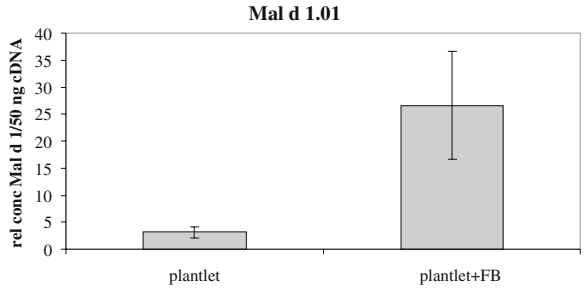

Fig. 2 Relative quantification of major apple allergen transcript (Mal d 1.01) expression in "Golden Delicious" plantlets after fireblight infection (+FB) as compared to non-infected controls performed by Real Time PCR

In both cultivars, "Topaz" and "Elstar", no obvious differences were observed in Mal d 1.01, Mal d 1.02 and Mal d 1.03 transcript levels between apples from trees with *E. amylovora* infection (+FB) versus apples from healthy trees. In contrast, in fruits from trees with fireblight and AP treatment (FB+F), Mal d 1.01, Mal d 1.02 and Mal d 1.03 transcript levels were reduced. In "Elstar", Mal d 1.01 was reduced by 41%, Mal d 1.02 by 38%, respectively. In "Topaz" Mal d 1.01 and Mal d 1.02 transcript levels were reduced by 53% and 45%, respectively (Fig. 3).

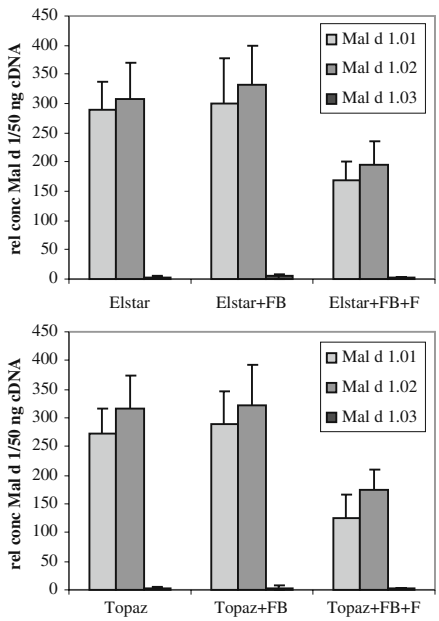

Fig. 3 Relative quantification of Mal d 1 isoform transcript expression in healthy apples and in fruits infected by fireblight (+FB) and treated with *Aureobasidium pullulans* (+F) application in cvs "Elstar" and "Topaz"

For investigations on the protein level, apple protein extracts (cultivar "Elstar", "Topaz": controls, +FB; +F; +FB+F) were prepared essentially as described by Bjorksten et al. (1980). Mal d 1-related allergenic reactivity of protein extracts was analysed by IgE immunoblot and IgE ELISA using sera from apple allergic patients (n=5; 1:5 diluted in 50 mmol/l sodium phosphate buffer/0.5% Tween 20+0.5% BSA, pH 7.5) according to established protocols (Jarolim et al. 1989; Ma et al. 2006). Briefly, in immunoblots, bound anti-Mal d 1 IgE antibodies were detected by ^{125}I-labelled antihuman IgE antibodies (1:40 dilution; MALT Allergy System Isotope Reagent, IBL, Hamburg, Germany) and visualized by autoradiography (Fig. 4a) and quantified by densitometry. The sera showed more intense IgE binding to Mal d 1 (18 kDa) from "Topaz" apples from trees with either fireblight infection (+FB) or fireblight and fungal AP-treatment (+FB+F) when compared to healthy control fruits (Fig. 4a; "Topaz" control sample: 100%, "Topaz"+FB: 146%, "Topaz"+FB+F: 165%).

In IgE ELISA Mal d 1 levels were quantified from apple protein extracts from cv. "Topaz" and "Elstar" (cultivar "Topaz": control, +FB, +F; +FB+F, cultivar "Elstar": control, +FB; concentrated, 1:10, 1:100 dilutions in 25 mM $NaHCO_3$, pH 9.5; Fig. 4b). In parallel, serial dilutions of purified recombinant Mal d 1.02 used as standard were applied (10–0.01 μg/well) (Oberhuber et al. 2005). Mal d 1 reactivity was expressed as percentage of total protein content. Different Mal d 1 levels were seen in the cultivars "Elstar" and "Topaz", with the highest values in "Elstar" fruits. This is in agreement with the literature, classifying "Elstar" as a more allergenic cultivar as compared to "Topaz" (Bolhaar et al. 2005). "Elstar" and "Topaz" apples from *E. amylovora* infected trees (+FB) had a 6.6-fold and 5-fold increase in Mal d 1 protein, respectively. In "Topaz", treatment with either AP alone ("Topaz"+F) or *E. amylovora* infection and AP treatment ("Topaz"+FB+F) led to comparable Mal d 1 protein levels, which were higher as the "Topaz" control sample but lower than the "Topaz"+FB sample (Fig. 4b).

