Identification of genes differentially expressed in resistance reaction to *Didymella pinodes* in *Pisum sativum* L. field experiment.

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Introduction

Ascochyta blight, caused by *Didymella pinodes*, is the most destructive foliar pathogen of peas in the temperate zones of Europe (Fig.1). This disease causes 10% yield losses as an average and can reach 50% under certain conditions. Current control practices are uneconomic and inefficient. Very little is known on the mechanisms or genes that control host plant resistance against this fungus. Flavonoids are compounds with antioxidant and antimicrobial properties. Isoflavone synthase is the key enzyme catalyzing the biosynthesis of isoflavonoids, which have demonstrated efficient antibacterial and antifungal activities (Fig.2). Herein, we investigated the expression change of isoflavone synthase in eight cultivated pea accessions before inoculation and in 6 time points after inoculation.

Fig.1. Lesions caused by *Didymella pinodes* on *Pisum sativum* (fot. by L. Boros)

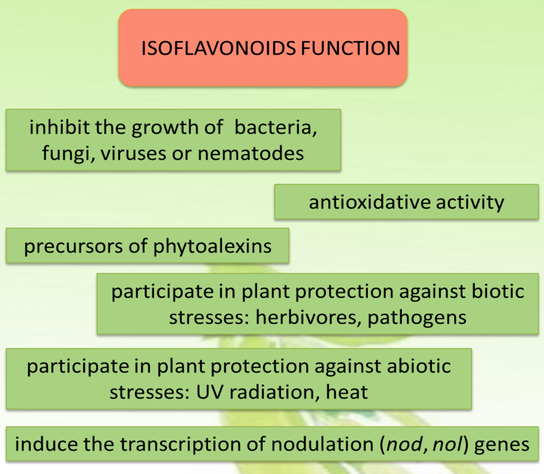


Fig. 2. Isoflavonoids function.

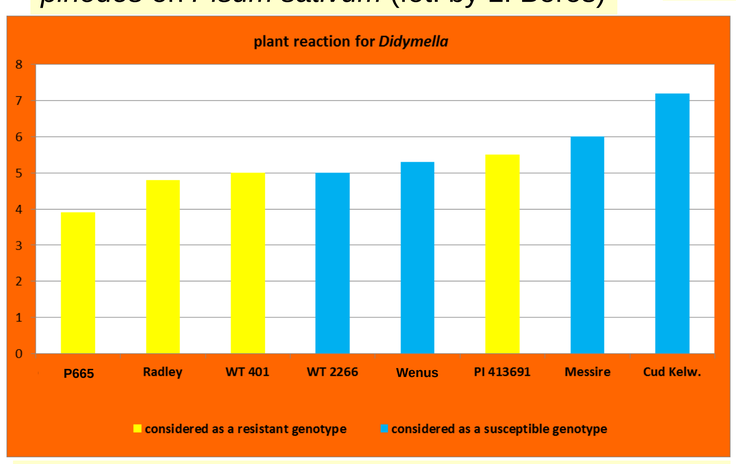


Fig. 3. Pea cultivars reaction for *Didymella* in the field experiment.

Materials and methods

2.1 Plant material and inoculation

Plants from eight pea cultivars were used for leaf tissue sample collection under field conditions in Radzików (52°21' N 20°38' E) (Tab.1). Cultivars were selected on the basis of observation disease severity over the years (data not shown).

2.1 Plant material and inoculation cont.

Leaf samples were collected in the 5-6 leaf stage (control) before inoculation and in 6 time points after inoculation (4, 8, 16, 24, 48 hours and 8 days) with a spore solution of 12 day culture of *D. pinodes.* The concentration of spores in the solution was adjusted to $2x10^6$ spores per milliliter. Five plant samples from each cultivar for each treatment (control/inoculated) were harvested and stored at −80 °C. Disease severity assessment was done, according to Xue et al., 1996 (the scale: 0 – resistant, 9 – susceptible). The assessment of plant reaction for *Didymella* was carried out 38 or 52 days after inoculation.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from 30-80 mg of leaf tissue by using SV Total RNA Isolation System (Promega). The concentration and purity of RNA samples were tested using NanoDrop ND-1000 spectrophotometer. Integrity of the RNA was tested by using 0,8% agarose gel electrophoresis with Midori Green Advance DNA Stain. The RNA samples were diluted with nuclease-free water to 1 µg in 12 µl and reverse transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche) and oligo dT primer according to manufacturer's protocol. The cDNA preparations were diluted 5-times with nuclease-free water to use as template in qPCR analysis. To confirm the total absence of genomic DNA, cDNA was used as a template for PCR amplification using primers to multiple *chitynase* (JI899851.1).

2.3 Gene of interest

Isoflavone synthase (*IFS*; AF532999.2) is a crucial enzyme in biosynthesis patchway of phenolic secondary metabolites - isoflavonoids.

