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The elicitor-induced oxidative processes in leaves of *Solanum* species with differential polygenic resistance to *Phytophthora infestans*

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Summary

Previous studies have shown that suspension-cultured cells of *Solanum* genotypes with various polygenic resistances to *Phytophthora infestans* differed in activities of early oxidative processes in response to culture filtrate (CF) from this pathogen. These studies have now been extended by analysing production of reactive oxygen species (ROS), lipid peroxidation and lipoxygenase (LOX, E.C.1.13.11.12) activity induced by CF in detached leaves of *S. tuberosum* cv Bzura and clone H-8105, polygenically resistant and susceptible, respectively, as well as *S. nigrum*, nonhost, completely resistant. The relative increase in the ROS production was higher in the susceptible clone H-8105 than in both resistant genotypes. Lipid peroxidation increased only in the nonhost *S. nigrum*. An increase in lipid peroxidation in *S. nigrum* leaves coincided with enhanced LOX activity. In both *S. tuberosum* genotypes, significant increases in LOX activity were delayed and unaccompanied by changes in the level of lipid peroxidation. LOX activity attained a higher level in both of the resistant genotypes than in the susceptible one. The present results suggest that the involvement of both ROS production and LOX activity in the defense strategy in *Solanum species*/*P. infestans* interactions.

Abbreviations: Avr gene, avirulence gene; CF, culture filtrate; HR, hypersensitive response; LOX, lipoxygenase; NBT, nitroblue tetrazolium (2,2′- di-*p*-nitrophenyl-5,5′-diphenyl-3,3,-[3-3′-dimethoxy-4,4′-diphenylene]-ditetrazolium chloride); R gene, resistance gene; ROS, reactive oxygen species; TBA, 2-thiobarbituric acid; TBARS, 2-thiobarbituric acid reactive substances; TRIS, 2-amino-2-(hydroxymethyl)-1,3-propanediol

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Introduction

Elucidation of the genetic and biochemical mechanisms involved in plant disease resistance is a matter of interest to many research groups. Attention is mostly focused on early events in plant/pathogen interactions and the hypersensitive response (HR), a rapid cell death at the site of infection and a typical feature of plant resistance. The HR is commonly accompanied by an oxidative burst, a rapid generation of reactive oxygen species (ROS), enhanced lipid peroxidation, and an increase in lipoxygenase (LOX) activity. Despite intensive studies, the interconnection among these events in defense strategy still remains obscure (Bolwell, 1999; Ebel and Mithöfer, 1998). Mittler et al. (1999) demonstrated that by manipulating ROS-scavenging mechanisms in nonhost tobacco plants, they could change the extent of cell death in response to bacterial infection. However, Dorey et al. (1999) documented that ROS are neither necessary nor sufficient for HR. On the other hand, recent results obtained by Buonaurio and Servili (1999), Rustérucci et al. (1999), and Jalloul et al. (2002) pointed to a LOX-dependent peroxidative pathway causing hypersensitive cell death. Furthermore, LOX activity was proposed to play an important role in the plant defense strategy (May et al., 1996; Rancé et al., 1998).

