Involvement of phospholipase A$_2$ in the response of *Solanum* species to an elicitor from *Phytophthora infestans*

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Changes in activity of phospholipase A$_2$ (PLA$_2$), a key enzyme in lipid metabolism and signal network in defence mechanisms, were investigated in Solanum species and Phytophthora infestans interaction. We have compared PLA$_2$ activity in response to an elicitor, a culture filtrate (CF) derived from P. infestans, in non-host resistant Solanum nigrum var. gigantea, field resistant Solanum tuberosum cv Bzura and susceptible S. tuberosum clone H-8105. To elucidate the contribution of specific forms of PLA$_2$ to plant defence mechanism reasonably selective PLA$_2$ inhibitors, haloenol lactone suicide substrate (HELSS) and p-bromophenacyl bromide (BPB), which discriminate between Ca$^{+2}$-independent PLA$_2$ (iPLA$_2$) and Ca$^{+2}$-dependent secretory PLA$_2$ (sPLA$_2$), were used. The in vivo and in vitro effects of the inhibitors on PLA$_2$ activity and on generation of reactive oxygen species (ROS) induced by CF in the studied plants were assayed. We found that PLA$_2$ activity increased in response to CF treatment, displaying various kinetics and intensity depending on the resistance status of a given genotype. Differences among the genotypes in the effects of each inhibitor on CF-induced PLA$_2$ activity and on ROS production may reflect the diversity of PLA$_2$ isoforms in plants. Contrary to BPB, the inhibitory effect of HELSS was observable mainly on CF-induced PLA$_2$ activity, which suggests that iPLA$_2$ participates in signal transduction in defence reactions. Various effects of the two inhibitors on PLA$_2$ activity and ROS production suggest different contribution of sPLA$_2$ and iPLA$_2$ to modulation of defence reactions in the interaction between Solanum genotypes and P. infestans.

Keywords: Phospholipase A$_2$

Phytophthora infestans
Resistance
Solanum
Abbreviations:

BPB  bromophenacyl bromide
CF   culture filtrate
FA   fatty acid
1,2-dilinoleoyl PC  1,2-dilinoleoyl phosphatidylcholine
HELSS haloenol lactone suicide substrate
HR   hypersensitive response
LA   linoleic acid
LaH  lipid acylhydrolase
LnA  linolenic acid
LOX  lipoxygenase
LPC  lysophosphatidylcholine
LPL  lysophospholipid
PLA  phospholipase A
cPLA cytosolic Ca\(^{2+}\)-dependent phospholipase A
iPLA\(_2\)  Ca\(^{2+}\)-independent phospholipase A\(_2\)
sPLA\(_2\)  secretory phospholipase A\(_2\)
PUFA polyunsaturated fatty acid
ROS  reactive oxygen species


**Introduction**

Recent literature shows that lipid metabolism is implicated in plant response to biotic and abiotic stresses, e.g., Ryu (2004), Muller and Berger (2009). Lipid metabolism is initiated by the release of polyunsaturated fatty acids (PUFAs) from structural membranes by the action of lipid acyl hydrolases (LAHs). Among multiple plant LAHs great interest has been focused on phospholipases A (PLAs), which generate linoleic (LA) and/or linolenic (LnA) acids and lysophospholipids (LPL). These products are themselves biologically active or serve as precursors of other compounds active in a complex signal network. PLAs consist of two types depending on positional specificity: phospholipase A\textsubscript{1} (PLA\textsubscript{1}) and phospholipase A\textsubscript{2} (PLA\textsubscript{2}), which hydrolyse phospholipids at \textit{sn}-1 or \textit{sn}-2 position, respectively. Based on sequence data and biological properties plant PLA\textsubscript{2}s are classified into two groups: the low molecular weight secretory PLA\textsubscript{2} (sPLA\textsubscript{2}), which is Ca\textsuperscript{+2}-dependent, and the patatin-like PLA, which is homologous in amino acid sequences to animal Ca\textsuperscript{+2}-independent PLA\textsubscript{2} (iPLA\textsubscript{2}); the patatin-like PLA combines PLA\textsubscript{1} and PLA\textsubscript{2} activity. The plant sPLA\textsubscript{2}s show significant similarity to animal sPLA\textsubscript{2}s in the active site and in the calcium-binding loop regions. The plant patatin-like PLAs, iPLA\textsubscript{2}, function as serine hydrolases and have an active Ser residue in the middle of the consensus sequence GXSXG, which is conserved in animal iPLA\textsubscript{2}s.

