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Erwinia amylovora-induced defense mechanisms of two apple species that differ in susceptibility to fire blight

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ABSTRACT

The bacteria *Erwinia amylovora*, the causal agent of fire blight, infects most members of the *Maloideae* including pear and apple. In this work the defense responses against this pathogen were monitored in two apple species grown *in vitro*, in the susceptible *Malus domestica* Borkh. cv. 'Idared' (later on 'Idared') and the resistant *Malus x robusta* (Carrière) Rehder var. *persicifolia* Rehder (*Mrp*). Our results indicate that the resistant plants might represent a less favorable environment for bacterial growth. At the same time, in these plants higher basic levels for some defense-related compounds such as salicylic acid or their activities such as the PAL enzyme activity can be found. In fire blight infected plants both the known pathways for SA synthesis as well as most of the phenylpropanoid genes examined were repressed due to disease, but apparently could be compensated by complex regulatory mechanisms. Not only the nature but also the quantity of defense-related compounds is likely to influence the outcome of plant–pathogen interactions.

1. Introduction

Fire blight is a disease of *Maloideae* of the family *Rosaceae*, e.g. apple (*Malus* spp.), pear (*Pyrus* spp.), and ornamentals such as *Cotoneaster* and *Pyracantha* spp. Infection occurs via natural plant openings such as nectaries in flowers and wounds on leaves or on succulent shoots. The causal bacterium *Erwinia amylovora* (*Ea*) multiplies and rapidly spreads through the plant *via* vascular tissues and can either cause cell dead (tissue necrosis) or can reside in symptomless tissue [1]. *Erwinia* harbors a pathogenesis mechanism known as the Type III secretion system [2,3] required for both hypersensitive resistance in non-host plants and pathogenicity (Hrp-TTSS). In addition, it carries genes for enzymes involved in the synthesis of capsular exopolysaccharide (EPS) - amylovoran and genes facilitating the growth in host plants. The Hrp secretory operon of *E. amylovora* is involved intranslocating virulence effector proteins into host cells where it interferes with regulation of many resistance genes following invasion. Although several of the 12 TTSS secreted proteins identified so far shares homologies with avirulence proteins [4], no race-cultivar interaction that involves matching of a bacterial avirulence (*avr*) gene and the corresponding plant resistance (*R*) gene has been reported in this pathosystem.

After recognition of the bacteria, plants induce a range of defense responses. In primarily infected cells are generated different reactive oxygen molecules, lipid peroxidation is induced and ion fluxes are also affected [5]. These changes often lead to hypersensitive response (HR) expressed as necrotic lesions of plant tissue. Adjacent cells surrounding the infection site are associated with localized acquired resistance that involves e.g. cell wall reinforcement, accumulation of phytoalexins and activation of different defense proteins such as antioxidant enzymes and pathogenesis-related (PR) proteins [6,7]. PR proteins can also be synthesized throughout the plant and lead to systemic acquired resistance (SAR), which represents the third line of defense mediated by salicylic acid [8,9]. Several authors have reported accumulation of different PR transcripts upon *E. amylovora* attack [9–11]. Many immune responses in

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plants to microbial pathogens (e.g. basal resistance and even gene-for-gene resistance) are associated with accumulation of salicylic acid (SA) that functions as a signaling molecule. SA is synthesized in plants either via isochorismate synthase [12] or though shikimate-phenylpropanoid pathway. However, *Erwinia* was shown to delay the expression of phenylpropanoid genes [9] to overcome the plant defense. It is assumed that the phenylpropanoid pathway leads to the synthesis of defense-related compounds such as lignin and flavonoid phytoalexins as well as SA [12,13]. The importance of the key enzyme of this pathway, the phenylalanine ammonia-lyase (PAL), in disease resistance has been demonstrated e.g. by increased disease susceptibility of tobacco plants in which PAL activity is suppressed [14].

In this work we used *in vitro* grown plants to study the molecular host responses induced by *Ea* in two apple species differing in susceptibility to this pathogen. We focused on changes in contents of salicylic acid and studied the possible ways of its synthesis. Further, changes in the phenylpropanoid pathway were monitored on the both transcriptional and protein level to evaluate its involvement in plant defense against *E. amylovora*. Levels of different carbohydrates were also analyzed to reveal the effect of fire blight bacteria on sugar metabolite contents.

2. Materials and methods

2.1. Plant material

In vitro apple shoot cultures of *Malus domestica* Borkh. cv. 'Idared' (later on 'Idared') and *Malus x robusta* (Carrière) Rehder var. *persicifolia* Rehder (*Mrp*) were micropropagated via shoot tip and nodal cuttings on MS-medium [15] with 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (both 1 mg l^{-1}). Plants were cultivated in plastic containers with 40 ml media supplemented with 3% sorbitol and 0.8% Daishin agar (Duchefa, Netherlands) at a temperature of 18 °C with a 16-h photoperiod. Plants were transferred to fresh medium 1 week before start of each experiment.