Our study concerns defense mechanisms in *Solanum* species in response to the pathogenic oomycete *Phytophthora infestans*, which causes late blight, the most devastating disease of potato (*S. tuberosum*). Recent phylogenic analyses suggest that oomycetes form a unique group of eukaryotic plant pathogens, which may display distinct genetic and biochemical mechanisms in interactions with plants (Kamoun et al., 1999). As is generally accepted, the race or cultivar-specific plant resistance requires the presence of both a dominant plant resistance (R) gene and a matching pathogen avirulence (Avr) gene, whereas nonhost and quantitative (partial) plant resistances are polygenically controlled (Keller et al., 2000). On the other hand, biochemical defense processes in host and nonhost resistance are very similar, although the latter does not apparently require a R/Avr gene combination (Somssich and Hahlbrock, 1998). According to Kamoun et al. (1999, 2001), in *Solanum*/*P. infestans* interactions nonhost resistance may be explained by the occurrence of an arsenal of R genes interacting with multiple Avr genes, whereas partial resistance could result from ''weak'' R gene–Avr gene interactions or gene-dosage effects. In the *Solanum*/*P. infestans* system, wild *Solanum* species often express nonhost resistance, whereas cultivated potatoes represent mostly quantitative resistance manifested by various levels of disease reduction (Kamoun et al., 1999; Vleeshouwers et al., 2000). In the case of cultivated potato, the presence of some unidentified R genes was not excluded (Vleeshouwers et al., 2000). Cytological studies of Vleeshouwers et al. (2000) indicated that different wild *Solanum* species, potato cultivars have various levels of resistance, and representative nonhost species infected with *P. infestans* displayed HR. The severity and timing of this response varied depending on the form and level of resistance exhibited by a given genotype. Therefore, the authors suggested that in *Solanum*/*P. infestans* interaction resistance is of a quantitative rather than qualitative nature.

In our previous work (Polkowska-Kowalczyk and Maciejewska, 2001), we investigated the oxidative burst and lipid peroxidation induced by elicitor from *P. infestans* in suspension-cultured cells of *Solanum* genotypes that differ in the type and level of resistance to this pathogen. We studied the cells of *S. tuberosum* cv Bzura, which are polygenically resistant and likely carrying unidentified R genes (Świeżyński et al., 1993), *S. tuberosum* clone H-8105, susceptible (E. Zimnoch-Guzowska, unpublished), as well as the cells of *S. nigrum* wild species, nonhost, completely resistant to *P. infestans* (Colon et al., 1993). In all genotypes examined the elicitor-induced reactions were the same, but they varied with respect to kinetics and intensity. The experimental model with suspension-cultured cells, though useful for analysis of the chronology of events triggered within minutes after elicitation, provides results that may not reflect strictly the complexity of the *in planta* system. Differences in responses of cell suspension or leaf discs in comparison to intact leaves were described for different plant/pathogen (elicitor) interactions (Baker et al., 1993; Dorey et al., 1999). Moreover, Dorey et al. (1997) found that defense reactions in tobacco leaves infiltrated with an elicitor varied spatially and temporally, depending on which part of the leaf was analysed. Therefore, as an extension to our previous work, we have undertaken studies on detached leaves to elucidate whether the previously found differences were the consequence of the resistances represented by the *Solanum* species studied or rather they could be attributed to the conditions of the cell suspension assay.

Materials and methods

Plant material and pathogen elicitor

Axenic shoots of *Solanum nigrum* var. *gigantea*, nonhost completely resistant, *S. tuberosum* L. cv

Bzura and clone H-8105, resistant and susceptible, respectively, grown in vitro on hormone-free MS medium (Murashige and Skoog, 1962), containing 2% (w/v) sucrose and solidified with 0.6% (w/v) agar, were propagated at 4–6 week intervals. The oomycete pathogen *P. infestans* (Mont.) De Bary (complex race MP346 with virulence factors: 1,2,3,4,6,7,8,10,11) was kindly provided by IHAR, Młochów Research Center (Poland). The pathogen was maintained on rye agar medium at 15°C in the dark. A culture filtrate (CF) that served as elicitor was prepared from the pathogen grown in liquid medium (Henniger, 1958) as previously described (Awan et al., 1997). After 6 weeks of growth at 20°C in the dark, the medium was separated from the oomycete, dialysed against water for 48 h and lyophilised. The CF residue was dissolved in distilled water to one-tenth of the initial volume and quantified as μg glucose equivalents ml^{-1}.