2.4 Reference gene validation

As reference genes for normalization, we used previously reported as suitable for normalizing mRNA expression in *P. sativum*: elongation factor (EF1) and actin (ACT; PsCam042218). Determination of the best reference gene or best gene pair was performed using NormFinder software (ver. 0.953) (http://moma.dk/normfinder-software/) (Andersen et al. 2004) based on Ct value.

2.4 Quantitative real-time PCR analysis

Primers were designed using OligoAnalyzer 3.1 and OligoCalc: Oligonucleotide Properties Calculator to create a product of 100 bp and avoid self complementarity and primer-dimer formation. The specificity of the primers was confirmed by SYBR Green melting curve analysis. The efficiency of each primer pair was calculated based on slope of the line ($E = 10^{-1/slope}$) considering an ideal value range of 0.95 to 1.5, by optimization primer: probe ratio. All qPCRs were carried out in 96 well plates LightCycler® using 200 ng of first strand cDNA templates, LightCycler® 480 Probes Master, and proper amount of each primer. The qPCR reactions were conducted using the PCR settings of pre-incubation at 95°C for 600 s, 45 cycles of 2 step amplification and 30s of cooling in 37°C. Fluorescence readings were obtained using FAM channel in LightCycler® 96 Roche. For each sample, two technical replicates were performed.

Results

The assessment of plant reaction for *Didymella* was carried out 52 days after inoculation, while the grade for the P665 form comes from 38th day after inoculation. The resistant cultivars exhibited 3.9-5 score. Susceptible genotypes showed 5-7.2 score. Initially line PI413691 was considered as a resistant genotype. However it behaved as a susceptible genotype in the field experiment (less resistant than Wt2266 and Wenus cultivars) (Fig. 3).

3.1 Reference gene validation

NormFinder algorithm indicated the *EF1* and ACT pair as the most stable genes in this case, the stability value for that combination was 3.64.

3.2 Quantitative real-time PCR analysis

The mean expression value for *IFS* before inoculation was 28.8 Ct, 4 and 8 hours after inoculation 23.9, 16 hours after inoculation 26.6, 24 hours after inoculation 27.7, 48 hours after inoculation 31.9 and 8 days 30.6. Fold change ($2^{-\Delta\Delta CT}$) control vs. inoculated samples, showed that the expression of *IFS* increased 9 times in the resistant cultivars and 7 times in the susceptible ones in 4th hour after inoculation. The expression decrease was 0.7 for resistant cultivars and the increase 1.0 for susceptible cultivars in 8th hour, the increase 1.7 for resistant cultivars and the decrease 0.8 for susceptible cultivars in 16th hour, the increase 1.7 for resistant cultivars and the decrease 0.6 for susceptible cultivars in 24th hour, the decrease 0.1 for resistant cultivars and 0.04 in 48th hour and decrease 0.9/0.8 in 8th day after inoculation (Fig.4). The negative correlation between $\log_2(2^{-\Delta\Delta CT})$ of *IFS* mRNA transcription levels and disease severity was the strongest in 24th hour. Pearson's correlation coefficient was r =-0.70 (Fig.5).

