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# Early Response of Defense Related Genes to Secondary Downy Mildew Infection in Sunflower Line with *Pl6* Gene

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**Abstract:** Sunflower line with resistance for downy mildew (Ha-26R) and susceptible line (Ha-26S) were inoculated with the suspension of *Plasmopara halstedii* zoosporangia, race 730, in the phase of first pair of leaves. The expression of defense related genes was investigated in the time period of 2 to 96 h after treatment. Several categories of defense-related genes: signalization (EDS1 and EDR1); $H_2O_2$ producing (Hacaox and Haoxox); antioxidative response (SODc and SODp), pathogenesis related (chi, PAL, PR5) were examined. Most examined defense related genes were constitutive with higher expression in resistant line. However, in response to secondary downy mildew infection six genes were upregulated. Upregulation of HaEDS1 signaling gene 2 h after infection indicates that SA mediated response is activated. Pathogenesis related genes: chitinase and PR5, were also upregulated in the earliest time point. Other defense related genes: SODp, Caox and OxOx were sequentially upregulated from 4 to 48 h after infection in resistant line. Our results indicate that the early response of defense related genes to secondary downy mildew infection, resembles to hypersensitive-like reaction and is connected with resistance conferred by *Pl6* gene. According to our results resistance to secondary infection is characterized with earlier upregulation of PR5 in comparison to primary infection.

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## Introduction

Sunflower (*Helianthus annuus* L.) belongs to the most important annual oil crops. However, sunflower production is endangered by a number of pathogens. Downy mildew (DM) caused by *P. halstedii* is one of the major problems. DM is one of the most important diseases of sunflower because this disease can decrease yield up to 85% (Göre, 2008). *P. halstedii* may induce disease symptoms of various kinds depending on age of tissue, level of inoculum, environmental conditions (moisture and temperature) and cultivar reaction. Economically and epidemiologically relevant sunflower DM incidences are considered to derive from primary soil born infections with *P. halstedii*.

However secondary infections can occur when zoosporangia are trasferred from infected plants to the leaves of healthy plants if adequate free moisture is present. Spring (2009) has shown that the epidemiological risk of airborne secondary infections, that may occur in all developmental stages of sunflower, has been underestimated. Control of this disease by the application of fungicides is not effective for secondary infections and actually contributed to the appearance of new virulent races. Over the past 7 years this number increased to a total of 24 races in Europe and 36 in America (Sedlarova et al., 2016; Virányi et al., 2015).

Two types of resistance to *P. halstedii* have been recognized in sunflower, resistance type I and type II. In type I resistance the growth of the pathogen is restricted in the basal region of the hypocotil, however in type II resistance pathogen can reach cotyledons after infection (Radwan et al., 2011). The recognition of biotic stress by plants starts by binding of signalling molecules for receptors on cell membranes, which initiates intracellular signalling network and induction of gene expression. Generally in resistant plants incompatible plant-pathogen interaction often results in a hypersensitive response (HR), resulting with programmed cell death at the site of attack within 12–24 h of inoculation (Levine et al., 1996), which can happen later during the days after *P. halstedii* has penatrated the sunflower host (Radwan et al., 2005a) Hypersensitive reaction (HR) response is characterized by numerous physiological and molecular changes and induces SAR (Systemic acquired resistance). The emergence of the SAR is associated with local and systemic increasing levels of salicylic acid as well as with the expression of specific sets of genes encoding the synthesis of PR proteins (Kombrink and Schmelzer, 2001).

Production of reactive oxygen species (ROS) and pathogenesis-related proteins are among early molecular changes in HR (Oliveira et al., 2016). It has been suggested that oxalate oxidase (Haoxox) and carbohydrate oxidase (Hacaox) could also have an important role in $H_2O_2$ production in defense response of sunflower (Hu et al., 2003; Custers et al., 2004). Stronger accumulation of antioxidative scavengers transcripts, superoxide dismutase (SOD) and glutathione peroxidase (GPX), indicate that these enzymes are involved in HR of sunflower to DM infection (Herbette et al., 2003). Increased expression of defense related genes phenylalanine ammonium-lyase (PAL) and chitinase (CHI) were shown to be good markers of resistance to secondary infection of sunflower in period up to 72 h after infection by *P. halstedii* (Mazeyrat et al., 1999). Pathogenesis related protein 5 gene (PR5) is induced at about 6 days post infection in both compatible and incompatible interaction when exposed to primary infection (Radwan et al., 2005a, b). However its relative transcript accumulation increases up to 20 times in type II resistant sunflower lines (Radwan et al., 2011).