Elicitor treatment of leaves

Leaves harvested from 4-week-old plants were placed on moist filter paper in Petri dishes. The CF was applied in droplets on the surface of each leaf at a concentration of 0.67 μg glucose equivalent g^{-1} fresh weight. The appropriate volume of the elicitor was applied with a micropipette in small droplets onto the abaxial leaf surface. In parallel, dishes with control leaves with an equal volume of distilled water in droplets were set up. Leaves were incubated for 6, 18 and 30 h at 25°C under continuous light 40 $\mu m\, m^{-2}\, s^{-1}$ supplied by fluorescent tubes (Pila, Poland).

Assay for ROS production

The determination of the $O_2^{\bullet -}$ generated by leaves was based on the reduction of nitroblue tetrazolium (NBT) according to the procedure described by Doke (1983). After treatment with CF or H_2O, the leaves were partially dried with blotting paper and immersed in a mixture containing 0.05% (w/v) NBT, 0.01 M potassium phosphate buffer (pH 7.8), and 10 mM NaN_3 (150 mg f.wt. per 2.5 ml of mixture). After 1 h of incubation, 2 ml samples of the mixture were heated at 85°C for 15 min, then cooled and absorbance at 580 nm was measured. The reduction of NBT in the mixture is expressed as $\Delta A_{580}\, g^{-1}$ fresh weight h^{-1}.

Peroxidation of lipids

Lipid peroxidation was measured as 2-thiobarbituric acid-reactive substances (TBARS), according to the modified method of Oteiza and Bechara (1993). The tissues were homogenised in 5% (w/v) trichloroacetic acid and centrifuged at 10,000*g* at 4°C for 10 min. The reaction mixture contained the cell extract, 0.3% (w/v) sodium dodecyl sulfate, 0.25% (w/v) TBA in 50 mM NaOH, and 6% (v/v) HCl in a total volume of 1 ml. After heating at 80°C for 40 min, TBARS were extracted with 1 ml of 1-butanol. The specific A_{532} of the organic phase was measured and the nonspecific A_{600} was subtracted. Measurements are expressed as A_{532}–$A_{600}\, g^{-1}$ fresh weight.

Lipoxygenase extraction and assay

Leaves treated with water or elicitor for 18 or 30 h and untreated ones were frozen in liquid nitrogen and stored at −80°C until use. For extraction of LOX (E.C.1.13.11.12), frozen leaves (0.5 g) were ground in 1.5 ml of ice-cold 0.25 M sodium phosphate buffer (pH 6.5) containing 5% (w/v) polyvinylpyrrolidone. The homogenate was stirred for 1 h at 4°C before being centrifuged at 20,000*g* (4°C, 30 min). LOX activity was determined in the supernatant (hereafter termed "leaf extract") using a Clark oxygen electrode as described by Fournier et al. (1993). An aliquot of leaf extract (0.2 ml) was incubated in 0.25 M sodium phosphate buffer (pH 6.5) in a final volume of 0.78 ml. The reaction was started by adding 20 μl linoleic acid to a final concentration of 1.6 mM. To determine the pH optimum of the enzymes extracted from the water- or elicitor-treated leaves, the assays of LOX activity were carried out in different buffers: 0.25 M citrate phosphate (pH 5.5–6.5) and Tris-HCl (pH 7.0–8.0). Appropriate controls with buffer alone and heat-denatured leaf extracts showed no consumption of O_2. Additional experiments with Tween-20 to detect peroxidase activity (Ben-Aziz et al., 1970) indicated no peroxidase activity under the assay conditions. The LOX activity is expressed in nkat g^{-1} fresh weight.

Presentation of data

The presented data are the mean values±SD from three independent experiments, with each sample tested at least in duplicate. The significance of differences between mean values was determined by the Student's *t*-test.