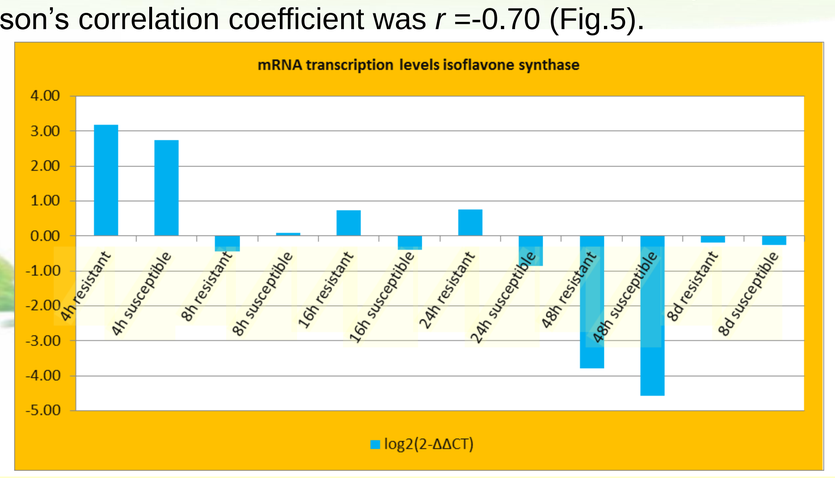
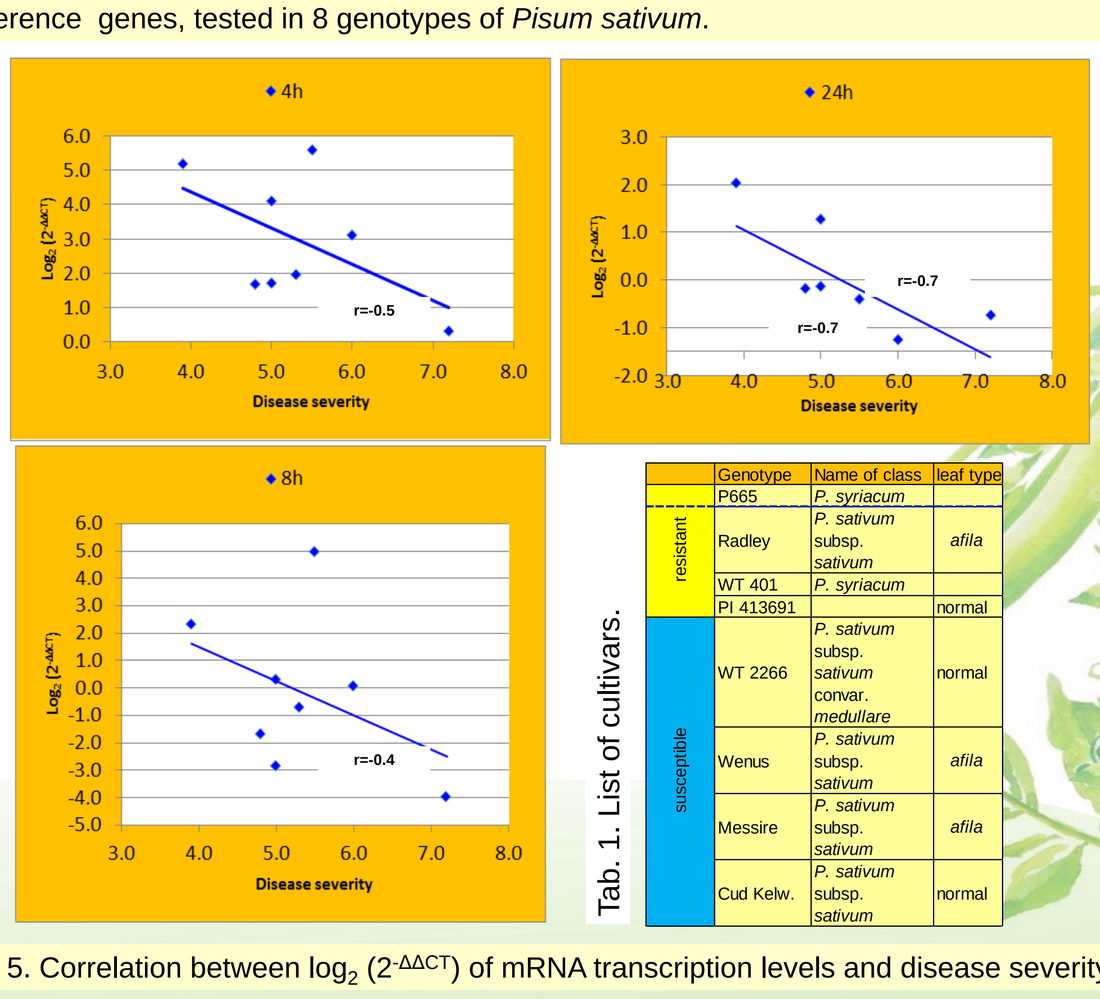


Fig. 4. mRNA transcription levels of ISF - isoflavone synthase and geometric mean of 2 reference genes, tested in 8 genotypes of *Pisum sativum*.



Tab. 1. List of cultivars.

	Genotype	Name of class	leaf type
resistant	P665	*P. syriacum*	
	Radley	*P. sativum* subsp. *sativum*	*afila*
	WT 401	*P. syriacum*	
	PI 413691		normal
susceptible	WT 2266	*P. sativum* subsp. *sativum* convar. *medullare*	normal
	Wenus	*P. sativum* subsp. *sativum*	*afila*
	Messire	*P. sativum* subsp. *sativum*	*afila*
	Cud Kelw.	*P. sativum* subsp. *sativum*	normal

Fig. 5. Correlation between $\log_2$ ($2^{-\Delta\Delta CT}$) of mRNA transcription levels and disease severity.

Conclusions.

Isoflavone synthase is overexpressed after infection with *D. pinodes* in all used pea cultivars and expression level is negatively correlated with disease severity. According to Fondevilla et al. (2014) expression values obtained by SuperSAGE experiment and qRT-PCR, showed that IFS expression in leaves of *P. sativum* line P665 increased (1.04; 0.85) after 24 hour after inoculation by *D. pinodes*, under control conditions. In our study the highest increase of ISF expression in the resistant genotypes and the decrease in susceptible ones was observed in 24th hour in field experiment.

As demonstrated in previous research by Shiraishi et al. (1978) *D. pinod*es is tolerant to pisatin (main isoflavone produced by *P. sativum*), but increase of ISF expression suggests that isoflavones take part in defence against thise fungus. Considering that isoflavonoids participate in plant protection against abiotic and biotic stresses and induce the transcription of nodulation genes the role of isoflavone synthase in response for *D. pinodes* infection deserve more detailed analysis in the future.

References:
Andersen et al. (2004) Cancer Res 64: 5245-5250
Fondevilla et al. (2014) Plant Mol Biol Rep 32:258–269
Shiraishi et al. (1978) Ann. Phytopath. Soc. Japan 44:641-645
Xue et al. (1996) Can J Plant Sci 78: 685–689.

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