The existence of cytosolic plant PLA\textsubscript{2}, homologous to animal cytosolic Ca\textsuperscript{+2}-dependent PLA (cPLA), has not yet been well documented (Holk et al. 2002; Lee et al. 2005; Mansfeld et al. 2007). Effective resistance to pathogens is often dependent on the host cell death at the infection site, defined as a hypersensitive response (HR), and on expression of defence genes followed by changes in activity of selected metabolic pathways. La Camera et al. (2005, 2009), using transgenic \textit{Arabidopsis} plants with modulated levels of patatin-like protein (PLP2) infected by pathogens with different lifestyles, observed that PLP2 influenced various forms of cell death in host as well as in the lesion-mimic mutant. Induction of PLA\textsubscript{2} activity in response to pathogen or elicitor treatment of plant tissues has often been reported, e.g., in potato tuber inoculated with \textit{Phytophthora infestans} (Kawakita et al. 1993), in tomato leaves after 15 min incubation with systemin, oligosaccharide
elicitors and chitosan (Narváez-Vásques et al. 1999). It has been found that in elicitor treated tobacco leaves transcripts of genes encoding patatin-like proteins (NtPat) and 9-Lox accumulated with similar profiles (Dhondt et al. 2002). Enhanced PLA2 activity in potato cells treated with an elicitor from P. infestans and increased levels of transcripts of 9-Lox and NtPat in elicited tobacco leaves preceded the biosynthesis of biologically active oxylipins, as demonstrated, respectively, Göbel et al. (2001) and Cacas et al. (2005). Hence, the key role of PLA2 in defence processes is thought to produce PUFAs, which after oxidation by 9- or 13-lipoxygenase (9-, 13-LOX) are further metabolised into oxylipins. In addition, free PUFAs are important cellular mediators (Scherer et al. 2010). Furthermore, the LPL, another product of PLA2 activity, has been shown to promote protein kinase activity and H+ - ATPase pumping, thereby affecting the intracellular pH, another important factor in plant defence response (Munnik et al. 1998).

Generation of reactive oxygen species (ROS) due to the action of NADPH oxidase, referred to as an oxidative burst, is one of early events in plant/pathogen interaction. The ROS produced in response to pathogen/elicitor are considered to contribute to HR. They can serve as signals for activation defence reactions or affect cell metabolism directly (Baker and Orlandi 1995; Mur et al. 2008). In contrast to studies on the involvement of PLA2s in the oxidative burst in mammalian cells (Dana et al. 1994; Levy 2006), reports concerning plant material are scarce. In studies, performed on cultured cells of soybean (Chandra et al. 1996) and tobacco (Piedras et al. 1998) treated with inhibitors of PLA2, chlorpromazine, p-bromophenacyl bromide (BPB) and quinacrine, the elicitor-induced ROS generation and PLAs activities were modified differently; hence, univocal evidence of a relationship between these activities remains to be addressed.

Elucidation the contribution of specific forms of PLA2 to plant defence mechanisms remains a challenging task. Generally, there are two basic approaches to this study: using genetically modified material with altered activity of the genes of interest, and, the most straightforward one, employment of specific chemical inhibitors of PLA2 to asses its implications on a given process. In both cases direct appraisal of the role of specific PLA2s is rather difficult due to multiple isoforms of PLA2 and the lack of completely specific inhibitors (Balsinde et al. 1999). One of the PLA2s
reasonably selective inhibitor is haloenol lactone suicide substrate (HELSS) which binds covalently at or near the active site of iPLA₂ and is specific for iPLA₂ but not for Ca²⁺-dependent sPLA₂ (Hazen et al. 1991). Holk et al. (2002) reported that HELSS inhibited in vitro activity of purified Arabidopsis PLA IVA, which by conserved sequence elements may represent patatin-iPLA gene family. On the other hand, HELSS at high concentrations affects also cPLA₂ and can inhibit phosphatidate phosphohydrolase, so that its specificity in vivo in intact cells is unclear. Another inhibitor is p-bromophenacyl bromide (BPB), which inactivates sPLA₂ by blockage of exposed His or Lys residues (Balsinde et al. 1999; Mansfeld and Ulbrich-Hofman 2007).

Our research interests concern the defence mechanisms in Solanum species in response to an elicitor, the culture filtrate (CF) from Phytophthora infestans, the pathogenic oomycete that causes late blight, the most destructive potato disease. Interestingly, in this plant/pathogen interaction HR may occur in all forms of resistance: non-host, host (vertical), field (horizontal) as well as in total susceptibility (Vleeshouwers et al. 2000; Tian et al. 2006). In our studies we have compared early metabolic events induced by the elicitor in Solanum nigrum var. gigantea, Solanum tuberosum cv Bzura and clone H-8105, representing, respectively, non-host resistance, field resistance and susceptibility to P. infestans. We have found that detached leaves from all examined genotypes treated with CF displayed HR spots, but the timing and intensity of this response varied depending on the resistance status of the plants. The elicitor-induced ROS production, lipid peroxidation and LOX activity in the resistant and the susceptible genotypes differed quantitatively. The relative increase in ROS production was higher in the susceptible H-8105 than in both resistant genotypes. An increase in lipid peroxidation coincided with enhanced LOX activity only in S. nigrum (Polkowska-Kowalczyk et al. 2004). Detailed studies on lipid peroxidation revealed that intrinsically elevated lipid metabolism may be correlated with resistance (Polkowska-Kowalczyk et al. 2008). This finding prompted us to undertake studies on the involvement of PLA₂ in defence mechanisms in Solanum. In the present work we have demonstrated changes in PLA₂ activity in CF treated leaves of Solanum genotypes. The experiments with PLA₂ inhibitors, HELSS or BPB, were performed in order to evaluate the involvement of iPLA₂ and sPLA₂ in defence strategy. The effects
of the inhibitors on PLA₂ activity and on generation of ROS in the studied interactions are described.