2.2. Inoculation

The *E. amylovora* strain 295/93 (Austrian Agency for Health and Food Safety) was grown overnight in King's B medium broth at 28 °C in a rotary shaker. Stems above growth media were inoculated using a sterile toothpick dipped into the bacterial suspension (~10^8 cfu ml^{-1}), while a single wound was applied by shoot. Control plants were was mock treated with sterile King's B medium. Microshoots were sampled after 6 h, 1, 2 and 3 days and immediately frozen in liquid nitrogen and stored at −80 °C until analysed. The experiments were repeated three times and included five plants for each time point.

2.3. RNA isolation and cDNA transcription

From three *in vitro* grown plants (leaves and stems) for both conditions the total RNA was isolated according to the method of Chang et al. [16]. Contaminating DNA was removed from RNA samples using DNA-free kit (Ambion, Austin, TX, USA). Total RNA (5 μg) were reverse transcribed with Super Script TM II Reverse Transcriptase (Invitrogen) using oligo dT primer (Invitrogen) in a total volume of 20 μl as recommended by the manufacturer. The cDNAs were diluted 1:5 with nuclease-free water. Aliquots of the same cDNA sample were used for real-time PCR.

2.4. Real-time PCR

Real-time PCR was carried out using an iCycler iQTM Multicolor Real-Time Detection System (BIO-RAD). For Light Cycler reactions a master mix of following reaction components was prepared: 0.75 μl forward primer (10 μM), 0.75 μl reverse primer (10 μM), 12.5 μl iQ TM SYBR Green Supermix (BIO-RAD), 10 μl water and 1 μl sample. For transcript analysis a 1:5 diluted cDNA was used. The PCR program started with polymerase activation at 95 °C for 3 min followed by 40 cycles of 15 s at 95 °C for denaturation and 45 s at 55 °C for annealing and extension. Design of gene specific primers and optimization in regard to primer dimmer, low self priming formation, low misspriming efficiency and primer melting temperature was done with the Primer 3 software [17]. The primers (Table 1) were purchased from MWG Biotech (Ebersberg, Germany).

2.5. Quantification of bacterial growth in the host tissue via RT-PCR

Inoculated microstems (~9 mm) were cut into three parts of similar sizes and used for detection of bacteria. Tissue was eluted in 100 μl PCR water. Dilutions of 1:10 and 1:50 were subjected to qRT-PCR analyses (primers in Table 1). The PCR program started with an initial step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 45 s. For each run a dilution series of genomic DNA from *Ea* was included as standard. All samples were measured in triplicates. The products were further analysed with a melting program after an initial denaturation step at 95 °C for 1 min followed by 100 cycles at 95–50 °C for 10 s each. A bacterial culture of measured optical density (OD_{600} = 0.9; 8.9×10^9 cfu ml^{-1}) was used for calibration of the number of bacterial cells by qPCR [19].

Table 1
Primer sequences used for expression analyses in qRT-PCRs.

Gene	GeneBank accession	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size (bp)
anr	DQ139835	GTTGCAACCCCTGTCAACTT	CTTTCACGCACGACTTCAGA	100
ans	DQ156905	TGGTGACATCTTCGAACAAGAG	CCCTGCATTTTGCTCTCACT	117
chi	AF494398	CATCGTTACAGGTCCGTTTG	TTTCAATGGCTTTGCCTTCT	152
chs	AF494401	AACGAGGCCTTCAAGCCTAT	GGGTGTGCAATCCAGAAGAG	108
ics1	CN996953	GCATTAGTCAAAAAGGTAAACCA	CGACCGTAGAATCCAGAAGG	114
ics2	CN996091	CGTAAATTCCCTCGAGTCCA	TGGAAATCCACAAACTGCTG	120
dfr	AF494392	TGCCAAAGAAAACAACATTGA	GTGAACATACTGCCCCTGCT	160
f3gt	AB 074489	GAAAACATCCGGAGGAGCTT	AACTCGTCAGCCAAGTGGAC	110
fht	AY691918	TGGAGGAGCCGATGACTTAC	AGTTGCTCCTTTGCATGCTT	94
fls	AF494389	CATGTCCTCGCCCTGATCT	TGACAAGGGCATTAGGGATG	157
pal	AF494403	TGACAAATGGCGAGAGTGAG	CTCTTCCTCGAAAGCTCCAA	79
pEA29[a]	-	CACTGATGGTGCCGTTG	CGCCAGGATAGTCGCATA	112
pr-1	A507974	CTTGACGTGGGATGACAATG	AGTGCTCATGGCAAGGTTTT	118
Ubq[b]	CX023931	TCTTTGTGAAAACCCTCACC	ATCCCTTCCTGGTCCTGGAT	117

[a] Harbours the ESP-encoding region on the bacterial chromosome [18].
[b] Housekeeping gene used for normalization [18].