Transcriptome analysis uncovers the genes regulating the apple rootstock response to *Phytophthora cactorum*

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Abstract

***Phytophthora cactorum* is a polyphagous oomycetal pathogen infecting many host plant species. It is the principal agent of root rot in the strawberry and collar rot in the apple trunk, causing inhibition of plant growth and root system development. The motile zoospores are spread through water in soil between the root system of plants, but also through splashing rainfall water on the above ground parts of plants. Within the research carried out at The National Institute of Horticultural Research (InHort) in Skierniewice, Poland, apple rootstocks M.9, PJ-173/2012, PJ-191/2016, P 59 and P 60 were inoculated with zoospores of *P. cactorum*. To confirm the effectiveness of inoculation, bait tests were done, for selective detection of the active fungal spore presence in the substrate in which plants were grown. The presence of the pathogen was confirmed for PJ-173/2012 rootstock. From infected and control plants of PJ-173/2012 root samples were collected for total RNA isolation addressed for the comparative Next Generation Sequencing analysis. Apple transcriptome sequencing was performed for both the control and the infected samples. A total of 34 478 raw reads were obtained and mapped on the reference genome of the cultivar 'Golden Delicious'. After normalizing the raw data, differentially expressed genes (DEGs) comprising 9 335 upregulated and 9 342 downregulated were identified by comparison assessment of infected and control samples. Functional Gene Ontology enrichment analysis, allowed to assign the DEG's into three main functional groups: biological processes (7 681); cellular components (2 724) and molecular function (4 938). Mapping of DEGs confirmed the largest number of genes in the endoplasmic reticulum protein processing pathway (mdm4141). Three of the selected genes, i.e.: *BiP*, *HsP70* (involved in ubiquitin ligase complex formation) and *sHSF*, were subjected for verification analysis (qRT-PCR) to assess their suitability as candidates for early selection of plants infected by *P. cactorum*.**

Keywords: differentially expressed genes, GO enrichment

INTRODUCTION

Phytophthora cactorum is the oomycete soil borne pathogen affecting over 200 plant species, mostly herbaceous (e.g. strawberry) and woody plants (most of Rosaceae family) (Chen et al., 2023). No single races have been reported to date, except of its speciation based on host preferences from which individual isolates were resolved, and for which significant different virulence were observed (Nellist et al., 2021; Chen et al., 2023). As it was reported by Nellist et al. (2021), the different plant species infested by single isolates, collected from infected strawberry or apple plants are not always be able to develop the disease symptoms. Moreover, in recently presented studies the authors emphasize the genetic diversity between *P. cactorum* isolates (recovered from different plant hosts and from different geographical locations), determined via AFLP (amplified fragment length polymorphism) and RAMS (random amplified microsatellite sequences) analyses (Eikemo et al., 2004).

They confirmed that *Phytophthor*a spp. should be regarded as a species complex (Nellist et al., 2021; Chen et al., 2023). The pathogen is extremely effective in host plant infection

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development, generally through the high ability to disperse and infect hosts via the asexual stage of life cycle (zoospores) as well as sexual oospores both having the capacity to survive for many years in unfavorable conditions and affecting new hosts (Ramasamy et al., 2021). *P. cactorum* occurs on most of apple growing world regions, causing the fruit yield reduction and often tree death (Alexander and Stewart, 2001). The pathogen causes collar rot of the grafted scions and ring rot root in apple rootstocks, also causing the necrosis of root system (root rot) and fruit rots (Harris, 1991). Taking into consideration that *P. cactorum* is self-fertile fungus, producing sexual oospores and can survive in the soil for a long time, establishment of plant growing material must include a fungicide protection, which may lead to losses of economic efforts (Chen et al., 2023). Additionally, when apple trees are planted on moisture regions in moderate temperatures and in heavy soils the disease severity may increase (Jeffers and Aldwinckle, 1988; Carisse and Khanizadeh, 2005).