**Materials and methods**

**Plant material and pathogen elicitor**

Axenic shoots of *S. nigrum* var. *gigantea*, non-host completely resistant, *S. tuberosum* cv Bzura, polygenically field resistant, and clone H-8105, susceptible to the oomycete pathogen *Phytophthora infestans* (Mont) De Bary, were cultured *in vitro* as described previously (Polkowska-Kowalczyk et al. 2004). The plants grew under controlled conditions: day light fluorescent lamp 150 µmol m⁻² s⁻¹ for 16 h, day/night temperature 22/18°C. The pathogen *P. infestans* (complex race 618 with virulence factors 1, 2, 3, 4, 6, 7, 10 and 11) received from IHAR, Młochów Research Center (Poland) was maintained on rye agar medium at 15°C in the dark. A culture filtrate (CF), which served as an elicitor, was prepared from the pathogen grown in liquid medium. After 6 weeks of growth the medium was separated from the oomycete, dialysed against water for 48 h and lyophilised. The CF residue dissolved in distilled water was quantified as µg glucose equivalents ml⁻¹, as described in detail by Polkowska-Kowalczyk et al. (2004).

**Treatment of leaves**

The CF was applied in droplets at a concentration of 0.67 µg glucose equivalent g⁻¹ FW on the surface of leaves detached from 4-week-old plants; control leaves were treated with distilled water. All leaves were placed on moist filter paper in Petri dishes and kept for 6, 18, 30 and 36 h at 25°C under continuous light, as described in detail by Polkowska-Kowalczyk et al. (2004). At given time intervals the leaves were taken for analysis. In experiments with the PLA₂ inhibitors applied *in vivo*, stock solutions of the inhibitors: HELSS (12 mM in 96% EtOH) and BPB (10 mM in 50 mM TRIS buffer pH 8.5 containing 60% EtOH) were diluted with 50 mM TRIS buffer pH 8.5, giving a final concentration of EtOH within the range of 0.2 - 0.6% in the case of HELSS and in the range of 0.6 - 2.4% in the case of BPB. Detached leaves were immersed with shaking for 1 h in the solutions of inhibitors, whereas the control leaves were kept in similarly diluted solvents of inhibitors.
Afterwards, all leaves were quickly rinsed and placed in Petri dishes on filter paper moistened with distilled water. In some experiments portions of leaves previously preincubated with the inhibitors or its solvents (the control ones) were additionally treated with CF or water (control). All leaves after preincubation were kept for 18 h under conditions described above. Subsequently, the leaves were used for PLA$_2$ activity assay or for determination of ROS production.

**Enzyme extraction**

After incubation samples of leaves were ground in extraction medium (1:4, f.wt : v) containing: 0.3 M NaCl, 0.1 M HEPES pH 7.5, 2 mM EDTA, 5 mM DTT, 2 mM SHAM (salicylhydroxamic acid), 1 mM PMSF (phenylmethanesulfonyl fluoride), 50 mM NaF and 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 13000 g for 20 min. The obtained supernatant was submitted to fractionation with ammonium sulfate (30 - 60%). After centrifugation the protein precipitate was dissolved in 1 mL of 50 mM TRIS buffer pH 8.5, 1mM PMSF, 5 mM DTT. Then, the enzyme extract was partially purified on Sephadex G-25 column eluted with 50 mM TRIS buffer pH 8.5 containing 1 mM PMSF. All procedures were conducted at 4°C. The protein content in the obtained extract was determined by the method of Bradford (1976).

**Determination of PLA$_2$ activity**

Phospholipase A$_2$ activity was measured by continuous spectrophotometric method developed for assaying patatin phospholipase activity (Jiménez-Atiénzar et al. 2003). In this method [1,2-dilinoleoyl] PC, prepared as described by Jiménez-Atiénzar et al. (2003), was used as a substrate with LOX serving as the coupling enzyme, which oxidised the linoleic acid released by phospholipase activity. The PLA$_2$ activity was then followed spectrophotometrically by measuring the increase in absorbance at 234 nm, resulting from formation of hydroperoxides due to the LOX action. In methodical experiments, PLA$_2$ from hog pancreas (EC 3.1.1.4, Fluka) as well as enzyme extracts obtained from the studied plants were used to optimize the procedure for our material. The established reaction medium contained: 50 mM TRIS buffer pH 8.5, 3 mM CaCl$_2$, 3 mM
deoxycholate, 60 µM [1,2-dilinoleoyl] PC, 400 U LOX (Lipoxidase Type I-B, EC 1.13.11.12; 1,000,000 U/mg, Sigma). Lipoxidase from soybean (Type I-B EC 1.13.11.12, Sigma) and appropriate amounts of exogenous LA (cis-9,cis-12-octadecadienoic acid, Sigma) were used to confirm oxidation of LA by LOX in the reaction medium. Each set of assays was preceded by controls without the enzyme extract and/or lipoxygenase to ascertain the absence of substrate oxidation by lipoxygenase or other compounds in the reaction medium. Inhibitors of PLA₂, HELSS and BPB, added to these samples, did not interfere with the LOX activity, hence appearing applicable to PLA₂ assay. Phospholipase A₂ from hog pancreas (EC 3.1.1.4, Fluka) was used as a standard to check the inhibitory effects of HELSS or BPB at different concentrations. In the case of BPB, PLA₂ as a standard was preincubated with the inhibitor in 50 mM TRIS buffer pH 8.5 and 3 mM CaCl₂ for 30 min at 25°C, following which PC, deoxycholate and LOX were added to initiate the enzymatic reaction.

Assay for ROS production

ROS production was evaluated by determining the reduction of nitroblue tetrazolium (NBT) in the medium by O₂⁻ released from leaf tissues (Doke 1983). The leaves treated with CF or the control ones treated with water were incubated for 1 h in a mixture containing NBT (nitroblue tetrazolium (2,2'-di-p-nitrophenyl-5,5'-diphenyl-[3,3'-dimethoxy-4,4’diphenylene]-ditetrazolium chloride). The mixture was then heated at 85°C, cooled and absorbance at 580 nm was measured as described by Polkowska-Kowalczyk et al. (2004).