Results

Symptoms in leaves after application of elicitor

Leaves used for analyses, detached from in vitro cultured plants, were of an average size of about 1.5 cm^2 (cv Bzura and clone H-8105) or 3–4 cm^2 (*S. nigrum*). Elicitor, CF from *P. infestans* or water (control) was applied on leaf surfaces in droplets, and observations were carried out for 72 h. In CF-treated leaves of the resistant, nonhost, *S. nigrum*, the first very rare, small HR spots, visible under 10× magnification, appeared 6 h after the treatment. They increased in number until 30 h (Fig. 1A). Afterwards, until 72 h of CF treatment, no further changes were noticeable. The resistant Bzura leaves displayed the first HR spots at 18 h of CF treatment. They increased in number and size over the subsequent 12 h (Fig. 1B). Later, the leaves began to display chlorosis, which was apparent in almost all leaves after 72 h of CF treatment. In leaves of the susceptible clone H-8105, initial HR spots appeared at 12 h of exposure to CF (Fig. 1C). Between 30 and 72 h of CF treatment, the necrotic spots multiplied, enlarged, and chlorosis developed in almost all elicited leaves. In the control leaves, no changes were observed during the whole experiment.

Biochemical assays

In preliminary biochemical experiments we have noted that untreated leaves of the studied genotypes showed large differences in the level of ROS and lipid peroxidation, probably as a consequence of their genetic features. The early detectable changes in reactions induced by CF could be recorded 6 h after the challenge; therefore, this time was chosen to start the assays. We have followed further changes in the leaves at 18 and 30 h after CF treatment. Changes occurring after prolonged exposure to CF (72 h) were excluded from analysis to avoid overlapping with possible effects of leaf senescence. In control leaves (i.e. treated with water) of each genotype, the activities of reactions examined were practically stable for the entire duration of the experiment (30 h); hence only values representing average levels of the activity in the respective controls are shown.

ROS production

ROS production was evaluated by determining the reduction of NBT present in the surrounding medium, which was caused by $O_2^{\bullet -}$ released from leaf tissues.

In leaves of the resistant *S. nigrum*, ROS production increased significantly (200% of the control level; $p<0.05$) after 18 h of CF treatment (Fig. 2A). Then, after 30 h of treatment, no significant change in ROS production was observed. In leaves of both *S. tuberosum* genotypes, significant increases ($p<0.05$) in ROS production took place at 6 h of CF treatment, reaching in cv Bzura about 150% and in clone H-8105 180% of the control level. In the resistant cv Bzura, the ROS production did not change significantly after subsequent 24 h

