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Research article

Erwinia amylovora affects the phenylpropanoid–flavonoid pathway in mature leaves of *Pyrus communis* cv. Conférence

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ABSTRACT

Flavonoids, which are synthesized by the phenylpropanoid–flavonoid pathway, not only contribute to fruit colour and photoprotection, they also may provide antimicrobial and structural components during interaction with micro-organisms. A possible response of this pathway was assessed in both mature and immature leaves of shoots of 2-year-old pear trees cv. Conférence, which were inoculated with the gram-negative bacterium *Erwinia amylovora* strain SGB 225/12, were mock-inoculated or were left untreated. The phenylpropanoid–flavonoid pathway was analysed by histological studies, by gene expression using RT-qPCR and by HPLC analyses of the metabolites at different time intervals after infection.

Transcription patterns of two key genes anthocyanidin reductase (*ANR*) and chalcone synthase (*CHS*) related to the phenylpropanoid–flavonoid pathway showed differences between control, mock-inoculated and *E. amylovora*-inoculated mature leaves, with the strongest reaction 48 h after inoculation. The impact of *E. amylovora* was also visualised in histological sections, and confirmed by HPLC, as epicatechin –which is produced via *ANR*– augmented 72 h after inoculation in infected leaf tissue. Besides the effect of treatments, ontogenesis-related differences were found as well.

The increase of certain key genes, the rise in epicatechin and the visualisation in several histological sections in this study suggest a non-negligible impact on the phenylpropanoid–flavonoid pathway in *Pyrus communis* due to inoculation with *E. amylovora*. In this study, we propose a potential role of this pathway in defence mechanisms, providing a detailed analysis of the response of this system attributable to inoculation with *E. amylovora*.

1. Introduction

Fire blight, caused by the gram-negative bacterium *Erwinia amylovora*, is characterised by a rapid dissemination and a systemic distribution in *Rosaceaous* plants, of which apple and pear both are economic important species. Due to its destructive character and the lack of effective control methods, sustaining a considerable fruit yield has become a major challenge in many parts of the world. Every year, moderately resistant to highly sensitive apple and pear orchards are decimated because of quarantine related measures. In Europe, fire blight is considered as a growing problem as higher temperatures, breeding of cultivars on susceptible rootstocks and the introduction of susceptible cultivars will probably enlarge the risk of infection in the near future [1]. Interestingly, the pathogenic cycle of *E. amylovora* is characterised by a reduction or even a total incapability of infecting and spreading of the bacteria in the adult leaves of the host plant, a phenomenon that is often reported in other pathosystems [2,3], such as *Pseudomonas syringae* in *Arabidopsis thaliana* [4], *Ramularia collo-cygni* on barley [5] and *Venturia inaequalis* on apple [6].

As a response to fire blight, a rapid production of reactive oxygen species (ROS) is one of the first reactions that will be triggered *in planta* [7,8], together with an induction of lipid peroxidation and a shift in ion fluxes [7]. Furthermore, an accumulation of phytoalexins [9–11], an activation of pathogenesis-related proteins [12–16] and a modification in plant hormone balances [17,18] is often observed during a fire blight infection.

Abbreviations: ROS, reactive oxygen species; PBS, Phosphate Buffered Saline; NA, Naturstoff reagent A; *PAL*, phenylalanine ammonium lyase; *CHS*, chalcone synthase; *FHT*, flavanon 3β hydroxylase; *FLS*, flavonol synthase; *F7GT*, flavonoid 7-O-glucosyltransferase; *DFR*, dihydroflavonol-4-reductase; *LAR1*, leucoanthocyanidin reductase 1; *ANS*, anthocyanidin synthase; *ANR*, anthocyanidin reductase; *Ea*-inoculated, *Erwinia amylovora* inoculated.

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An important pathway in several fruit crops is the phenylpropanoid–flavonoid pathway, which uses the amino acid L-phenylalanine as a precursor and is able to produce a large amount of various secondary metabolites [19], which in turn could fulfil crucial roles in UV protection, colouring and the development or ontogenesis of a plant or tree [20–28]. In strawberry fruits, it is even shown that developmental cues play a key role in flavonoid metabolism and are predominant over genotype and environmental factors [29].

However, in different plant species such as apple, pear, poplar,… [15,18,30–35] it is often reported that specific secondary metabolites increase or decrease during an interaction with a plant pathogen, assuming a direct or indirect role in defence mechanisms.

Hence, in the present work we assessed the involvement of the phenylpropanoid–flavonoid pathway after an inoculation with *E. amylovora* in leaves of different ontogenetic age of *Pyrus communis* cv. Conférence. Therefore, several genes coding for enzymes of the phenylpropanoid–flavonoid pathway were studied with RT-qPCR, as well as the profiles of soluble phenolic compounds. Furthermore, histological studies were performed to characterize the infection process in this specific plant–pathogen interaction.

2. Material and methods

2.1. Experimental design

Two-year-old trees (*P. communis*) of the moderately susceptible cultivar Conférence on Quince C rootstock were grown in a quarantine protected greenhouse (PC Fruit, Kerkom, Belgium) in a controlled environment with a temperature of 22 °C, a relative humidity of 60% and a minimal light intensity of 150 μmol $m^{-2}s^{-1}$. Trees were grown until they contained enough active growing shoots with an average length of 25 cm and a minimum of eight leaves on each shoot. Next, each tree with corresponding shoots was subjected to only one of the following treatments: (a) untreated immature leaves, (b) mock-inoculated immature leaves, (c) *Ea*-inoculated immature leaves, (d) untreated mature leaves, (e) mock-inoculated mature leaves and (f) *Ea*-inoculated mature leaves. Eight biological repetitions were applied, as eight leaves (trees) were used per variant and sampling date.

