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PHENOLIC COMPOUNDS AND PEROXIDASES IN SUNFLOWER NEAR-ISOGENIC LINES AFTER DOWNY MILDEW INFECTION

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SUMMARY

Two near-isogenic lines of sunflower, resistant (+*Pl6* gene) and susceptible (-*Pl6* gene) to downy mildew were used in this paper. Secondary infection with a suspension of *Plasmopara halstedii* spores was done on the plants in the phase of first pair of leaves. In the samples taken 12 h after infection, content of phenolic compounds in methanolic extracts from frozen leaves was analysed by HPLC. POD activity was determined spectrophotmetrically and POD isoforms by isoelectrophoresis.

Constitutive level of phenolic compounds and their accumulation after infection were higher in the susceptible than in the resistant line. Increased POD amount in leaf, constitutively present in NS-H-26R, was in correlation with increased guaiacol-dependent POD activity and low total phenolics contents. After infection, guaiacol- and chlorogenic acid-dependent POD activity significantly increased in both lines. Scopoletin-dependent POD activity was induced upon infection only in NS-H-26R. IEF electrophoresis revealed existance of four anionic isoforms of peroxidase in leaves of both lines. The main isoform with pI 5 was particularly intesified in the resistant line.

In conclusion, scopoletin-dependent POD activity that was induced upon infection only in resistant NIL indicates a specific role of POD in coumarin metabolism that is possibly connected with the presence of *Pl6* gene.

Key words: ***Helianthus annuus* L., *Plasmopara halstedii*, disease resistance, *Pl6* gene**

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INTRODUCTION

Sunflower (*Helianthus annuus* L.) is an important crop species that is grown primarily as a source of oil, as well as a source of high protein meal, for birdseed and ornamental purposes. One of the diseases that can severely decrease the yield of sunflower is downy mildew. It is caused by oomycetes of *Plasmopara halstedii* (Farlow) Barlese & de Toni, that has evolved quickly and presently consists of more than ten races. As new races can be only partially controlled by fungicides, genetic resistance to specific races of *P. halstedii* is one of the approaches in breeding for resistance to downy mildew (Škorić, 1992; Jan *et al.*, 2004). Among the twelve genes that confer the resistance to *P. halstedii* (Rahim *et al.*, 2002), *Pl6*, introduced from wild *Helianthus*, confers the resistance to four most abundant races (Miller and Gulya, 1991). Sunflower inbred line Ha26 S, with high general combining ability (Škorić *et al.*, 2000), was used as a recipient of the *Pl6* gene, and after several cycles of backcrosses was converted to a resistant NIL Ha-26R (Panković *et al.*, 2004, 2006; Saftić-Panković *et al.*, 2005). The fine structure map of the *Pl6* genomic region indicates that this locus is large and complex (Slabaugh *et al.*, 2003). Bouzidi *et al.* (2002) have shown that the presence of *Pl6* resistance gene analogues confers resistance to several downy mildew races, but their function is still not understood.

It is known that in response to biotic and abiotic stresses plants accumulate phenolic compounds that may be oxidized to brown compounds (polymerized phenolics) or to more reactive species, quinons, by phenol oxidases and peroxidases (Takahama and Oniki, 2000). Phenolics from the group of hydroxicoumarins *i.e.,* scopoletin, scopolin and ayapin, that were shown to accumulate in sunflower plants, exhibit antimicrobial activity (Tal and Robeson, 1986a,b; Gomez-Vasquez *et al.*, 2004; Prats *et al.*, 2006). Increase in peroxidase activity during incompatible plant-pathogen interactions is well documented and often correlates with the appearance of new isoforms (Bestwick *et al.*, 1998; De Gara, 2004; Kawano, 2003). In sunflowers resistant to sunflower stalk rot, scopoletin peroxidase activity is correlated with high levels of scopoletin, which supports the hypothesis of scopoletin peroxidase induction or activation by its substrate (15).

Therefore we examined the content of phenolics and peroxidase level and activity in order to investigate their role in hypersensitive response to sunflower downy mildew conffered by *Pl6* gene.