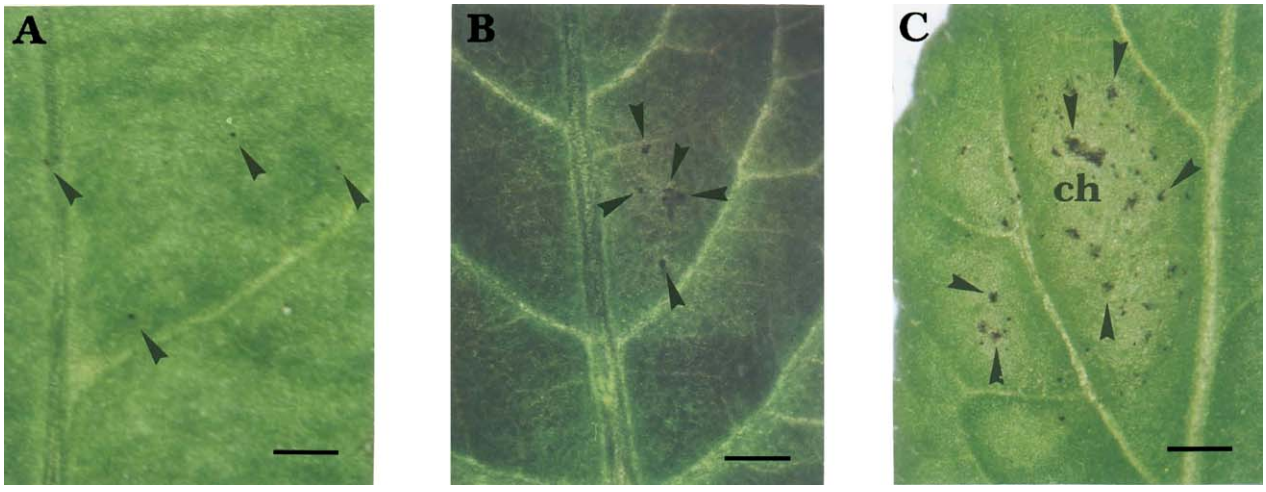

Figure 1. Detached leaves of *S. nigrum* (A), *S. tuberosum* cv Bzura (B), and *S. tuberosum* clone H-8105 (C) treated with culture filtrate (CF) from *P. infestans* for 30 h. The CF was applied on leaf surfaces in droplets. HR necrosis (arrows) and chlorosis (ch) are indicated. Scale bar = 1 mm.

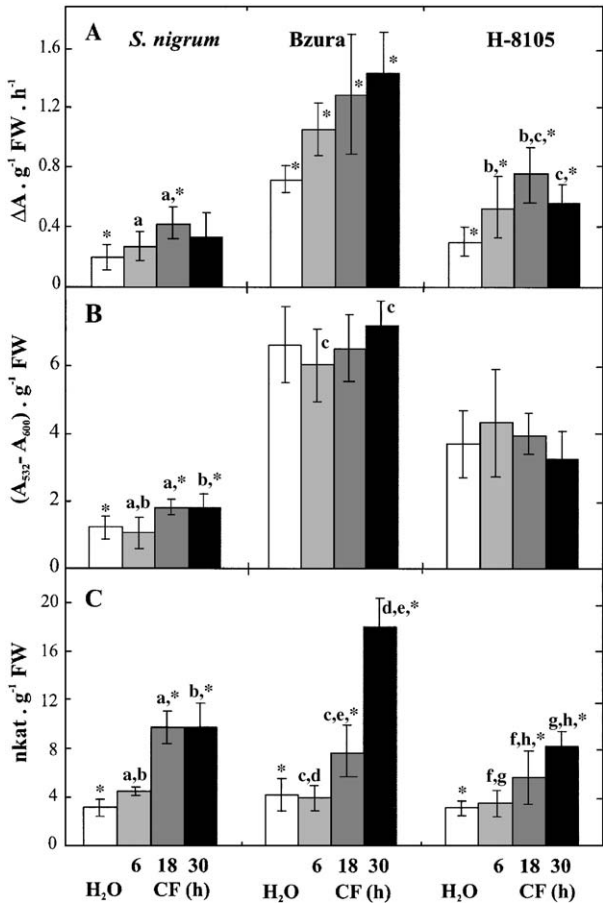

Figure 2. Changes in ROS production (A), lipid peroxidation (B) and LOX activity (C) in leaves of *Solanum* species treated with culture filtrate (CF) for 6 (▒), 18 (▓) or 30 h (■). Empty columns denote average values represented by control leaves treated with water (H_2O). The values are mean±SD ($n = 6$ at least). The same letters indicate significant differences ($p < 0.05$) between mean values for CF-treated leaves in respective genotypes for each activity, (*) significant differences ($p < 0.05$) between mean values for CF-treated and control leaves.

of CF treatment. In contrast, in the susceptible clone H-8105, the ROS production increased further up to 260% ($p < 0.05$) of the control at 18 h of CF treatment, and after 30 h, it decreased significantly to 190% ($p < 0.05$) of the control. Thus, the genotypes studied differed in the kinetics and intensity of CF-induced ROS production. The relative increase in the ROS production was higher in the susceptible clone H-8105 than in resistant cv Bzura and *S. nigrum*.

Lipid peroxidation

ROS activity is frequently considered to cause membrane damage through peroxidation of fatty acids. To check this possible effect of ROS on membranes in CF-treated leaves, in parallel to the ROS assays we have estimated lipid peroxidation by determining accumulation of TBARS.

In CF-treated *S. nigrum* leaves, lipid peroxidation increased significantly ($p < 0.05$) to 150% of the control at 18 h after the challenge and remained at this level after another 12 h (Fig. 2B). The enhanced lipid peroxidation coincided with an increase in ROS production. In contrast, in leaves of cv Bzura and clone H-8105, despite ROS production, no significant changes in lipid peroxidation were observed during 30 h exposure to CF.