In this work we have investigated the early response of ten defense related genes to DM infection with race 730 in sunflower line with *Pl6* gene in order to reveal molecular reactions supporting resistant response to secondary infection.

# Materials and methods

## Sunflower genotypes and infection procedure

Sunflower inbred line Ha26 S, with high general combining abilities (Škorić et al., 2000) was used as a recipient of *Pl6* gene from the initial cross with Ha336 (Panković et al., 2007) and after several cycles of backcrosses converted to a resistant line. Sunflower seed of two sunflower line, with and without *Pl6* gene, i. e. resistant and susceptible to DM (race 730) were surface sterilized by dipping in 10 % sodium hypochlorite for 5–10 min, thoroughly rinsed in tap water, and incubated in the dark under saturated humidity in a germinator at 25 °C for 24 to 36 h. Germinated, healthy and uniform seedlings were transfered to trays with a mixture of peat:sand (3V:1V). Plants were grown in climate chamber at light intensity 10 000–12 000 lux (16 h), temperature 17–19 °C and 70 % humidity (Tourvieille de Labrouche et al., 2000). When the first pair of true leaves appeared plants were spreyed with water (control) or with suspension of spores of *P. halstedii* (race 730). In the time period of 2 to 96 h after treatment leaves of sixteen plants per time point were harvested, bulked and immediately frozen in liquid nitrogen, and stored at -70 °C until use. Remaining plants were

**Table 1:** Defense related genes: primer sequences (F-Forward, R-Reverse), Tm (°C) and number of cycles used for polymerase chain reaction.

| Gene | Description | Gene category | Primer sequence | Tm (°C) | Number of cycles | Reference | Accession no. |
|---|---|---|---|---|---|---|---|
| Ha-EDS1 | Enhanced Disease Susceptibility | Signalling gene | F 5'-GTCCCTCGGATAATGCTTGCTC-3'<br>R 5'-CATGCAGCATAACTTGCAACAG-3' | 53 | 36 | Radwan et al. (2005b) | AY645209 |
| Ha-EDR1 | Enhanced Disease Resistance | Signalling gene | F 5'-TTATACAAACTGCTCCATCGTAC-3'<br>R 5'-TTGGAGTCTTCAAATCCCTGTGC-3' | 55 | 33 | Radwan et al. (2005b) | AY645208 |
| Haoxox | Oxalate oxidase | $H_2O_2$ producing | F 5'-GTTCAAGAGGGTGGACTTCG-3'<br>R 5'-TTTCTCCCCATTCACTTGCT-3' | 59 | 30 | Hu et al. (2003) | AU718274 |
| Hacaox | Carbohydrate oxidase | $H_2O_2$ producing | F 5'-TCTTCTGCAACTTCCCGTTC-3'<br>R 5'-AACATGTTCGGCTGTGATGA-3' | 54 | 33 | Custers et al. (2004) | AAL77103 |
| HaSODc | Cytosolic Cu/Zn – superoxide dismutase | Antioxidative response | F 5'-AATCCTCATGGAAAGGAGCA-3'<br>R 5'-TCATCAGGATCAGCATGGAC-3' | 59 | 30 | Herbette et al. (2003). | AY172569 |
| HaSODp | Plastidic Cu/Zn – superoxide dismutase | Antioxidative response | F 5'-CAAACGGGTGCATATCAACA-3'<br>R 5'-AAGCTCATGAACCACCAAGG-3' | 59 | 30 | Herbette et al. (2003) | AY172568 |
| HaPR5 | Pathogenesis related protein 5 | Pathogenesis related | Forward 5'-GGATGCAACAATCCTTGCACGG-3'<br>Reverse 5'-TGGTCGGGTCATCTTGAGGG-3' | 59 | 30 | Radwan et al. (2005a) | AF364864 |
| HaPAL | phenylalanine ammonia-lyase | Pathogenesis related | F 5'-ATAACAATGGGTTGCCTTCG-3'<br>R 5'-GTTTTCCTTGCGGAAATCAA-3' | 59 | 30 | Mazeyrat.et al. (1999) | Y12461 |
| Hachi | Chitinase | Pathogenesis related | F 5'-TTGGGTCTGTGGCTAACAAA-3'<br>R 5'-GATTTGCATGGGGATATTGC-3' | 59 | 30 | Mazeyrat et al. (1999). | U96640 |
| Ha-EF1 | Constitutive elongation factor | House-keeping gene | F 5'-AGGCGAGGTATGATGAAATTGTCA-3'<br>R 5'-GTCTCTTGGGCTCATTGATTTGGT-3' | 59 | 30 | Körösi et al. (2011) | AAM19764 |

grown up to phase of 3 pairs of true leaves, in order to evaluate the symptoms of diseases (Tourvieille de Labrouche et al., 2000).