In apple orchards, *Phytophthora* spp. often has been reported as component of apple replant disease complex (Utkhede and Smith, 2009; Choi et al., 2021). Till now, some plant fumigation methods have been successfully applied to hamper the pathogen propagules dispersion, but an investigation of genetic tolerance or resistance of apple genotypes seems to be the best strategy to overcome this disease (Luberti et al., 2021; Nellist et al., 2021; Chen et al., 2023). Simultaneously, as the fungicide resistance increased and fungicide application become more restricted by legalization, the growing of apple cultivars on disease-resistant rootstocks is the most effective in managing soil borne diseases (Brown and Mircetich, 1993; Luberti et al., 2021; Fazio et al., 2022). Since the donor of crown rot resistance is *Malus sieviersi*, the research conducted by Smit et al. and Fazio et al. led to develop the germplasm pool, which could increase the overall seedling's resistance in the apple breeding programs (Smit and Labuschagne, 2004; Fazio et al., 2022).

Considering the plant molecular mechanism of apple rootstock response to *P. cactorum* is not clear the main aim of presented study was to identify specific genes involved in plant pathogen resistance regulation. In this research we have used the Next Generation Sequencing technique to uncover new genes potentially involved in the mechanism of rootstock response to apple collar and root rot disease.

MATERIAL AND METHODS

Inoculum preparation and plant inoculation

P. cactorum isolate PCLukMich, stored as an agar plug with mycelium in water in the plant pathogen collection of the Department of Plant Protection of The National Institute of Horticultural Research, was used for apple rootstock inoculation. The strain was revived by transferring the plug into fresh PDA medium (Czapeks Agar, Duchefa), incubated at 25°C for 14 days and then transferred on oatmeal medium (5 g of oat flakes/15 mL of water). After 14 days of incubation, the medium with fungus culture was fractionated (Zelmer blender) and mixed with a soil. The prepared substrate was left in plastic bags for another 10 days (at temp. 23-25°C). After this time, selected rootstocks (10 plants per genotype): new Inhort selections with low susceptibility to *P. cactorum*: PJ-173/2012, PJ-191/2016, standard M.9, as well as P 59, P 60 (differ in plant response to *P. cactorum*), growing in the tunnel, were planted into the substrate soil with *P. cactorum* inoculum, and additionally were watered with a solution of pathogen suspension. After planting (June 21, 2022), the plants were pruned and maintained to continue regrowth and development of new shoots.

Plant rNA isolation and reverse transcription

Total RNA was isolated from five above mentioned, apple rootstocks genotypes according to the method described by Zeng and Yang (2002). The leaf tissue ground in liquid nitrogen was incubated with preheated (65°C) extraction buffer (CTAB) for 20 min. (65°C). After centrifuging the samples in a mixture of chloroform and isoamyl alcohol (24:1, v/v) RNA was collected from the upper fraction and precipitated with 10 M $LiCl_2$ (overnight incubation, 4°C). Finally, the centrifuged RNA precipitate was dissolved in RNAse-free water (DEPC). The quality, degree of integration and concentration of preparations were assessed by micro-flow

electrophoresis using the Agilent 2100 Bioanalyzer (Perlan Technologies) and the Expert 2100 software. Then, the RNA (1 µg) was reverse transcribed into stable cDNA using the AffinityScript QPCR cDNASynthesis Kit (Agilent). The reaction was carried out in the presence of the universal oligo-dT primer (0.1 µg μL^{-1}) and the enzyme – reverse transcriptase (RT) under optimized thermal conditions: 25°C/5 min., 42°C/5 min. – oligo-dT connection, 55°C/ 15 min – RT, 95°C/ 5 min. - enzyme inactivation (Biometra Basic thermal cycler). The stable cDNA served as a template for transcriptome sequencing and the quantitative amplification reaction (RT-qPCR).