Presentation of data

At least two independent series of experiments, each with at least two portions of about 300 mg tissue (8-25 leaves, depending on their size) were performed. The data presented are the mean values ± SD. The significance of difference between mean values was determined by the Student’s t-test.
Results

PLA$_2$ activity

In all Solanum genotypes PLA$_2$ activity increased in response to CF treatment but varied with respect to intensity and timing (Fig. 1). In S. nigrum leaves, the PLA$_2$ activity of up to 290% (p<0.05) in relation to water treated control was noted at 18 h of CF treatment, decreasing slightly after 30 h of treatment. In elicited Bzura leaves, PLA$_2$ activity rose at 6 h of treatment to about 160% (p<0.05) of the control, and afterwards remained at the elevated level. In contrast, in the susceptible clone H-8105, after an early and transient increase to 170% (p<0.05) of the control values, PLA$_2$ activity decreased to the initial level.

In vivo and in vitro effects of HELSS and BPB on PLA$_2$ activity

To our knowledge data on the effects of PLA$_2$ inhibitors on defence processes in plant tissues are scarce. Moreover, Holk et al. (2002) demonstrated that several PLA$_2$ inhibitors effective in vitro were ineffective in vivo in auxin-induced hypocotyl elongation growth of Arabidopsis. Therefore, experiments were performed with HELSS and BPB applied in vivo (leaves incubated in the presence of the PLA$_2$ inhibitors) and in vitro (inhibitors added to enzyme extracts). In experiments in vivo the detached leaves were preincubated with HELSS at concentrations of 24, 48, 72 and 96 µM vs control (see Material and methods). No symptoms of injury appeared in leaves treated with HELSS at concentrations up to 72 µM or in controls. In leaves of all genotypes HELSS applied in vivo did not inhibit PLA$_2$ activity in a dose dependent manner (Table 1). An inhibitory effect of HELSS was about 10–20% in resistant S. nigrum and Bzura, and up to 46% in the susceptible H-8105. When BPB was applied in vivo at concentration of 200 µM PLA$_2$ activity was affected slightly, whereas at concentration of 400 µM leaves were injured. Based on these observations HELSS at the concentration of 72 µM and BPB at 200 µM were used in subsequent in vivo assays.

In experiments in vitro, when HELSS was added to extracts from leaves pretreated in vivo with HELSS or its TRIS buffer solvent (control), changes in PLA$_2$ activity varied among the genotypes studied. In S. nigrum HELSS inhibited PLA$_2$ activity significantly (p<0.05) in a dose dependent
manner, independently of the leaf pretreatment in vivo (Fig. 2). In Bzura HELSS applied in vitro at all used concentrations caused about 50% (p<0.05) inhibition of PLA₂ activity. On the contrary, in the susceptible H-8105, in the presence of 24 or 48 µM HELSS PLA₂ activity decreased about 20% in extracts from the control leaves and those preincubated with HELSS in vivo whereas at the highest dose of HELSS, PLA₂ activity declined about 90% (p<0.05), regardless of the previous in vivo treatment of leaves.

In all genotypes the inhibitory effect of BPB applied in vitro to enzyme extracts from leaves pretreated in vivo with the TRIS solvent, appeared mostly at 400 µM concentration, leading to reduction of PLA₂ activity from 86 to 67% (p<0.05) of control (Fig. 3). In extracts from leaves pretreated in vivo with 200 µM BPB, PLA₂ activity was inhibited by BPB in a dose dependent manner in the resistant S. nigrum and Bzura. In contrast, in the susceptible H-8105, in extracts from leaves pretreated with BPB or its solvent the activity of PLA₂ increased in the presence of 100 or 200 µM BPB, and decreased to 72% (p<0.05) of control only at the concentration of 400 µM.

**Effect of HELSS and BPB on PLA₂ activity in CF treated leaves**

In order to assign fractions of PLA₂ that responded to CF, and were thereby possibly involved in defence mechanisms, the effects of HELSS and BPB inhibitors, which discriminate between iPLA₂ and sPLA₂, on CF-induced PLA₂ activity were examined. Detached leaves were preincubated with TRIS solvents of the inhibitors (samples 1) and in 72 µM HELSS or 200 µM BPB (samples 2) for 1 h (Figs. 4 and 5, respectively). All leaves were then rinsed and one portion of leaves from samples 1 and 2 were treated with CF (samples 3 and 4, respectively), whereas the rest of the leaves treated with water served as respective reference for samples 3 and 4. After 18 h of treatment, the activity of PLA₂ was determined in all samples. In the case of HELSS (Fig. 4), comparison of samples 1 and 3 revealed an increase in PLA₂ activity in response to CF treatment only in S. nigrum (about 50%, p<0.05), whereas in the other genotypes PLA₂ activity practically did not change. Thus, in this experiment, when leaves were preincubated with TRIS solvent, the effect of CF on PLA₂ activity was less pronounced than that in experiments without the PLA₂ inhibitors, when control leaves
remained in water all the time (compare Figs. 1 and 4). Possibly, the change in the pH of the TRIS preincubation medium (pH 8.5) in comparison to water (pH 6) may have affected the response of leaves to CF. Piedras et al. (1998) demonstrated that ROS accumulation in Cf9 tobacco cell suspension challenged with Avr9 depended upon extracellular pH values.