### Gene transcript accumulation analysis

Gene transcript accumulation analysis was performed by Reverse Transcription-PCR. Total RNA was isolated by RNAeasy kit (Quiagen). cDNA synthetised by RevertAid First Stand cDNA Synthesis Kit (Fermentas), was used as template in PCR to examine the expression pattern of several defense related genes. (Table 1). PCR conditions for all primers used in this study were as follows:

PCR amplifications were carried out with one µl of cDNA in the presence of 0.4 mM of each dNTP, 1 U of Taq DNA polymerase (Fermentas), 1XTaq polymerase buffer, 0.6 µM of each primer, 3 mM $MgCl_2$, 2 µg/µl BSA. PCR with all primers (Table 1.) were carried out in a Mastercycler gradient Eppendorf thermocycler under the following conditions: 4 min at 95 °C, 30 cycles for 10 s at 95 °C, 30 s at annealing temperature given in Table 1, 30 s at 67 °C (primer extension), and 5 min at 67 °C (final extension). PCR products were separated using 1% TBE agarose gel electrophoresis. PCR reactions were done in 2 replications.

Gel images were stored with the Bio-print system (Vilber Lourmat, France). The signals from ethidium bromide-stained gels of investigated genes were quantified using BioCapt program (Vilber Lourmat, France), normalized over the signals from constitutive probes (Ha-EF-1α- sunflower elongation factor 1α) and presented as relative transcript accumulation as in Radwan et al. (2005).

## Results

In this study we have investigated the early response of defense related genes, up to 96 h after secondary infection with the suspension of *P. halstedii* zoosporangia, in sunflower plants at the stage of first pair of true leaves. However, the symptoms of the disease were evaluated when plants were in the stage of 3 pairs of true leaves. Susceptible line developed typical disease symptoms, i. e. leaf chlorosys with or without sporulation, which was not the case with resistant line (Figure 1).

Leaf samples taken from 2 to 96 h after inoculation, were used to examine the expression pattern of several defense related genes. The expression of a gene involved in signaling, HaEDS1, was mainly constant in susceptible line and lower in comparison to resistant line. This gene was upregulated in resistant


**Figure 1:** (A) Symptoms of chlorosis on the reverse side of leaves in susceptible line (Ha26 S); (B) plants of resistant (Ha26 R) sunflower line without disease symptoms after inoculation with race 730 of *Plasmopara haldstedii*.

sunflower line 2 to 4 h after infection (Figure 2), which was followed by down-regulation. These results indicate that the increased expression of this signaling gene and synthesis of stress proteins, occurs only in the early stages after sunflower infection with spores of *P. halstedii*.

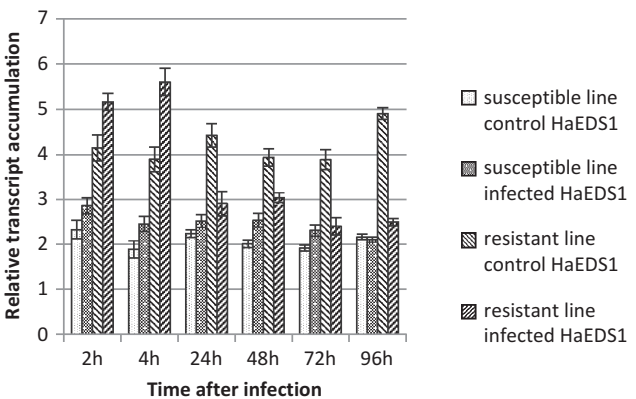


**Figure 2:** Accumulation of defence-related gene transcript (HaEDS1) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

The expression of second signaling gene HaEDR1 was constitutive and decreased by infection in similar manner in both examined lines (results not presented).

Constitutive expression of carbohydrate oxidase (Hacaox) in both analyzed sunflower lines, was observed up to 48 h after treatment. Expression was approximately 2 times higher in the resistant line. However a reduction the expression of this gene occurred in both sunflower lines 72 and 96 h after infection (Figure 3).