Transcriptome sequencing and qRT-PCR tests

Regarding that the inoculated plants did not show any disease symptoms, bait tests were carried out, as a result of which the presence of *P. cactorum* zoospores was recorded only for the rootstock PJ-173/2012. Transcriptome analyses were performed for RNA preparations of the root samples collected from control and inoculated plant (despite being colonized by the pathogen, it did not show any disease symptoms). After checking the degree of RNA integrity and estimating its concentration (Bioanalyzer, Agilent 2100, Perlan Technolgies), the preparations were sent to the commercial company for transcriptome sequencing approach in the Illumina system (Genomed S.A.). Sequence quality check of the raw data from two constructed cDNA libraries – control K_PJ173/2012 and infected Z_PJ173/2012 root samples, and a number of reads counts was performed using Trimmomatic software. Sequence raw reads, were mapped to the reference genome of the 'Golden Delicious' (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCF_002114115.1/) (Htseq software). The gene expression level was calculated as FPKM (fragments per kb per million reads), giving the number of fragments per kilobase length of a protein-coding gene per million reads mapped. Based on compilation of the list of all protein coding genes and differential protein-coding genes (counted based on FPKM), we calculated the representative GO function set (hypergeometric distribution, p-value). Then the genes at different levels of expression (log2 fold-change >2) were mapped on Kyoto Encyclopedia of Genes and Genomes https://www.genome.jp/kegg/pathway.html (KEGG).

Specific oligonucleotides, complementary to the differentially expressed genes (DEGs), uncovered by transcriptome analysis, were designed for the selected sequences and dedicated for the RT-qPCR reaction (Primer3Plus program, https://www.primer3plus.com/index.html) (Table 1). qRT-PCR test were performed for cDNA (collected in two consecutive seasons 2022, 2023) isolated from the root tissue of control and infected rootstock of PJ-173/2012 and control and infected M.9 rootstock as a standard. Tests were optimized in RotorGene 6000, Corbett Life Science thermocycler and the following thermal profile was used: 95°C for 5 min of cDNA initial denaturation, followed by 30 cycles with 95°C for 30 s denaturation, 60°C for 30 s – oligonucleotide annealing and 72°C for 20 s – PCR product elongation and reading the fluorescence acquisition in each reaction cycle.

Table 1. Oligonucleotides complementary to the sequence of DEGs from endoplasmic reticulum protein processing pathway (mdm4141).

Gene ID	Forward primer	Revers primer	Gene
LOC103435785	ACACCAGACACCAGACACTG	CCCCATTTTCCAACTTGGCG	553 bp
LOC103412527	CCACACCCATGGCTCAATCT	GCAGCTGTCATGAGAAGGGT	544 bp
LOC103411069	GTGCTGCAGCCAGTACTACT	CAATGCTGATGAGCTTGGGC	526 bp
Ref. Md18sRNA	GACTGTGAAACTGCGAATGGCT	CATGAATCATCAGAGCAACGGG	658 bp

qRT-PCR data and statistical analysis

The relative expression of the selected genes was calculated in relation to the sequence of the reference *Md18sRNA* gene, with stable activity in the tested experimental layout and using the RotorGene 6000 Series Software v. 1.7 (Corbett Life Science). The relative change in gene activity was calculated using the $2^{-\Delta\Delta Ct}$ method. Graphs showing the expression profiles of the selected genes were prepared in the GraphPad PRISM 8.1 software, considering the

average relative gene expression, compared to the controlled samples and standard M.9 rootstock and normalized to the *Md18sRNA* gene (showing stable expression in the experimental setup), assigned as the average standard error±SEM.

RESULTS AND DISCUSION

The first assessment of infected plants was performed 14 days after inoculation, and subsequent observations were conducted every 20 days (September-December 2022). Due to the long process of disease development (Brown and Mircetich, 1993) no significant symptoms were observed for inoculated plants (M.9, P 60, P 59, PJ-191/2016 and PJ-173/2012) during the research period. Finally, in the next season (April-May 2023) bait tests were carried out, allowing selective detection of the presence of active *P. cactorum* propagules in soil samples in which single plants were grown. The tests confirmed the presence of the pathogen in three single soil samples in which the PJ-173/2012 rootstock grew. The long period of development of disease symptoms in inoculated plants is probably genotype dependent, which could favored the death of the mycelium of *P. cactorum*, also by the development of other antagonistic factors, inhibiting its growth (Chen et al., 2023). As it was reported, there are many factors that may hamper the pathogen growth or indirectly enhance the plant defense such as: chemical components of phosphonate or matrix-based selenium nanocomposites (Weiland et al., 2009, Perfileva et al., 2021), as well as some biological control agents like: *Pseudomonas fluorescens* (Barahona et al., 2011), *Serratia plymuthica* (Kurze et al., 2001) or *Trichoderma* spp. (Porras et al., 2007). Since soil composition was not analyzed in details within conducted study we cannot verify the mechanisms affecting the selective presence of the pathogen.