Interestingly, the inhibitory effect of HELSS on PLA$_2$ activity was pronounced only in CF treated leaves from $S. nigrum$ and Bzura (Fig. 4, compare samples 3 and 4); in the absence of CF practically no such effect was observed (compare samples 1 and 2). In $S. nigrum$ CF-induced PLA$_2$ activity (sample 4) decreased about 30% (p<0.05) and in Bzura about 20% (p<0.05) in relation to that in leaves without preincubation with HELSS (sample 3). In contrast, BPB (Fig. 5) practically did not inhibit PLA$_2$ activity in control (compare samples 1 and 2) and in CF treated leaves (compare samples 3 and 4) of all genotypes except $S. nigrum$. In the latter the PLA$_2$ activity even increased slightly.

Effect of HELSS and BPB on ROS production

To ascertain a possible relationship between ROS production and PLA$_2$ activity, as reported for human cells, we have compared ROS production in response to CF in leaves preincubated with the inhibitors of PLA$_2$ for 1 h and those without this treatment. When leaves were treated with CF alone ROS production increased in all genotypes studied (Table 2). In leaves preincubated with HELSS before CF treatment, ROS production did not change in comparison with that in leaves without HELSS treatment. When leaves were preincubated with BPB and then treated with CF, ROS production surprisingly increased in $S. nigrum$, to 180% (p<0.05) of that in leaves without BPB treatment. On the contrary, in Bzura and H-8105 leaves ROS production decreased by about 40% (p<0.05) due to inhibitor treatment.
Discussion

Identification of the function of a specific PLA among multiple isoforms as well as their products in a complex signalling network is a great challenge for researchers. The present results contribute to some degree to the knowledge on the involvement of PLA$_2$ in plant defence response. Our studies on Solanum genotypes revealed: i) an increase in PLA$_2$ activity in response to CF treatment, ii) various effects of HELSS and BPB applied in vivo and in vitro on PLA$_2$ activity, iii) different modification of CF-induced PLA$_2$ activity by HELSS and BPB, iii) differences in the effects of HELSS and BPB on CF-induced ROS production. The increase in CF-induced PLA$_2$ activity varied depending on the resistance status of the genotypes. The highest increase in PLA$_2$ activity was noted in S. nigrum, non-host completely resistant to P. infestans, and the lowest one in the susceptible H-8105. This observation is in concert with our previous findings showing that intense lipid metabolism in the early phase of response to the elicitor may be related to resistance (Polkowska-Kowalczyk et al. 2008). An increase in PLA$_2$ activity in different plant/pathogen interactions was also reported by other authors, e.g., Kawakita et al. (1996), Narváez-Vásquez et al. (1999) and Göbel et al. (2001).

The doses of PLA$_2$ inhibitors, HELSS and BPB, applied in vivo were selected at levels at which they affected PLA$_2$ activity without symptoms of leaf injury. In all genotypes studied the in vivo effects of each inhibitor were ambiguous, but PLA$_2$ activity decreased markedly when high doses of the inhibitors were added in vitro to leaf enzyme extracts. Surprisingly, at low BPB concentrations PLA$_2$ activity increased in H-8105 leaf extract. Various response of PLA$_2$ activity to HELSS and BPB, which discriminate between iPLA$_2$ and sPLA$_2$, suggested differences in pools of PLA$_2$ isoforms and/or their sensitivity to the inhibitors in the studied genotypes. The observed different effectiveness of the inhibitors in vivo and in vitro may be due to either various accessibility of the inhibitors entering the leaf tissues or to their metabolism in the tissues. It is also conceivable that in vivo some fractions of the enzyme might be inactive under surrounding conditions and/or be unavailable to the inhibitors because of their intracellular localization. According to Senda et al. (1996) PLA$_2$ activity of potato patatin is inactive under acidic conditions in vacuoles and becomes active under basic conditions in the cytosol, where the enzyme is translocated upon pathogen attack.
It has been found that both inhibitors influenced mainly CF-induced PLA$_2$ activity. The HELSS applied \textit{in vivo} before CF treatment inhibited PLA$_2$ activity mostly in the non-host \textit{S. nigrum} and the field resistant Bzura. In contrast, when BPB was added, CF-induced PLA$_2$ activity increased slightly in \textit{S. nigrum} and practically did not change in the other genotypes. The observation that an inhibitory effect of HELSS but not of BPB manifested itself on CF-induced PLA$_2$ activity, i.e., fraction responding to the elicitor, suggests that iPLA$_{2S}$ are involved in defence signalling, whereas the contribution of sPLA$_{2S}$ remains questionable. Potential function of PLA$_2$ in plant signal transduction in response to auxin, elicitor or wounding was also suggested by Holk et al. (2002) and Scherer (2002). The stimulation of CF-induced PLA$_2$ activity in the presence of BPB in \textit{S. nigrum} was rather unexpected. Explanation of this observation is difficult because knowledge on crosstalk among different plant PLA$_2$ fractions is very limited (Scherer et al. 2010). If one presumes that the observed increase in PLA$_2$ activity originates mainly from iPLA$_2$ activity (not inhibited by BPB), it is conceivable that it might result from compensation of sPLA$_2$ activity likely lowered due to BPB action. On the other hand, since plant iPLA2 are reported to be upregulated by multiple stresses (Scherer et al. 2010 and ref. therein), in our experiment BPB applied \textit{in vivo} might serve as a toxic factor leading to enhanced activity of iPLA$_2$. Alternatively, it is also conceivable that in \textit{S. nigrum} iPLA2 activity enhanced in the presence of CF might result in high production of LPC. According to Viehweger et al. (2002) the action of LPC might lead to a shift in cellular pH. Lee et al. (2005) reported that plant sPLA$_{2S}$ differ in their pH and Ca$^{2+}$ concentration optima, so that some of them might be inactive in their subcellular locations, requiring a change in pH and Ca$^{2+}$ concentration to achieve maximum activity. Therefore, CF-induced changes in the cellular environment might lead to some activation of sPLA$_2$ and, in consequence, allow to overcome the inhibitory effect imposed by the BPB applied \textit{in vivo}.

Simultaneous inhibition of PLA$_2$ activity and oxidative burst induced by elicitor or pathogen may imply a relationship between these two responses. Contrary to mammalian cells, studies in plants on the involvement of PLA$_{2S}$ activities in the generation of ROS by NADPH oxidase in response to elicitation are scarce. Chandra et al. (1996) demonstrated that in soybean cells chlorpromazine, a
nonspecific inhibitor of PLA$_2$, inhibited both PLA$_2$ activity and ROS generation induced by extract from the pathogenic fungus *Verticillium dahliae*, which suggested a correlation between these responses. Similarly, Piedras et al. (1998) demonstrated that inhibitors of PLA$_2$, BPB, quinacrine and chlorpromazine, reduced ROS production in Cf9 tobacco cells treated with Avr9. We have found that in response to CF treatment alone ROS production increased in all genotypes similarly as it has been demonstrated previously (Polkowska-Kowalczyk et al. 2004). The HELSS applied *in vivo* before CF treatment had practically no effect on ROS production, whereas BPB in similar circumstances led to an increase in ROS production in *S. nigrum* leaves but inhibited this process in Bzura and H-8105. These results suggest that ROS production in response to CF may be independent of iPLA$_2$ activation, while the contribution of sPLA$_2$ to this process is plausible. Different effects of the PLA$_2$ inhibitors on CF-induced PLA$_2$ activity and ROS production in the non-host *S. nigrum* in comparison with the field resistant Bzura and the susceptible H-8105 may imply various signal transduction pathways in defence mechanisms employed by *S. nigrum* and the other genotypes. On the other hand, considering the obtained results, particularly the data concerning *S. nigrum*, there might be an interplay between iPLA$_2$s and sPLA$_2$s. Perhaps change in iPLA$_2$ activity in the presence of CF and alteration in cellular environment might lead to enhanced sPLA$_2$ activity and possible stimulation of ROS production. Different possibilities of interplay between various forms of PLA$_2$ have been reported for mammalian cells (Balboa and Balsinde 2006). Kim et al. (2002) showed that sPLA$_2$s, acting on the outer membrane of human neutrophils, release FA and LPC. This in turn, can lead to an increase in cytosolic free Ca$^{2+}$ level and induce phosphorylation of cPLA$_2$$\alpha$, which then result in cPLA$_2$$\alpha$-dependent leukotriene biosynthesis. Han et al. (2003) in studies on the effects of expression of genes of Group IIa or V sPLA$_2$ and cPLA$_2$$\alpha$ on H$_2$O$_2$-induced arachidonic acid (AA) release in murine mesangial cells found that both groups of sPLA$_2$ amplified the cPLA$_2$$\alpha$-mediated response to H$_2$O$_2$, resulting in increased release of AA from phospholipids. In the light of the similarities between plant and neutrophile oxidative burst (Piedras et al. 1998) as well as some homologies between plant and animal PLA$_2$s an assumption of an interplay between plant iPLA$_2$s and sPLA$_2$s seems warranted.
Overall, the results presented here show that in the studied *Solanum* species there occur various fractions of PLA₂, which differ in response to elicitor treatment. The iPLA₂s probably participate in signal transduction in defence mechanisms whereas the involvement of sPLA₂s is unclear. However, due to a possible interplay among various fractions of PLA₂, all of them might modulate the defence response in interaction between *Solanum* and *P. infestans*. Detailed elucidation of these mechanisms needs further investigation.

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Table 1.
PLA₂ activity ($\Delta A_{234} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}\text{FW}$) in leaves treated \textit{in vivo} with HELSS or BPB

| Genotype                | HELSS (µM) |          |          |          |          |          |          |          |          |
|-------------------------|------------|----------|----------|----------|----------|----------|----------|----------|----------|
|                         | TRIS (control) | 24       | 48       | 72       | TRIS (control) | 200      | 100      | 100      | 100      |
|                         | 1.12±0.13  | 1.01±0.29 | 0.88±0.06 | 0.98±0.11 | 1.10±0.31  | 1.06±0.10 |
| \textit{S. nigrum var. gigantea} | 1.11±0.12  | 0.96±0.02 | 0.92±0.04 | 0.91±0.07 | 0.91±0.08  | 0.88±0.16 |
| Bzura                   | 1.24±0.08  | 0.69±0.01 | 0.83±0.02 | 0.90±0.04 | 0.90±0.05  | 0.88±0.08 |
Table 2. Effect of inhibitors of PLA$_2$ on production of ROS ($\Delta A_{580}\text{g}^{-1}\text{FW}\cdot\text{h}^{-1}$)

| Treatment       | S. nigrum var. gigantea | Bzura   | H-8105  |
|-----------------|-------------------------|---------|---------|
| TRIS (control)  | 0.08 ± 0.023            | 0.36 ± 0.070 | 0.16 ± 0.013 |
| TRIS + CF       | 0.25 ± 0.116$^a$        | 1.23 ± 0.299$^b$ | 1.94 ± 0.188$^c$ |
| HELSS + CF      | 0.24 ± 0.092            | 1.70 ± 0.422 | 2.00 ± 0.203 |
| BPB + CF        | 0.45 ± 0.040$^a$        | 0.73 ± 0.076$^b$ | 1.15 ± 0.067$^c$ |

Leaves were incubated in 72 µM HELSS or 200 µM BPB for 1 h before treatment with CF. The values obtained for control leaves differed significantly (p<0.05) from those for treated leaves. The same letters indicate significant differences (p<0.05) between values obtained from treated leaves. Data represent mean values ± SD (n ≥ 4).
Legend to Figures

**Fig. 1** Changes in phospholipase A$_2$ (PLA$_2$) activity in leaves of *Solanum* species treated with culture filtrate (CF) for 6, 18, 30 and 36 h in relation to controls treated with H$_2$O. The mean activities in the control were: *S. nigrum* - 0.44 ΔA$_{234}$·min$^{-1}$·mg$^{-1}$ protein, Bzura - 0.37 ΔA$_{234}$·min$^{-1}$·mg$^{-1}$ protein, H-8105 - 0.25 ΔA$_{234}$·min$^{-1}$·mg$^{-1}$ protein. The values are mean ± SD (n ≥ 6). The same letters indicate significant differences (p<0.05) between mean values for CF treated leaves and controls in respective genotypes.

**Fig. 2** Changes in phospholipase A$_2$ (PLA$_2$) activity in *Solanum* species in response to HELSS added *in vitro* (vtr) to leaf enzyme extracts obtained from leaves treated *in vivo* (v) with TRIS □ or with 24 µM □, 48 µM ■, 72 µM ■ HELSS for 1 h. Data are means ± SD (n ≥ 4).

**Fig. 3** Changes in phospholipase A$_2$ (PLA$_2$) activity in *Solanum* species in response to BPB added *in vitro* (vtr) to leaf enzyme extracts obtained from leaves treated *in vivo* (v) with TRIS □ or 200 µM BPB ■ for 1 h. Data are means ± SD (n ≥ 4).

**Fig. 4** Changes in phospholipase A$_2$ (PLA$_2$) activity in leaves of *Solanum* species preincubated *in vivo* (v) for 1 h in TRIS or 72 µM HELSS (samples 1 and 2) and then treated for 18 h with culture filtrate (CF): samples 3 and 4 or H$_2$O: samples 1 and 2. Data are means ± SD (n ≥ 5). The same letters indicate significant differences (p<0.05) between mean values for samples 1 and 3; 3 and 4.

**Fig. 5** Changes in phospholipase A$_2$ (PLA$_2$) activity in leaves from *Solanum* species preincubated *in vivo* (v) for 1 h in TRIS or 200 µM BPB (samples 1 and 2) and then treated for 18 h with culture filtrate (CF): samples 3 and 4 or H$_2$O: samples 1 and 2. Data are means ± SD (n ≥ 5). The same letters indicate significant differences (p<0.05) between mean values for samples 1 and 3; 3 and 4.
References

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. Ann Rev Phytopathol 33:299-321

Balboa MA, Balsinde J (2006) Oxidative stress and arachidonic acid mobilization. Biochim Biophys Acta 1761:385-391

Balsinde J, Balboa MA, Insel PA, Dennis EA (1999) Regulation and inhibition of phospholipase A2. Annu Rev Pharmacol Toxicol 39:175-189

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Cacas J-L, Vailleau F, Davoine C, Ennar N, Agnel J-P, Tronchet M, Ponchet M, Blein J-P, Roby D, Triantaphylidès Ch, Montillet J-L (2005) The combined action of 9 lipoxygenase and galactolipase is sufficient to bring about programmed cell death during tobacco hypersensitive response. Plant Cell and Environ 28:1367-1378

Chandra S, Heinstein PF, Low PS (1996) Activation of phospholipase A by plant defense elicitors. Plant Physiol 110:979-986

Dana R, Malech HL, Levy R (1994) The requirement for phospholipase A2 for activation of the assembled NADPH oxidase in human neutrophils. Biochem J 297:217-223

Dhondt S, Gouzerh G, Müller A, Legrand M, Heitz T (2002) Spatio-temporal expression of patatin-like lipid acyl hydrolases and accumulation of jasmonates in elicitor-treated tobacco leaves are not affected by endogenous levels of salicylic acid. Plant J 32:749-762

Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. Physiol Plant Pathol 23:345-357

Göbel C, Feussner I, Schmidt A, Scheel D, Sanchez-Serrano J, Hamberg M, Rosahl S (2001) Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. J Biol Chem 276:6267-6273
Han WK, Sapirstein A, Hung CC, Alessandrini A, Bonventre JV (2003) Cross-talk between cytosolic phospholipase A\(_2\alpha\) (cPLA\(_2\alpha\)) and secretory phospholipase A\(_2\) (sPLA\(_2\)) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells. J Biol Chem 278:24153-24163

Hazen SL, Zupan LA, Weiss RH, Getman DP, Gross RW (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipaseA\(_2\). J Biol Chem 266:7227-7232

Holk A, Rietz S, Zahn M, Quader H, Scherer GFE (2002) Molecular identification of cytosolic, patatin-related phospholipases A from Arabidopsis with potential functions in plant signal transduction. Plant Physiol 130:90-101