MATERIAL AND METHODS

Plant material and infection

Two near-isogenic sunflower lines, NS-H-26R with the *Pl6* gene conffering resistance to downy mildew and NS-H-26S susceptible to downy mildew, were investigated. After surface sterilization, seeds were germinated at 25°C from 24-

48 h. Seedlings with radicula length ≅ 1cm were sown in plastic trays with a mixture of peat : sand (3V:1V). Plants were grown at light intensity of 10 000-12 000 lux (16 h), temperature 17-19°C and 70% humidity (Tourvieille de Labrouche *et al.*, 2000). When plants developed the first pair of leaves, they were sprayed with a suspension of *P. halstedii* spores (40-70 × 10^3 zoosporangia/ml) or with water (control plants). Leaves from 4 plants were bulked, sampled 12 h after treatment, and frozen in liquid nitrogen.

HPLC analysis of phenolics

Frozen leaf samples were ground in liquid nitrogen and extracted in methanol for 5 min in ultrasonic bath. After centrifugation, the supernatant was analyzed in HPLC Agilent 1100 with diode array detector (measuring λ 280 nm; referent λ 600 nm). Samples were separated in Agilent ODS Hypersil column (length 200 mm; internal 2r 2.1 mm, cat. Num. 79916OD-572). The mobile phase was A: 5% CH_3COOH in water and B: methanol, the mobile phase gradient was 7-80%, with the rate of 0.5 ml/min and injection volume of 5 μl. Following standards were used: scopoletin, esculin, chlorogenic, caffeic and cinnamic acids (Sigma Aldrich Chemie) and ferrulic acid (Fluka Chemie).

Peroxidase activity

Several substrates such as guaiacol (A_{470}; ε=26,6 $mM^{-1}cm^{-1}$); chlorogenic acid (A_{410}) and scopoletin (A_{595}) were used as hydrogen donors and the absorbance increase was measured. The reaction mixture consisted of an aliquot of extract diluted 200 times and 1 mM H_2O_2 in 100 mM K-phosphate buffer (pH 6.0), with 30 mM guaiacol, or 4 mM chlorogenic acid, and 0.1 mM scopoletin. Reaction at 30°C was started by addition of H_2O_2.

Peroxidase level

Native electrophoresis was performed in 10% polyacrylamide gel and reservoir buffer consisting of 0.025 M Tris and 0.192 M glycine (pH 8.3) at 24 mA for 120 min. Isoelectric focusing was carried out in 7.5% polyacrylamide gel with 3% ampholite in a pH gradient from 3 to 9. To determine peroxidase activity, the gel was incubated with 10% 4-chloro-α-naphthol and 0.03% H_2O_2 in 100 mM K-phosphate buffer (pH 6.5).

Statistics

The results were analyzed by the two factorial analysis of variance with replications. The significance of means differences between treatments was determined by the LSD test.

RESULTS

Twelve days after infection with *P. halstedii,* the susceptible plants (NS-H-26S) developed typical disease symptoms (Tourvieille de Labrouche *et al.*, 2000), *i.e.,* leaf chlorosis with or without sporulation, which was not the case with the resistant plants (NS-H-26R) (results not presented).

Table 1: The average content of phenolic compounds and their percentage in total phenolic compounds (values in brackets) in the samples taken 12 h after infection (n=3).

Genotype[a]	Content of phenolic compounds[b] (mg/g FW[c])						
	Chlorogenic acid	Ferulic acid	Cinnamic acid	Esculin	Caffeic acid	Scopoletin	Total phenolic compounds
NS-H-26 SC	196.730 (82%)	25.300 (11%)	7.800 (3%)	10.340 (4%)	0.240 (0.1%)	0.110 (0.05%)	240.530
NS-H-26 SI	248.27* (84%)	31.580* (11%)	7.510 (3%)	9.270 (3%)	0.320* (0.1%)	0.100 (0.03%)	297.05*
NS-H-26 RC	171.990 (83%)	18.430 (9%)	8.200 (4%)	7.830 (4%)	0.240 (0.1%)	0.100 (0.05%)	206.800
NS-H-26 RI	183.610 (83%)	18.810 (9%)	8.270 (4%)	8.970 (4%)	0.260 (0.1%)	0.110 (0.05%)	220.030
LSD 0.05	23.210	2.440	1.200	4.760	0.041	0.027	29.830