Simultaneously, it has been notified that moisture content and particularly temperature of the soil have substantial effect on the survival of the *P. cactorum* mycelium and reproductive structures (oogonia and sporangia). These reports showed that the periods or prolonged exposure to the temperatures much higher than optimal for *P. cactorum* growth, together with low moisture content in soil, have strongly reducing or even destroying effect on the oomycetal structures, thus its survival could be compromised. We assume, that the uneven temperature conditions, including the local exposure on the high temperature during the summer in particular, could influence the survival of the *P. cactorum* in some but not all of the pots included in the experiment.

After sequence normalization (quality check, summarization of high-quality reads, and reference genome assembling) of cDNA libraries - control: K_PJ173/2012 and infected: Z_PJ173/2012, over 34Gb of clean data from the NGS reads were revealed. Regarding these 'in silico' analysis, we identified genes with different activity in the infected sample, in comparison to the control. In the conducted studies 9 335 genes were activated and 9 342 were inhibited. After performing gene ontology enrichment analysis (KEGG enrichment analysis), the extracted genes were assigned to three functional groups: biological processes, BP (7 681, 50%), cellular components, CC (2 724, 18%) and molecular factors, MF (4 938, 32%). Out of the 7 304 DEGs uncovered from BP group, 323 were mapped in protein processing of endoplasmic reticulum pathway (mdm4141). Based on performed calculation of significance level ($p<0.05$) of the fold change value in the number of transcripts, single genes with the highest degree of differentiation in comparison to control and infected samples were selected from the resolved pathway.

Three uncovered genes belonging to the general group of regulating Genetic Information Process: *BiP*, *HsP70* and *sHSF*, were selected (Table 2, Figure 1) for validation and expression profiling. Calculation of the expression profiles of selected genes by the qRT-PCR tests allowed to validate their activity in two different type of reference apple rootstocks: M.9 (low susceptible to root rot (Choi et al., 2021)) and newly bred – PJ-173/2012 (Figure 2).

Table 2. Biological function of genes from protein processing in endoplasmic reticulum pathway, and their localization in *Malus* genome.

Gene name	Gene function	Genome locus/localization
BiP	Binding protein 5-like endoplasmic reticulum chaperone BiP, ubiquitin ligase formation. Genetic Information Processing.	LOC103412527 (chromosome 2)
sHSF	17.9 kDa class II heat shock protein-like, HSP20 family protein. Genetic Information Processing	LOC103411069 (chromosome 8)
Hsp70	22.0 kDa class IV heat shock protein-like, mediator of RNA polymerase II transcription subunit. Ubiquitin ligase formation. Genetic Information Processing.	LOC103435785 (chromosome 5)