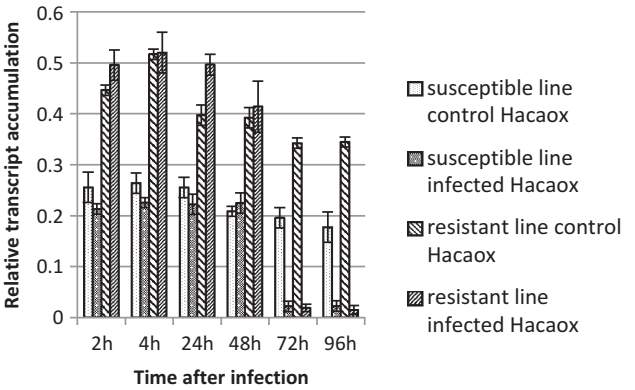


**Figure 3:** Accumulation of defence-related gene transcript (Hacaox) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

Expression of the oxalate oxidase (Haoxox) is about of 3 times higher in the resistant line up to the 24 h after infection.The expression of this gene was stable in the course of measurements both in control and infected plants. However, in the resistant line, 48 and 72 h after the infection, this gene is upregulated in infected plant, to be decreased at 96 h (Figure 4).

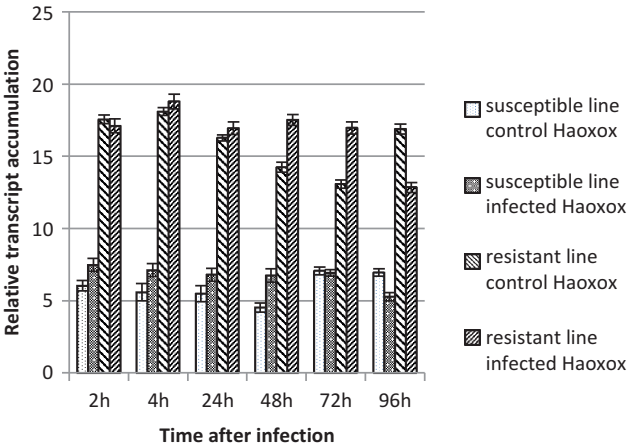


**Figure 4:** Accumulation of defence-related gene transcript (Haoxox) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

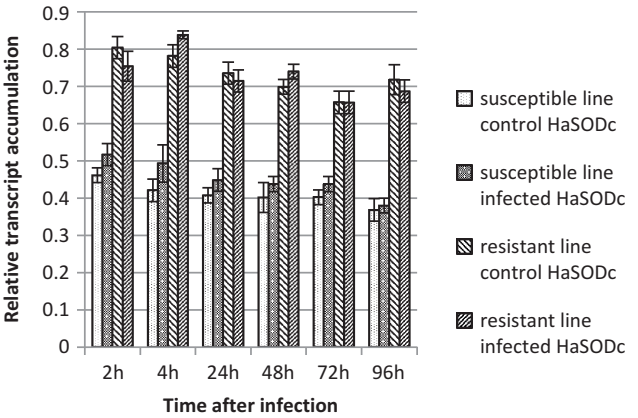


**Figure 5:** Accumulation of defence-related gene transcript (HaSODc) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

In both control and infected leaves, transcript of antioxidative enzyme SODc was about 2 times higher in resistant line than in susceptible line (Figure 5).

The expression of another antioxidative scavenger gene, SODp was generally higher, in resistant line in particular (Figure 6). The tendency of higher expression in infected leaves of resistant line was observed throughout the investigated period after infection.

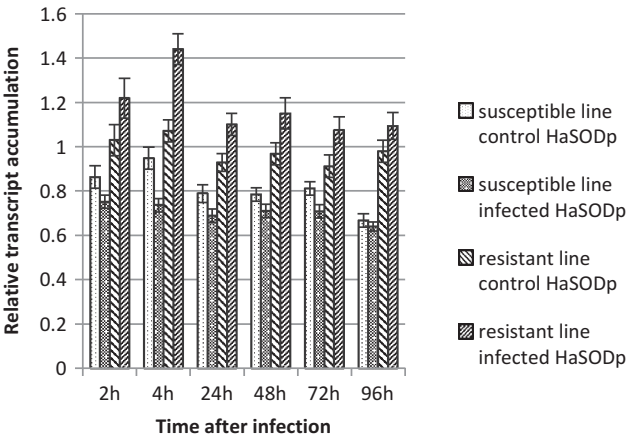


**Figure 6:** Accumulation of defence-related gene transcript (HaSODp) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

PAL was highly expressed in all samples (results not shown).

Expression of chitinase (chi) one of the PR (Patogenesis Related) genes, was also up to 2 times higher in the resistant line. Infection induced expression of chitinase in resistant line already 2 h after treatment (Figure 7). This induction was the most pronounced 24 h after infection in resistant line, followed by the decrease of this transcript in all samples.

The only gene that was induced early after infection only in resistant line was HaPR5 (Figure 8). In time period 2 h and 4 h after infection, HaPR5 gene was

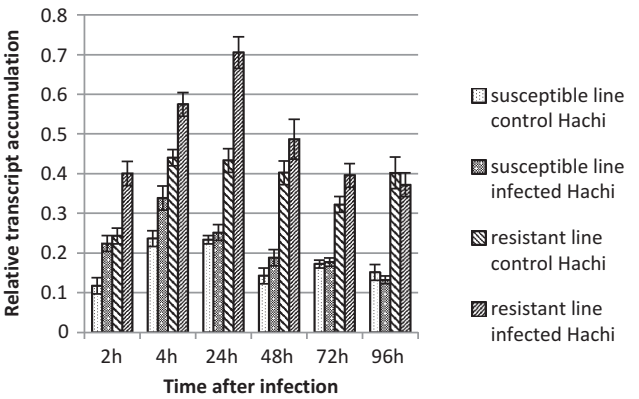


**Figure 7:** Accumulation of defence-related gene transcript (Hachi) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

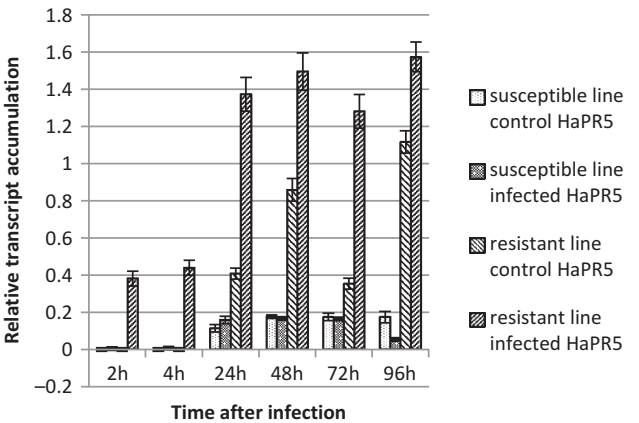


**Figure 8:** Accumulation of defence-related gene transcript (HaPR5) in susceptible and resistant sunflower line 2–96 h after infection with race 730 of *P. halstedii* or control treatment with water. The means of the relative transcript accumulation is presented ± SD ($n = 2$).

expressed only in the infected resistant sunflower line. In all the time intervals, expression of HaPR5 gene in resistant line was significantly higher in infected, compared with control treatment. 96 h after infection HaPR5 gene expression was more then 15 times higher in infected resistant than in susceptible infected line.

## Discussion

The processes that determine the outcome of an interaction between plants and pathogens are complex. Identification of genes differentially expressed in the compatible and incompatible interaction allow understanding of the molecular mechanism of interaction in order to design new strategies to improve plant resistance (Baldo et al., 2010). By comparing patterns of host defense-related gene expression in the two sunflower lines, we monitored several categories of defense-related genes: signalization (EDS1 and EDR1); $H_2O_2$ producing (Hacaox and Haoxox); antioxidative response (SODc and SODp), pathogenesis related (chi, PAL, PR5). Changes in gene expression, in response to secondary infection with suspension of spore of *P. halstedii* (pathotype 730), were investigated in two sunflower lines in the time interval 2–96 h after infection, in order to follow the early response of these genes to the infection with this pathogen. So far the defense related genes in sunflower were mainly investigated in response to primary infection of seedlings with *P. halstedii*, i. e. at earliest 18 h after infection (Radwan et al., 2005a, 2011).

In this work we have used resistant sunflower line with *Pl6* gene (Panković et al., 2007). The extensive study of Radwan et al. (2008) indicates that *Pl6* locus is complex with many different functional genes conferring resistance to several *P. halstedii* races. Indeed, another TIR-NBS-LRR candidate gene for resistance to race 300, was recently found at this locus (Franchel et al., 2013). Radwan et al. (2011) have suggested that sunflower resistance to downy mildew is supported by constitutive expression of TIR-NBS-LRR genes accompanied by hypersensitive response. In general NBS-LRR class of resistance proteins are receptors that might interact with pathogen effectors and/or detect the effector action (Dangl and Jones, 2001). Results on the mechanisms of recognition between TIR-NBS-LRR proteins and pathogen effectors in sunflower are scarce to our knowledge.

EDS1 signalling gene was shown to be a regulator of salycilic acid (SA) mediated resistance response required for TIR-NBS-LRR gene based resistance in Arabidopsis (Brodersen et al., 2006), and is strongly induced by pathogen infection (Bilgin et al., 2010). However EDR1, was reported as negative regulator of

HR cell death (Tang et al., 2005). Radwan et al. (2005b) observed constitutive expression of EDS1 and EDR1, i. e. these genes were not induced with *P. halstedii* race 300 primary infection. Our experimental system of secondary infection provided analysis of early changes in gene expression. We have seen that HaEDR1 was constitutive and decreased upon infection in both lines, however EDS1 signalling gene was upregulated already 2–4 h after infection in resistant line. Upregulation of EDS1 gene indicates that SA mediated response is activated (Brodersen et al., 2006).

In many plant pathogen reactions $H_2O_2$ production is an early event. Beside direct antimicrobial activity $H_2O_2$ is a signal leading to activation of pathogenesis related genes (Guidetti-Gonzalez et al., 2007). Hu et al. (2003) have shown that the expression of Hacaox in sunflower is significantly induced by $H_2O_2$. However the results of Custers et al. (2004) indicate that it can also be induced by fungal pathogens in sunflower and is probably responsible for the generation of extracellular $H_2O_2$. Overexpression of this gene in sunflower conffered enhanced resistance to oxalic acid generating fungus *S. sclerotiorum* (Hu et al., 2003). It has been suggested that Haoxox could also be involved in $H_2O_2$ production in response to biotic and abotic stress (Hu et al., 2003; Custers et al., 2004). In our experiment the expression of both genes was about two times higher in resistant line. Hacaox and Haoxox were upregulated 24 h and 48–72 h after infection in resistant line, which indicates that they are not involved in early reactive oxyge species production.

The increased expression of antioxidative scavenger genes was often noticed in case of HR. Herbette et al. (2003) have observed upregulation of HaSODc and HaSODp after secondary DM infection indicating increased potential for antioxidative response (Herbette et al., 2003). In their experiment SODc transript transiently increased 3–6 h after secondary infection of resistant sunflower plant. We have observed the upregulation of SODp gene in resistant sunflower line after infection throughout the experiment, with maximum at 4 h of treatment.

Literature data indicate that genes for PAL and chitinase might be good markers for resistance in the period up to 72 h after secondary infection of sunflower with *P. halstedii*. In our experimental system the expression of PAL was constitutive and similar in both lines and was not upregulated by infection. Chitinase was however upregulated in resistant line 2–72 h after infection in confirmation to previously mentioned results (Mazeyrat et al., 1999).

There are 17 known classes of PR proteins that are synthesised in plants after pathogen attack PR-1 to PR-17 (Saboki et al., 2011). PR-5 class of proteins have antifungal properties. It was shown that PR-5 gene expression was induced from the 6th day after infection with *P. halstedii* race 300 in both resistant and susceptible lines, however relative accumulation of this gene was up to 15 times

bigger in sunflower lines with type II resistance to DM (Radwan et al., 2005a, b, 2011) We have observed early induction of this gene, already 2 to 4 h after infection only in resistant line. One day after treatment this gene is expressed in both lines. However the expression of this gene is about 15 times bigger in resistant line 96 h after infection, which is in accordance with results of Radwan et al. (2011).

## Conclusions

Most examined defense related genes were constitutive with higher expression in resistant line. However, in response to secondary downy mildew infection six genes were upregulated. HaEDS1 signaling gene and pathogenesis related genes: chitinase and PR5, were upregulated in the earliest time point. i. e. 2 h after infection. Other defense related genes: SODp, Caox and OxOx were sequentially upregulated from 4 to 48 h after infection in resistant line. Our results indicate that the early response of defense related genes to secondary downy mildew infection, resembles to hypersensitive-like reaction and is connected with resistance conferred by *Pl6* gene. Presented results indicate that resistance to secondary infection is characterized with earlier upregulation of PR5 in comparison to primary infection.

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