LOX activity

The weak correlation between the ROS production and the increase in lipid peroxidation in *S. nigrum* leaves (correlation coefficient 0.46) suggested that in addition to free-radical-mediated reactions, LOX activity may also be responsible for this oxidative process.

We have established that in control and CF-treated leaves of the studied genotypes, the pH optimum for LOX activity was in the range of 6.5–7.0, and practically did not change during the whole period of exposure to CF (data not shown). Based on these data, LOX activity was routinely measured at pH 6.5.

The control leaves of the studied genotypes showed rather low and comparable LOX activities during the entire monitored period (Fig. 2C). In *S. nigrum* leaves, LOX activity increased to 300% that of the control ($p < 0.05$) after 18 h of CF treatment, and remained at this level after the subsequent 12 h of treatment. In both *S. tuberosum* genotypes, LOX activity reached about 180% that of the control ($p < 0.05$) at 18 h of CF treatment. Prolonged exposure to CF for up to 30 h resulted in a further significant ($p < 0.05$) increase in LOX activity in the resistant cv Bzura (420% of control) and to a lesser extent (260% of control) in the susceptible clone H-8105.

Discussion

In the present study, as an extension to our previous work on suspension-cultured cells (Polkowska-Kowalczyk and Maciejewska, 2001), we have characterised oxidative processes induced by elicitor from *P. infestans* in detached leaves of three *Solanum* genotypes expressing different types and levels of resistance against this pathogen. The leaves of all genotypes that were treated with the

elicitor displayed HR symptoms, but the timing and intensity of this response varied depending on the resistance of the plants. A similar conclusion was drawn by Vleeshouwers et al. (2000) from cytological studies on leaves of different *Solanum* species infected with *P. infestans*.

In CF-treated leaves the oxidative processes took a longer time (30 h) than in the cultured cells (maximum 3 h), as noted by Polkowska-Kowalczyk and Maciejewska (2001). The slow and prolonged kinetics of responses *in planta* system contrasting with the rapid and transient reactions in suspension cells was also noted by other authors (May et al., 1996; Dorey et al., 1999). This effect may result from the differences in physiological environment in leaf tissues and cultured cells that modify the accessibility of elicitors to the targeted cells and the level of detectable extracellular ROS in either experimental material.

Here, similar to previous results (Polkowska-Kowalczyk and Maciejewska, 2001), we have demonstrated that at the leaf level, the CF-induced processes varied with respect to kinetics and intensity, depending on the type and level of resistance exhibited by the plants (Table 1). The relative increase in ROS production in response to CF treatment was higher in leaves of the susceptible clone H-8105 than in the resistant cv Bzura and *S. nigrum* (Fig. 2A). Differences in ROS production between these resistant genotypes might be due to differences in the expression of R genes. This suggestion seems to be supported by May et al. (1996) who showed that two tomato lines whose resistance was conferred by interactions of two unlinked specific Cf-Avr genes differed in their defense responses.

Contrary to the previous results obtained on cell suspensions, where lipid peroxidation was detected immediately after the oxidative burst (Polkowska-Kowalczyk and Maciejewska, 2001), in leaves an increase in lipid peroxidation was found only in the resistant nonhost *S. nigrum*. The increment in lipid peroxidation coincided with a high LOX activity and increased ROS production. This observation suggests that in *S. nigrum*, apart from free-radical-mediated reactions, LOX activity was also responsible for lipid peroxidation. In the leaves of cv Bzura and clone H-8105, no increase in lipid peroxidation was observed, despite enhanced ROS production and LOX activity. This may suggest that their antioxidant mechanisms were effective, thereby preventing intracellular accumulation of free radicals and, consequently membrane damage.