A highly aggressive *E. amylovora* strain (BG16, isolated from *Malus sylvestris* (Bulgaria) with collection number SGB 225/12 [36]) was cultivated at a temperature of 25 °C on Yeast Peptone Glucose Agar growth medium. After 24 h, a suspension liquid of these bacteria was prepared in Phosphate Buffered Saline (PBS) at a density of 10^8 CFU ml^{-1} and used for inoculations.

For inoculation, both the second and third (immature leaves) or the sixth and seventh leaf (mature leaves) starting from the apex were cut perpendicular to the main vein with scissors dipped in the bacterial suspension liquid. For mock-inoculated leaves, scissors were dipped in PBS. No cutting was performed for the control.

Leaf samples were taken 6, 24, 30, 48 and 72 h after inoculation. Leaf samples were immediately used for histological analysis or were stored at −80 °C for further analysis with RT-qPCR or HPLC.

2.2. Histological study

2.2.1. Light microscopy

Leaf samples taken directly at the inoculation site were fixed for 4 h at 4 °C in 2% glutaraldehyde and malachite green 0.1%, buffered in 0.05 M sodium PIPES (1,4-piperazinediethanesulfonic acid) (pH 7.5). After dehydration in graded ethanol series, the tissues were impregnated and embedded in paraffin. Sections (8 μm) were obtained using a Leica MS 2000R rotary microtome equipped with a steel knife. The sections were stained with safranin and astra blue and mounted on DePex. The tissues were examined using a Polyvar Reichert-Jung interference microscope and the images were digitalized with an Olympus C-5050 zoom digital camera.

2.2.2. Fluorescence microscopy

Histochemical localization of flavonoids on 8 μm thick sections (see previous section Light microscopy) was performed by the protocol proposed by Pina and Errea [37]. The sections were deparaffinized in xylene, rehydrated in ethanol series for 2 min (100%, 70% and 40%), washed in distilled water, stained for 5 min in 1% (*w*/*v*) Naturstoff reagent A (NA; diphenylboric acid 2-aminoethyl ester, Sigma) in ethanol, and then repeatedly washed with ethanol. NA-stained sections were examined under a Fluorescence Microscope Nikon Eclips 80i with a filter cube comprised of a 540/25 nm excitation filter, a 605/55 nm barrier filter and a 565 dichroic mirror. Fluorescence micrographs were taken with an Imaging Source DFK H1AF02 camera coupled to the microscope. The yellow fluorescence of the stained material was consistent with the autofluorescence of polyphenolic compounds due to the NA-staining.

2.2.3. Transmission electron microscopy

Leaf parts taken directly at the inoculation site were fixed for 20 h at 4 °C using vacuum infiltration in 2% *v*/*v* glutaraldehyde and 0.01% *w*/*v* malachite green, buffered in 0.05 M sodium PIPES (pH 7.5). Samples were rinsed 3 times for 30 min in 0.05 M sodium PIPES (pH 7.5) and postfixed in 2% *w*/*v* osmium tetroxide, buffered in 0.2 M sodium cacodylate and twice in distilled water before staining in 2% *w*/*v* uranyl acetate overnight at 4 °C. After dehydration, the fixed tissues were imbedded in Spurr's epoxy resin. Ultra thin sections (65 nm), obtained using a Leica Ultracut UCT ultramicrotome, were mounted on 0.7% *w*/*v* formvar coated copper grids 50 mesh. The sections were contrasted with uranyl acetate (4% *w*/*v* in 50% ethanol) followed by lead citrate (4% *w*/*v* solution) and examined in a Philips EM 208 transmission electron microscope operated at 80 kV. Images were digitalized with a MORADA 10/12 camera (Olympus, Germany).

2.3. Quantitative reverse transcription PCR

Frozen and not necrotic leaf tissue adjacent to the infection site was grinded using two stainless steel beads in each sample and the Retsch Mixer Mill MM2000. Seven independent samples per treatment were used.

RNA was extracted using the protocol of Gasic et al. [38], which is optimized for trees belonging to the family of *Rosaceae*. The concentration and purity of RNA was tested spectrophotometrically with a Nanodrop 1000 machine (Thermo Fischer Scientific Inc). All RNA samples were adjusted to an identical concentration (1 μg/μl) and incubated in gDNA wipe-out buffer at 42 °C for 2 min to remove contaminating gDNA. First strand cDNA synthesis was primed with a combination of oligo(dT)-primers and random hexamers according to the manufacturer's instructions using a QuantiTect reverse transcription kit (Qiagen). A ten-fold dilution of the cDNA was made using 1/10 diluted TE buffer (1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) and stored at −20 °C.

RT-qPCR was performed with the ABI Prism Fast 7500 (Applied Biosystems), SYBR Green Chemistry. Primers were designed and optimized by Primer3 (Whitehead Institute for Biomedical Research) starting from the Ncbi database, were checked for similarities between biological sequences using BLAST (Ncbi) and were then verified for their quality, stability and efficacy using NetPrimer (Premier Biosoft). Specificity of the primers was checked by verifying the occurrence of single peaks on the melting curve. The amplification efficiencies of all primer sets were investigated by a 2-fold serial dilution over 6 dilution points and were approved