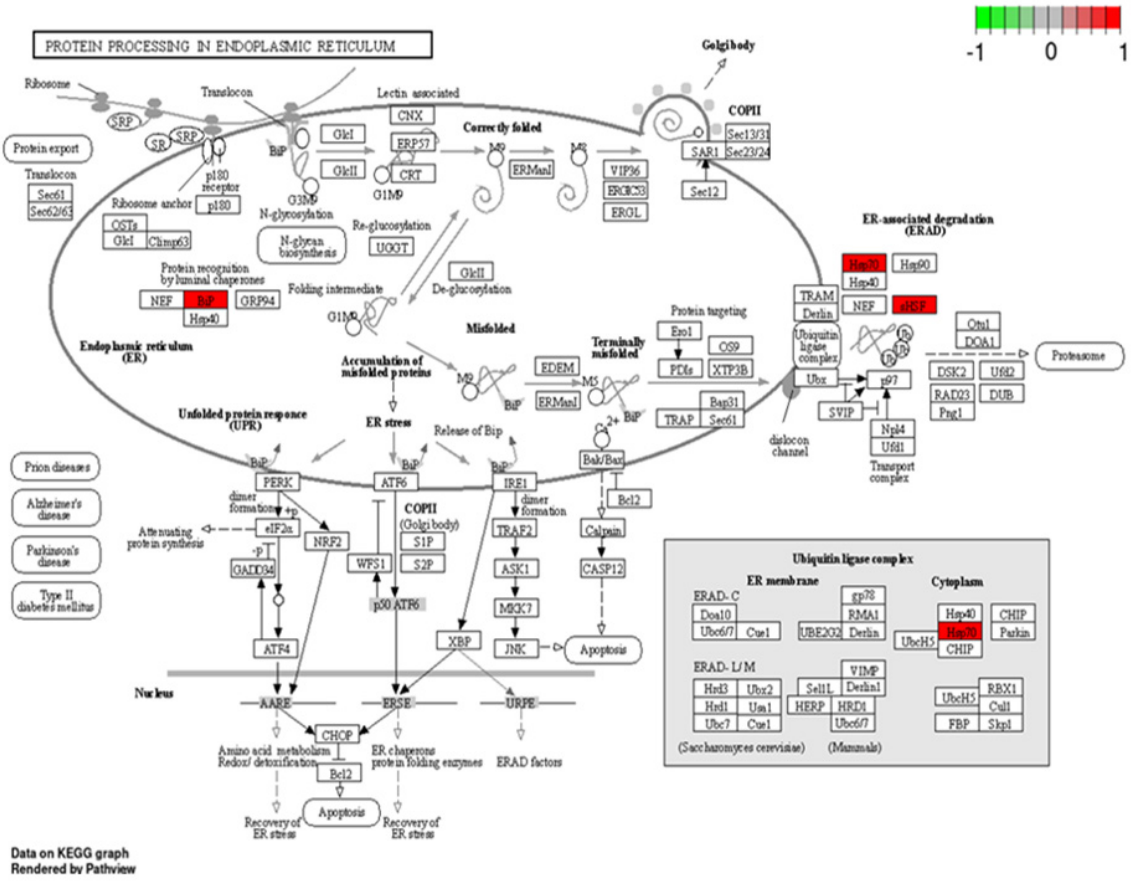

Figure 1. Scheme of the protein processing in endoplasmic reticulum pathway. Genes with the highest activity are marked in red.

Significant high expression level of genes *sHSF* and *BiP* (expression fold change 27x and 5x, respectively) in comparison to the uninfected plants was noted in inoculated root samples of PJ-173/2012 evaluated in season 2023. Additionally, upregulation of all selected genes was observed in M.9 standard rootstock, collected in the season of 2022. Only in case of *Hsp70* downregulation was calculated for newly selected apple rootstock PJ-173/2012 infected with *P. cactorum* and collected in the season of 2022. To summarize, for genes *sHSF* and *BiP* significant upregulation, in comparison to the controls, was noted in the genome of infected PJ-173/2012 in season 2023, while in the same season, downregulation of those genes was observed for immunized M.9 (Figure 2). GO enrichment analysis confirmed the involvement

of selected genes in the plant Genetic Information Processing, thus possibly explaining their role in general initiation of plant disease response process.

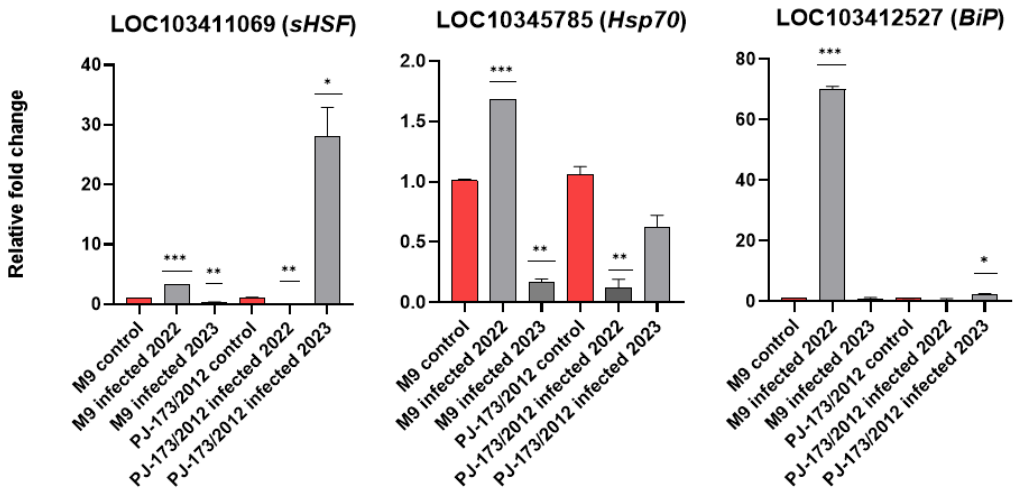

Figure 2. Expression profiles of genes from the protein processing pathway of endoplasmic reticulum calculated in the genomes of two apple rootstock genotypes. Diagrams present an average relative gene expression data with standard error of the mean (±SEM) compared to control not infected by *P. cactorum* (red bars) and t-test significance calculation level p<0,05*, 0,01**, 0,001***, normalized to *Md18sRNA* gene (showing stable expression in the experiment layout). The relative expression of gene of interests was calculated using mathematical Equation $2^{-\Delta\Delta CT}$ (RotorGene 6000 Series software 1.7) and visualized with GraphPad Prism10.0.3 software.

Moreover, our results also confirm the insights of other authors explaining the fact that plant disease response is a complex trial and one and the first plant defense mechanism is a programmed outbreak in transcription and translation of pathogenesis-related proteins, rely on ER processing (Korner et al., 2015). So far only several ER stress genes were described as upregulated during early stages of immune responses, and enhancing ER capacity is needed for plant cell immunity. This response, so called the unfolded protein response (thus the genes encoding binding protein or ER-located molecular chaperones), requires the transduction of a signal from the ER to the cell nucleus (Koizumi et al., 2001). Releasing new gene sequences belonging to this group complement the previous knowledge and gives new insights in understanding the complexity of resistance mechanism of pathogen in apple rootstocks that still remains unclear.

The conducted year to year observations explained the root rot disease symptoms occurs on trees between 3 and 8 years old and mainly grown on Malling (M) group of rootstocks (Ellis, 1997, Treviotdale and Gubler, 1997). Our test, based on the gene activity measurements (also verified in the years of the experiment) confirmed the upregulation of only one of the identified genes (BiP) in the genome of M.9 seedlings. In addition, we have observed that this gene was highly activated in the RNAseq experiment of infected PJ-173/2012 plant, and the type of regulation observed in qRT-PCR test (between the evaluated seasons 2022 and 2023) was different. A similar observation of inconsistencies between RNA-seq and RT-qPCR comprehensive analysis was reported by Everaert et al. (2017). The authors highlighted, that depending on the analysis workflow, 15-20% of genes usually are considered as 'non-concordant' in regard to the results obtained with RNA-seq and RT-qPCR (Everaert et al., 2017).

Moreover, we have observed, that the recognized genes in the genome of M.9 showed less activity in comparison to the selected PJ-173/2012, thus leading to the disease symptoms development. However, at the present stage of the research, we could consider the selected

apple rootstock PJ-173/2012 as less susceptible to *P. cactorum* infection b in comparison to the M.9 standard. This also confirms the previous characterization of this rootstock, being classified as moderately susceptible. Our preliminary results give new insight in the characterization of new apple rootstock selection, which may be consider as good resource for commercial application in apple breeding programs.

In woody plants, the root rot disease develops slowly, even during 2-3 seasons. The genes, identified based on the transcriptome reads comparison and involved in the protein processing pathway in endoplasmic reticulum, can be considered as potentially regulating plant response at early stage of disease development (detection of gene expression/no disease symptoms). Additionally, it should be noted that changes in the expression of the studied genes showed significant differences in individual research seasons. So, the molecular plant response to the disease is probably modulated by varying climatic conditions (Ramasamy et al., 2021). Researchers also underline, that if rootstocks with high/moderate susceptibility are stressed by the prolonged flooding conditions, they may become more susceptible to infection (Carisse and Khanizadeh, 2005), which make the mechanism of trait regulation more complex.

CONCLUSIONS

Our preliminary results confirm that genes, selected from the protein processing pathway of endoplasmic reticulum, could be considered as being the first activated in the plant response to the infestation by *P. cactorum*. They may be applied as potential functional molecular markers for the selection of newly developed apple rootstocks in breeding programs. However, further genetic and breeding research will enable verification of the possibility of their use for the early selection process of rootstocks for apple trees resistant to root rot disease, assisted by molecular markers (MAS, Marker Assisted Selection).

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