Recent data from the literature point to a link between hydrogen peroxide and LOX activity, the two factors considered to play a key role in plant HR. Maccarrone et al. (2000) showed that hydrogen peroxide-induced activation of LOX and consequent membrane peroxidation led to programmed cell death in lentil root protoplasts. Macrì et al. (1994) documented that hydrogen peroxide at low concentration stimulated LOX activity, while at higher concentrations the stimulation was reduced. Pérez-Gilabert et al. (1996) found that tomato LOX was inactivated by hydrogen peroxide in a time- and dose-dependent manner. The authors proved that the substrate of the LOX reaction as well as its product, the hydroperoxy octadecadienoic acid, displayed a protective effect against this inactivation. We have observed (Table 1) that in *S. nigrum* a high LOX activity was preceded by an insignificant increase in ROS production at the early phase of elicitation. In both *S. tuberosum* genotypes, enhanced ROS production occurred already at the early phase of elicitation, while the highest levels of LOX activity appeared after prolonged (30 h) CF treatment, when ROS production became stable (cv Bzura) or decreased (clone H-8105). Furthermore, hydrogen peroxide added to LOX extracts from leaves of the examined plants inhibited the enzyme activity (data not shown). Therefore, it might be conceivable that the ROS level modified the LOX activity in the genotypes studied, and thereby influenced their defense response.

The observation that in the resistant cv Bzura the relative increase in LOX activity was higher than in the susceptible clone H-8105 (Fig. 2C) seems to be supported by the results of Weber et al. (1999). The authors reported that indirect products of 9-LOX activity, divinyl ether fatty acids, accumulated more rapidly in potato cultivar Matilda with

Table 1. Time course of reactions analyzed in three *Solanum* genotypes

Reaction time (h)	ROS production			Lipid peroxidation			LOX activity		
	6	18	30	6	18	30	6	18	30
S. nigrum	—	↑+	—	—	↑+	—	—	↑++	—
Cv Bzura	↑	—	—	—	—	—	—	↑	↑++
Clone H-8105	↑	↑+	↓	—	—	—	—	↑	↑+

(—) no significant change, (↑) increase, (↓) decrease, (+) indicator of intensity.

increased resistance to late blight than in the susceptible cultivar Bintje. Our results are also in concert with the observation that *POTLX*-3, the leaf LOX gene of potato, proposed to be specifically involved in defense responses, was induced early and continuously in incompatible *S. tuberosum*/*P. infestans* interaction, whereas its induction was inconsistent and delayed in susceptible plants (Kolomiets et al., 2000). It seems also noteworthy that in the resistant *S. nigrum* leaves, the increased lipid peroxidation and enhanced LOX activity preceded the cessation of extension of HR symptoms. On the contrary, in *S. tuberosum* genotypes, particularly in the susceptible clone H-8105, HR and disease symptoms developed further, despite high LOX activity. However, in the case of the susceptible genotype the effects of toxins present in the elicitor cannot be excluded (Galal et al., 1991). The obtained results suggest that LOX activity may be a requisite for successful resistance in the *Solanum* species/*P. infestans* interactions. It has been demonstrated that in potato cells the activity of 9-LOX and its products increased preferentially in response to elicitor from *P. infestans* (Göbel et al., 2001). Rustérucci et al. (1999) reported that in cryptogein-treated tobacco leaves, 9-LOX activity was specifically induced. To elucidate the role of LOX activity in resistance in *Solanum* species/*P. infestans* interactions, studies on specificity of LOX and lipid peroxidation products in CF-treated leaves are currently being undertaken.

Overall, the results presented here, supplementing our previous work, revealed new aspects of defense response in the elicited plants. It has been demonstrated for leaf tissue, as was previously done for suspension-cultured cells, that CF-induced reactions in the resistant and susceptible *Solanum* species examined were the same but quantitatively different. Our results suggest that, at least in the system studied, successful resistance requires a tightly controlled balance among inducible reactions. They point to a possible role of LOX activity in resistance response in the studied interactions.

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