[a]SC-susceptible line control; SI-susceptible line inoculated; RC-resistant line control; RI-resistant line inoculated.
[b]The significant differences between examined treatments was validated with LSD test and marked with asterisks.
[c] FW- fresh weight

We have analyzed methanolic extracts 12 h after infection (Table 1). The content of chlorogenic acid in sunflower leaves ranged from 172 to 248 μg/g fresh weight (FW). The abundance of other phenolic acids was lower: ferulic (18.43-31.58 μg/g FW) and cinamic acid (7.8-8.27 μg/g FW), esculin (7.83-10.34 μg/g FW) and caffeic acid (0.24-0.32 μg/g FW). The content of scopoletin was the lowest (≈ 0.100 μg/g FW). The ratio of chlorogenic, ferulic, cinnamic and caffeic acids to total phenolic compounds was about 83%, 10%, 4% and 0.1%, respectively. The content of total phenolic compounds in control conditions was significantly higher in the susceptible than in the resistant line. In the infected susceptible plants, all examined phenolic compounds, except cinammic acid, esculin and scopoletin, were significantly accumulated. Conversely, the infection did not lead to significant accumulation of any phenolic compound in the resistant plants (Table 1).

Total peroxidase activity depended on the reducing substrates used for measurements (Table 2). Constitutive quaiacol-dependend POD activity was lower in the susceptible line. Conversely, constitutive CGA-dependent POD activity was lower in the resistant line. We found an increased activity of guaiacol- and chlorogenic acid-dependent peroxidase in leaf of both infected sunflower lines. This increase was higher for CGA-depended POD activity (>70%). Interestingly, scopoletin-dependent POD activity was induced upon infection with *P. halstedii* only in NS-H-26R.

Table 2: Total peroxidase (POD) activity in the presence of different reducing substrates (guaiacol, chlorogenic acid-CGA, and scopoletin) in leaf samples taken 12 h after infection (n=3). Samples are mixture obtained from four leaves. The significant differences in the POD activities between examined treatments was validated with LSD test and marked with asterisks.

Genotype[a]	Peroxidase activity[b]		
	guaiacol-POD (μmol/min g FW[c])	CGA-POD (ΔA_{410}/min g FW[c])	scopoletin-POD (ΔA_{595}/min g FW[c])
NS-H-26 SC	6.1	103	nd[d]
NS-H-26 SI	8.9*	179*	nd[d]
NS-H-26 RC	11.0	60	nd[d]
NS-H-26 RI	13.5*	107*	3.7±0.2*
LSD 0.05	2.31	33.7	-

[a]SC-susceptible line control; SI-susceptible line inoculated; RC-resistant line control; RI-resistant line inoculated.
[b]The significant differences between examined treatments was validated with LSD test and marked with asterisks.
[c] FW- fresh weight
[d] nd- not detected

IEF electrophoresis revealed the existance of four anionic isoforms of peroxidase in leaves of all investigated lines. The main form with pI 5 was particularly intesified in the resistant line, NS-H-26R, both in control conditions and particularly 12 hours after the infection (Figure 1).