Jiménez-Atiénzar M, Cabanes J, Gandía-Herrero F, Escribano J, García-Carmona F, Pérez-Gilabert M (2003) Determination of the phospholipase activity of patatin by a continuous spectrophotometric assay. Lipids 38:677-682

Kawakita K, Senda K, Doke N (1993) Factors affecting in vitro activation of potato phospholipase A\(_2\). Plant Science 92:183-190

Kim YJ, Kim KP, Han SK, Munoz NM, Zhu X, Sano H, Leff AR, Cho W (2002) Group V phospholipase A\(_2\) induces leukotriene biosynthesis in human neutrophils through the activation of group IVA phospholipase A\(_2\). J Biol Chem 277:36479-36488

La Camera S, Balagué C, Göbel C, Geoffroy P, Legrand M, Feussner I, Roby D, Heitz T (2009) The Arabidopsis patatin-like protein 2 (PLP2) plays an essential role in cell death execution and differentially affects biosynthesis of oxylipins and resistance to pathogens. MPMI 22:469-481

La Camera S, Geoffroy P, Samaha H, Ndiaye A, Rahim G, Legrand M, Heitz T (2005) A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. Plant J 44:810-825

Lee HY, Bahn SC, Shin JS, Hwang I, Back K, Doelling JH, Ryu SB (2005) Multiple forms of secretory phospholipase A\(_2\) in plants. Prog Lipid Res 44:52-67

Levy R (2006) The role of cytosolic phospholipase A\(_2\)-\(\alpha\) in regulation of phagocytic functions. Biochim Biophys Acta 176:1323-1334
Mansfeld J, Ulbrich-Hofmann R (2007) Secretory phospholipase A<sub>2-α</sub> from *Arabidopsis thaliana*: functional parameters and substrate preference. Chem Phys Lipids 150:156-166

Mueller MJ, Berger S (2009) Reactive electrophilic oxylipins: Pattern recognition and signalling. Phytochem 70:1511-1521

Munnik T, Irvine RF, Musgrave A (1998) Phospholipid signalling in plants. Biochim Biophys Acta 1389:222-272

Mur LAJ, Kenton P, Lloyd AJ, Ougham H, Prats E (2008) The hypersensitive response; the centenary is upon us but how much do we know? J Exp Bot 59:501-520

Narváez-Vásquez J, Florin-Christensen J, Ryan CA (1999) Positional specificity of a phospholipase A activity induced by wounding, systemin, and oligosaccharide elicitors in tomato leaves. Plant Cell 11:2249-2260

Piedras P, Hammond-Kosack KE, Harrison K, Jones JDG (1998) Rapid, *Cf*-9- and Avr9-dependent production of active oxygen species in tobacco suspension cultures. MPMI 11:1155-1166

Polkowska-Kowalczyk L, Wielgat B, Maciejewska U (2004) The elicitor-induced oxidative processes in leaves of *Solanum* species with differential polygenic resistance to *Phytophthora infestans*. J Plant Physiol 161:913-920

Polkowska-Kowalczyk L, Montillet J-L, Agnel J-P, Triantaphylidès C, Wielgat B, Maciejewska U (2008) Changes in the initial phase of lipid peroxidation induced by elicitor from *Phytophthora infestans* in *Solanum* species. J Plant Physiol 165:1929-1939

Ryu SB (2004) Phospholipid-derived signalling mediated by phospholipase A in plants. Trends in Plant Sci 9:229-235

Scherer GFE (2002) Secondary messengers and phospholipase A<sub>2</sub> in auxin signal transduction. Plant Mol Biol 49:357-372

Scherer GFE, Ryu SB, Wang X, Matos AR, Heitz T (2010) Patatin-related phospholipase A: nomenclature, subfamilies and functions in plants. Trends in Plant Science 15:693-700

Senda K, Yoshioka H, Doke N, Kawakita K (1996) A cytosolic phospholipase A<sub>2</sub> from potato tissues appears to be patatin. Plant Cell Physiol 37:347-353
Tian ZD, Liu J, Wang BL, Xie CH (2006) Screening and expression analysis of *Phytophthora infestans* induced genes in potato leaves with horizontal resistance. Plant Cell Rep 25:1094-1103

Viehweger K, Dordschbal B, Roos W (2002) Elicitor-activated phospholipase A$_2$ generates lysophosphatidyl cholines that mobilize the vacuolar H$^+$ pool for pH signaling via the activation of Na$^+$-dependent proton fluxes. Plant Cell 14:1509-1525

Vleeshouwers VGAA, van Dooijeweert W, Gouvers F, Kamoun S, Colon LT (2000) The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210:853-864
**Figure 3**

- **S. nigrum**
- **Bzura**
- **H-8105**

**PLA₂ activity (% of control)**

**BPB concentration (µM) in vivo**

BPB applied *in vitro* (vtr) 100 µM, 200 µM, 400 µM
Figure 4

![Bar chart showing PLA2 activity (ΔA* mg protein⁻¹ *min⁻¹) for S. nigrum, Bzura, and H-8105.](image)

Legend:
- No. 1
- No. 2
- No. 3
- No. 4
- TRIS v
- HELSS v
- TRIS v+CF
- HELSS v+CF
Figure 5

PLA$_2$ activity (ΔA$^*$ mg protein$^{-1}$ min$^{-1}$)

S. nigrum

Bzura

H-8105

no. 1 2 3 4

TRIS v BPB v TRIS v+CF BPB v+CF