These results illustrate that *E. amylovora* infection induced an upregulation of Mal d 1 transcripts in infected plantlets grown in the greenhouse. This effect could not be observed when analysing fruits from outdoor grown trees. This discrepancy could be due to

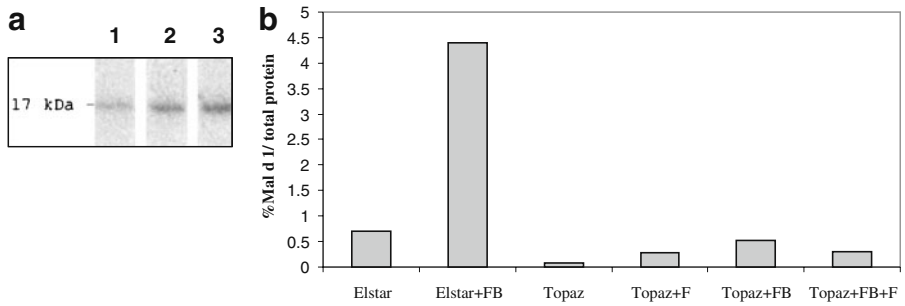

Fig. 4 **a**: Immunodetection of major apple allergen, Mal d 1 in apples with *E. amylovora* infection by specific IgE antibodies. Equal amounts of protein extracts from apples (cv. "Topaz") from non-infected trees (lane 1), from apples from *E. amylovora*-infected trees (+FB, lane 2) and from apples from trees with *E. amylovora* and *A. pullulans* treatment (+FB+F; lane 3), respectively, were separated by SDS-PAGE and transferred to nitrocellulose. After incubation with serum pool from apple allergic patients bound specific IgE was visualized by radiolabelled anti-IgE antibodies. **b**: Relative quantification and comparison of major apple protein levels (Mal d 1) in apples from trees with fireblight infection. Apple protein extracts (cvs "Elstar" and "Topaz") from non-infected fruits, from apples with fireblight (+FB), from apples after fungal treatment (+F), and from apples with fireblight and fungal treatment (+FB+F) were prepared. Mal d 1 concentration was determined in IgE ELISA using a serum pool from allergic patients

several reasons. Firstly, the systemic disease observed in the trees of the commercial orchard did not induce a signal strong enough in the fruits that are usually less affected by *E. amylovora* infection, compared to the plantlets showing severe symptoms caused by the pathogen. Furthermore, while greenhouse experiments provide controlled conditions, additional environmental factors influencing Mal d 1 levels in the fruits grown in orchards cannot be ruled out. Secondly, the apple fruits were stored for 3 months before analysis, the usual time span after which the potential allergic consumers come into contact with the fruits. Sancho et al. (2006) showed that Mal d 1 transcript levels peak at month 2 and 5 during storage, depending on the climate conditions applied. This general increase in the mRNA level during storage might overrule the initial pathogen-induced upregulation of Mal d 1. Mal d 1 is regarded as a protein highly susceptible to protease degradation and thus reliable quantification of the native protein is difficult to perform (Vieths et al. 1995; Sancho et al. 2006; Zuidmeer et al. 2006). Thus, absolute data have to be treated with caution. Mal d 1 quantification based on IgE reactivity from allergic patients might therefore be the method of choice when analysing allergenicity from apples. According to the allergenicity ranking of apples as shown by Bolhaar et al. (2005), the apple cultivars "Elstar" (medium-allergenic) and "Topaz" (low-allergenic) were selected for analysis in the present study. IgE reactivity was lowered and elevated towards protein extracts from apples from healthy trees and infected trees, respectively.

This study demonstrated that there are differences in Mal d 1-related allergen levels between samples from fruits with *E. amylovora* infection (+FB), and those samples treated with two biocontrol strains of *A. pullulans* (+FB+F), and healthy controls. In addition, it has provided support for the efficacy of fungal treatments in reducing the allergen load in apple fruits with *E. amylovora* infection.

This knowledge helps apple breeders to distinguish between high and low Mal d 1 content in apple fruits. According to the ranking of high- and low-allergenic apple cultivars, it can be assumed that low-allergenic apples (i.e. "Topaz"), harvested from infected trees, still have lower Mal d 1 levels than high-allergenic apples (i.e. "Golden Delicious") from non-infected trees. However, in the case of high-allergenic apple cultivars, *E. amylovora* infections could considerably increase the level of allergens even above expected levels. Detailed analysis of allergen levels in fruits from infected trees represents a useful contribution for improved food allergy prevention strategies. Moreover, this test system can be used to study the effect of conventional versus organic production or other plant diseases such as powdery mildew and apple scrab on the level of allergenic PR proteins. It can assist in designing appropriate prevention strategies for diseases aiming at reduced allergen levels in fruits.

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