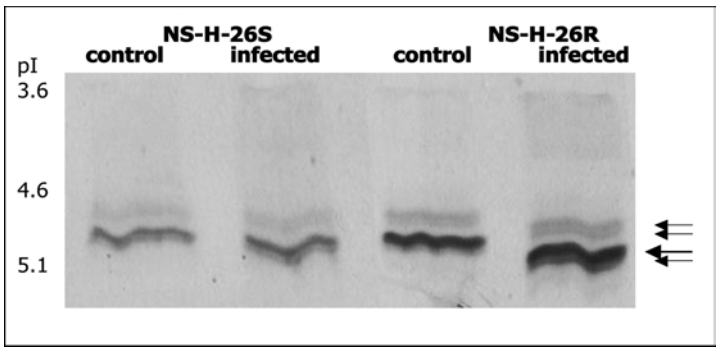

Figure 1: IEF electrophoresis of soluble proteins stained for peroxidase activity in control and infected sunflower leaves with P. halstedii after 12 hours. NS-H-26S is line susceptible and NS-H-26R resistant to P. halstedii. Isoelectrofocusing was done in a pH gradient of 3-9. Arrows indicate different POD isoforms with different pI values. An amount of 50 μmol of total proteins was applied to each well.

DISCUSSION

As shown previously, the resistance to downy mildew in NIL NS-H-26R is conferred by *Pl6* gene (Panković *et al.*, 2004, 2006; Saftić -Panković *et al.*, 2005).

The accumulation of phenolics, particularly the coumarins scopoletin, scopolin and ayapin, in response to fungal infections was often shown in sunflower (Tal and Robeson, 1986 a; Prats *et al*., 2003, 2006). Tal and Robeson (Tal and Robeson, 1986 b) found that the coumarin scopoletin accumulates 18 h after the infection of sunflower stem with pathogenic fungus *Alternaria helianthi*. Twelve hours after infection with *P. halstedii*, we observed the accumulation of several investigated phenolic compounds, precursors in the biosynthetic pathway of scopoletin (Cabello-Hurtado *et al*., 1998), in the susceptible line only. However, there were no significant changes in scopoletin amount. Prats *et al*. (2006) determined the same amount of scopoletin in sunflower leaves in a similar plant developmental stage.

Peroxidases are enzymes adapted to a wide spectrum of substrates, capable of oxidizing hydroxycinnamic derivates and other phenolic compounds with different specificities (Bernards *et al*., 1999). Although we found no differences in POD isoforms between the resistant and the susceptible line (Figure 1), several results indicate that there are significant differences in their abundance and substrate affinity between lines (Table 2). First, the increased POD amount in leaves that is constitutevely present in NS-H-26R was in correlation with the increased guaiacol-dependent POD activity and low total phenolics contents. Second, the lower constitutive CGA-dependent POD activity in leaves of NS-H-26R was connected with the lower specific activity for CGA, and possibly with the presence of different POD isoforms. Third, scopoletin-dependent POD activity was induced upon infection with *P. halstedii* only in the resistant line. It had been shown previously that the effect of scopoletin on POD activity can differ for individual POD isoforms of tobacco (Schafer *et al*., 1971). Edwards *et al*. (1997) described scopoletin-degrading peroxidase from sunflower leaves. They suggested that the induced peroxidative metabolism of scopoletin either has a direct defensive function and/or simply protects plants from potential phytotoxic effects of scopoletin. Prats *et al*. (2006) showed that scopoletin-dependent POD activity was low in a sunflower line susceptible to *Sclerotinia sclerotiorum*, both constitutively and after the infection, which was not the case with a resistant line. In their paper, increased scopoletin-dependent POD activity was in correlation with high levels of scopoletin, which did not happen in our experimental system. In our work we did not measure the amount of scopolin, glycoside of scopoletin, as commercial standards are not available. Scopolin, a potent antifungal metabolite with 40 fold higher content then scopoletin, is considered to be a metabolic pool for synthesis of ayapin and/or scopoletin compunds with increased phytotoxic effect (Prats *et al*., 2006). In further experiments, determination of scopolin content should contribute to the explanation of scopoletin-dependent POD activity. Changes of phenolic compounds and POD activity and amount should also be examined on a longer time scale, as there are results on the increase of scopoletin in sunflower leaf discs only 162 h after the infiltration of *P. halstedii* sporangia (Brandle and Spring, 2003).

Induction of POD activity together with the observed increase in the amount of total phenolics, particularly in the susceptible line, indicates that infection of plants with downy mildew leads to oxidative stress conditions and thereby to activation of antioxidative defense.

In conclusion, the scopoletin-dependent POD activity that was induced upon the infection only in the resistant NIL indicates a specific role of POD in coumarin metabolism that is possibly connected with the presence of the *Pl6* gene.

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PAPEL DE COMPUESTOS FENÓLICOS Y PEROXIDASIS EN LA RESPUESTA DE DIFERENTE-RESISTENTES LÍNEAS ISOGÉNICAS DE GIRASOL A LA INFECCIÓN POR EL CAUSANTE DE TIZÓ

RESUMEN

En el trabajo fueron investigadas dos líneas de girasol isógenas (NIL), una resistente (+*Pl6* gen), y otra sensible (-*Pl6* gen) a Tizón. Las plantas jóvenes en el estadio de primer par de hojas fueron expuestas a la infección secundaria con suspensión de esporas *Plasmopara halstedii*. En las muestras tomadas 12 horas después de la infección, fue analizado el contenido de los compuestos fenólicos por el método HPLC. La actividad POD fue determinada espectrofotométricamente, y las isoformes POD fueron separadas mediante isoelectroforesis.

El contenido constitutivo de los compuestos fenólicos y su acumulación tras la infección, fueron más altos en la línea sensible que en la línea resistente. La mayor cantidad de POD en las hojas NS-H-26R en las condiciones de control, en correlación con la aumentada actividad de POD guayacol-dependiente, y también con el contenido más bajo de fenoles totales. Tras la infección, las actividades POD, dependientes de guayacol y el ácido clorogeno, significativamente se incrementan en ambas líneas. La actividad POD Scopoletina-dependiente, es inducida tras la infección sólo en NS-H-26R. Cuatro isoformes POD aniónicas se separan por medio de electrofóresis IEF en las muestras de ambas líneas. La isoforme principal (pI 5) está especialmente intensificada en la línea resistente.

La actividad POD, Scopoletina-dependiente, que fue inducida tras la infección sólo en NIL resistente, indica el papel específico de POD en el metabolismo de cumarina, que está probablemente vinculada con la presencia del gen *Pl6*.

RÔLE DES COMPOSÉS PHÉNOLIQUES ET DES PEROXYDASES DANS LES LIGNÉES QUASI-ISOGÉNIQUES DU TOURNESOL RÉSISTANTES À L'INFECTION DU MILDIOU

RÉSUMÉ

Deux lignées quasi-isogéniques de tournesol, l'une résistante (gène *+Pl6*) et l'autre sensible (gène *–Pl6*) au mildiou ont été examinées dans cet article. Les jeunes plantes au stade de la première paire de feuilles ont été soumises à une infection secondaire avec une suspension de spores *Plasmopara halstedii*. Dans les échantillons recueillis 12 heures aprs l'infection, le contenu de composés phénoliques dans les extraits méthanoliques des feuilles congelées a été analysé par la méthode HPLC. L'activité POD a été déterminée spectrophotométriquement et les isoformes par isoélectrophorèse.

Le contenu constitutif des composés phénoliques et leur accumulation après l'infection étaient plus élevés dans la lignée sensible que dans la lignée résistante. La plus grande quantité POD dans les feuilles NS-H-26R était en corrélation avec une activité augmentée de POD dpendant de gaïacol et avec de faibles totaux de contenus phénoliques. Après l'infection, l'activité POD dépendante de gaïacol et d'acide chlorogénique a augmenté de manière significative dans les deux lignées. L'activité POD dépendante-scopolétine a été induite après infection seulement dans NS-H-26R. L'électrophorèse IEF a révélé l'existence de quatre isoformes anioniques de peroxydase dans les feuilles des deux lignées. L'isoforme principale (pI 5) est particulièrement intensifiée dans la lignée résistante.

L'activité dépendante scopolétine qui est induite après infection seulement dans les lignées NIL résistantes indiquent un rôle spécifique du POD dans le métabolisme de la coumarine qui est probablement lié à la présence du gène *Pl*6.

Presented at: