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Research article

Involvement of phospholipases C and D in early response to SAR and ISR inducers in *Brassica napus* plants

B. Profotová, L. Burketová [1], Z. Novotná, J. Martinec [1], O. Valentová *

Department of Biochemistry and Microbiology, Institute of Chemical Technology Prague, Technická 3, 166 28 Prague 6, Czech Republic

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Abstract

Phospholipid signaling is an important component in eukaryotic signal transduction pathways. In plants, it plays a key role in growth and development as well as in responses to environmental stresses, including pathogen attack. We investigated the involvement of both phospholipase C (PLC, EC 3.1.4.11) and D (PLD, EC 3.1.4.4) in early responses to the treatment of *Brassica napus* plants with the chemical inducers of systemic acquired resistance (SAR): salicylic acid (SA), benzothiadiazole (BTH), and with the inducer mediating the induced systemic resistance (ISR) pathway, methyl jasmonate (MeJA). Rapid activation (within 0.5–6 h treatment) of the in vitro activity level was found for phosphatidyl inositol 4,5 bisphosphate (PIP_2)-specific PLC (PI-PLC) and three enzymatically different forms of PLD: conventional PLDα, PIP_2-dependent PLD β/γ, and oleate-stimulated PLDδ. The strongest response was found in case of cytosolic PIP_2-dependent PLD β/γ after BTH treatment. PLDδ was identified in *B. napus* leaves and was very rapidly activated after MeJA treatment with the highest degree of activation compared to the other PLD isoforms. Interestingly, an increase in the amount of protein was observed only for PLDγ and/or δ after ISR induction, but later than the activation occurred. These results show that phospholipases are involved in very early processes leading to systemic responses in plants and that they are most probably initially first activated on post translational level.

Keywords: Brassica napus; Induced resistance; Phospholipase C and D; Isoforms

1. Introduction

Plants, as well as pathogens, possess genes whose products are involved in determining host–pathogen specificity. This "gene-for-gene" interaction triggers a series of physiological changes not only at the site of infection, but also at distant tissues of the plant initiating systemic acquired resistance (SAR) [1–3]. This type of resistance is induced after a local hypersensitive response (HR) accompanied by the accumulation of salicylic acid (SA), burst of reactive oxygen species (ROS), synthesis of phytoalexins, and accumulation of pathogenesis-related proteins (PR proteins).

More recently it has been demonstrated that resistance phenotypically similar to SAR can be stimulated by some non-pathogenic rhizobacteria [4] and, as different signaling pathways are probably involved, it was named induced systemic resistance (ISR). SAR is dependent on the accumulation of SA whereas ISR requires appearance of both jasmonic acid (JA) and ethylene [5]. Thus appropriately stimulated plants are able to substantially enhance their defensive capacity in either SA-dependent or SA-independent manner.

Besides pathogens or ISR-inducing bacteria, exogenously applied SA or its analogues, such as 2,6-dichloroisonicotinic acid (INA) [6] and benzothiadiazole (benzo [1,2,3]thiadiazole-7-carbothioic acid S-methyl ester, BTH) [7], or JA result in the induction of resistance.

Among a number of induced long-term defense responses, PR proteins occupy a particular place due to their suggested role in SAR against a wide range of pathogens. PR proteins

Abbreviations: BTH, benzothiadiazole; ISR, induced systemic resistance; MeJA, methyl jasmonate; PI-PLC, phosphatidylinositol 4,5 bisphosphate specific PLC; PIP_2, phosphatidylinositol 4,5 bisphosphate; PLD, phospholipase D; PR proteins, pathogenesis-related proteins; SA, salicylic acid; SAR, systemic acquired resistance.

* Corresponding author. Tel.: +420 20 44 5102; fax: +420 20 44 5167.
E-mail address: olga.valentova@vscht.cz (O. Valentová).
[1] Present address: Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

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are currently classified in 17 groups according to their biochemical and molecular-biological properties (for review see [8]). The most important groups are considered to be PR-2 proteins with β-1,3-glucanase activity and PR-3 with chitinase activity.

Early events involved in defense responses as well as the mechanisms of the signal transduction are still not well characterized. In the last few years, signs of the involvement of a phospholipid (or lipid) signaling pathway in these processes were foreshadowed, but much remains unclear.

Phospholipases, enzymes hydrolyzing phospholipids, are divided to groups according to the site of substrate molecule cleavage (phospholipase A, C and D; PLA, PLC and PLD). PLD is the predominant family of phospholipases in plants and the biochemical properties, domain structure and genome organization of plant PLDs are much more diverse than those of other organisms. Based on domain structure there are two principal subfamilies of plant PLDs in *Arabidopsis*: C2-PLDs and PXPH-PLDs [9,10]. C2-containing PLDs are further distinguished according to their biochemical properties to four types designated α, β, γ and δ. These groups show different requirements for substrate, Ca^{2+} and phosphatidylinositol-4,5-bisphosphate (PIP_2).

Different members of the phospholipase family were shown to be activated in response to various cellular and environmental cues both on the protein and gene level, and the phospholipase network is obviously also involved in mechanisms of plant systemic responses to pathogen attack.

Both symbiotic and pathogenic interactions are thought to involve PLC signaling. *OsPI-PLC1* expression was induced by chemical inducers SA, BTH, JA, methyl jasmonate (MeJA) and these treatments induced resistance in rice against blast disease caused by *Magnaporthe grisea* [11]. Although evidence is increasing for the involvement of PLC, the function of DAG in plants has not been established. It was shown by Munnik et al. [12] that DAG is rapidly phosphorylated to phosphatidic acid (PA) by ubiquitous DAG kinase and thus forms the same product as PLD. PA and diacylglycerol pyrophosphate (DAGPP) are rapidly and transiently accumulated in tomato culture cell suspensions treated with the general elicitors tetraacetylchitotetraose, xylanase and flagellin-derived peptide, flg22. PA derived from PLD action was detected for xylanase treatment [13]. Rapid and specific expression of *PLDβ1* was later observed in tomato [14]. Different isoforms of *AtPLD* were expressed in response to wounding and other stress cues including SA and MeJA treatment of *Arabidopsis* leaves. Treatment of detached leaves with MeJA significantly increased expression of *PLDβ* but not *PLDα* and γ1 [15]. On the contrary, SA induced expression of *PLDγ1* in *Arabidopsis* leaves and this isoform was also upregulated during the incompatible interaction with *Pseudomonas syringae* pv. tomato [16]. Antisense abrogation of *PLDα* decreased the wound induction of PA, JA and JA-regulated gene for vegetative storage protein (*VSP*), and wound-induced lipoxygenase 2(*LOX2*) expression, when compared to the wild type plants.

PLDδ, the oleate-stimulated PLD has been reported for the first time in *Arabidopsis* by Katagiri et al. [17] and by Wang and Wang [18] as well. McGee et al. [19] cloned and characterized several *PLD* genes in rice cells. One of them, *RPLD5*, was most closely related to *Arabidopsis PLDδ*. Katagiri et al. [17] suggested the role of this enzyme in dehydration and salt stress. Wang and Wang [18] have characterized PLDδ biochemically and showed its association with plasma membrane. Activation of PLD by H_2O_2 was shown in *Arabidopsis* [20] and rice cells [21]. In *Arabidopsis* it was clearly demonstrated that plasma membrane-bound PLDδ is activated after H_2O_2 treatment and that *PLDδ* knockout plants are more susceptible to H_2O_2-promoted programmed cell death. Accordingly, PLDδ likely plays a role in mediating the plant response to oxidative stress [20].

The basic knowledge of the molecular mechanisms involved in the process leading to induced plant resistance is obviously of great importance. Whereas the long-term demonstration of this type of resistance including production of PR-proteins is relatively well characterized, the early events involved in this process and mechanisms of the signal transduction are still unclear.

PLC and PLD are supposed to be the modulators of phospholipid signaling network but their involvement in many processes was investigated separately. Here we show, that both PLC and PLD are involved in the process of induced resistance in crop plant *Brassica napus* at the level of enzyme activity and/or protein level.

2. Results and discussion

2.1. Long-term response of B. napus plants to SAR and ISR inducers

In the introductory experiments we intended to verify that the treatment of *B. napus* plants with chemical inducers of resistance (SA/BTH or MeJA) activates defense responses related to SAR in our experimental material.

Both SA and BTH induced accumulation of the proteins cross-reacting with antisera raised against basic sugar beet β-1,3-glucanase (Glu2) and basic sugar beet chitinase class IV (Ch4) in apoplast of treated leaves (Fig. 1). On the other hand, MeJA did not induce the same protein pattern, only several slightly induced proteins of higher molecular weights cross-reacted with the antibodies in immunoblot (data not shown).

Mimicking the effect of pathogen attack by chemical compounds has been reported earlier in a number of plant species. Similarly to the real infection, such treatment was followed by the establishment of SAR in non-treated parts of the plant, as was demonstrated by exogenous application of SA or its functional analogue BTH [7,22]. Despite the assumed identical mode of action of SA and BTH, it has been shown, that while SA induces direct gene expression, BTH often rather potentiates plants to quicker defense response [23]. On the contrary, in our experiment, both compounds induced the studied PR-proteins in the same way, and the response to BTH was stronger, indicating the dependence of the response in particular plant species.

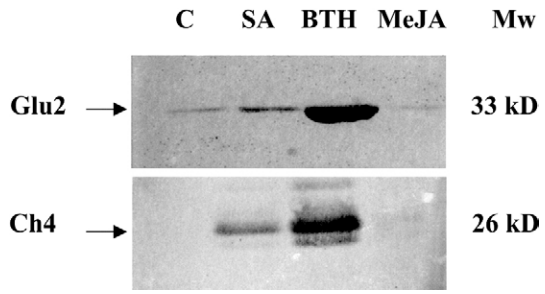

Fig. 1. Accumulation of PR-proteins in leaf apoplast induced by exogenously applied SAR and ISR inducers. Three-week-old plants of *B. napus* were sprayed with 1 mM SA, 0.3 mM BTH and 1 mM MeJA. Five days after the treatment an EF was prepared as described in "Section 3" and equal volumes corresponding to equal amounts of leaf tissues were subjected to immunoblot analysis. The proteins cross-reacting with polyclonal anti-Glu2 and anti-Ch4 serum prepared against sugar beet basic β-1,3-glucanase (Glu2) and basic chitinase class IV (Ch4), respectively, were detected. Compared to SA and BTH, no effect on the induction of the proteins mentioned above was recorded in plants treated with MeJA. The lane labeled "C" is the control.

In *B. napus* plants, the accumulation of PR-proteins or expression of *PR* genes was studied earlier. In accordance with our results, Rasmussen et al. [24] detected basic chitinase class IV (Ch4) in plants infected with fungal pathogen *Leptosphaeria maculans*.

On the other hand, among SA-induced genes, which have been isolated from rape seed using subtractive hybridization, the only representative of PR proteins was a *PR-1a* homologue gene along with cytochrome P450, glutathione S-transferase, and lipase [25]. Similarly, exogenously applied SA did not induce accumulation of apoplastic proteins cross-reacting with antisera raised against PR-2 (β-1,3-glucanase), PR-Q (chitinase class IV) and PR-S (thaumatin-like protein) of tobacco in *B. napus* [26], even though they represent proteins from the same PR groups as were investigated in our study. This discrepancy with our results could be caused by higher similarity of rape seed PR proteins with sugar beet PR-proteins.

Gene expression initiated by MeJA was reported earlier. MeJA activated the expressions of several genes referred to as jasmonate-induced proteins (JIPs) and VSPs. It seems that JIPs may protect the plant from various stresses, mainly mechanical damage, insect attack or some pathogens [27]. In *B. napus* plants, MeJA, when supplemented in nutrient solution or sprayed on the leaves, promoted the accumulation of 23 kDa VSP in taproots and stems [28]. None of SA-dependent proteins under study was induced by MeJA treatment in *B. napus* in our experiment. That is in accordance with the assumption that different signaling pathways are involved.

We can conclude that SA, BTH and MeJA treatment led to the induction of different PR protein patterns and that the process leading to induced resistance was established in plant material used for further experiments.

2.2. Phospholipase activities and cell localization in the leaves of B. napus

The subcellular distribution of phospholipases and their in vitro activities in the leaves of 3-week-old plants of *B. napus*, the experimental material used in all of the following experiments, are shown in Table 1. PI specific PLC is known to be a plasma membrane associated enzyme, and the activity measured in microsomal fractions of matured leaves was on average one order of magnitude lower than in developing plants [29]. Three biochemically different isoforms of PLD could be distinguished by different assay conditions. PLD_α was detected using phosphatidyl choline (PC) vesicles as the substrate in the presence of millimolar concentration of Ca^{2+} [30], whereas PLDβγ (PIP_2-dependent) was active at micromolar concentration of Ca^{2+} [31]. PLDδ was assayed in the presence of unsaturated acid (oleic acid) as described by Wang and Wang [18].

Concerning PLDα, in all experiments a higher activity was found in the membrane fraction than in the cytosol fraction, in the reverse ratio to that found in developing plants as described previously in [29]. The same changes in the distribution of this conventional type of PLD within the cells were observed for young and old plants of *Ricinus communis* [32,33]. PIP_2-dependent activity was predominantly associated with membrane fraction. Oleate-stimulated PLD activity (PLDδ) was detected for the first time in *B. napus*. Nevertheless the activities were approximately 10 times lower than found in *Arabidopsis thaliana* [18]. This form was about 2.5 times more abundant in microsomal fraction then in cytosolic fraction of fully expanded leaves of *B. napus*. Such a finding does not correspond well to the results published for *Arabidopsis* where PLDδ was found almost solely associated with microsomal fraction [18]. However, immunoblot analysis of cytosolic and microsomal proteins from 14-day-old rice plants showed the predominant presence of a protein cross-reacting with antibodies generated to a peptide derived from the rice *RPLD5* sequence (similar to *Arabidopsis PLDδ* gene) in the cytosolic fraction [19].

2.3. Involvement of phospholipases in the early response to SAR inducers

We intended to investigate simultaneous changes of in vitro PLC and PLD activities in the process triggered by SA and BTH.

In time course experiments we measured in vitro activities of PI-PLC and three biochemically different isoforms of PLD. To analyze the stimulation of enzymes, plants were sprayed with 1 mM SA or 0.3 mM BTH and activities were measured

Table 1
Specific activities and subcellular distribution of phospholipases in the leaves of *B. napus*

Microsomal and cytosolic fractions from leaves of 3-week-old plants of *B. napus* were prepared from the extracts by centrifugation at 100,000 × *g*. Activities of different phospholipases were determined as described in "Section 3"

Enzyme	Specific activity (nmol min^{-1} mg^{-1} protein)	
	Microsomal fraction	Cytosolic fraction
PIP_2-PLC	2 ± 0.2	–
PLDα	150 ± 20	100 ± 20
PIP_2-dependent PLD	1.55 ± 0.25	0.55 ± 0.15
PLDδ	0.80 ± 0.09	0.31 ± 0.01

in the soluble and membrane fractions. The results are summarized in Fig. 2.

PLDα activity rapidly increased within 3 hours after treatment (Fig. 2A), which can be explained in this case by the activation of pre-existing protein. No significant changes in protein level (immunoblots not shown) were observed. This finding is consistent with the knowledge that PLDα is a constitutively expressed isoform, thus activation on the post translational level can be presumed.

Rapid stimulation within 3 hours was also observed for PIP_2-dependent PLD activities in response to both SAR activators applied. The increase of membrane-bound forms was more pronounced after BTH treatment, but the final degree of activation of about a 50% increase was approximately the same for SA and BTH (Fig. 2B).

Involvement of forms *PLD β* and/or γ in plant defense against pathogen attack was shown after inoculation of *Arabidopsis* leaves with *P. syringae* pv. tomato [16] or tomato cells

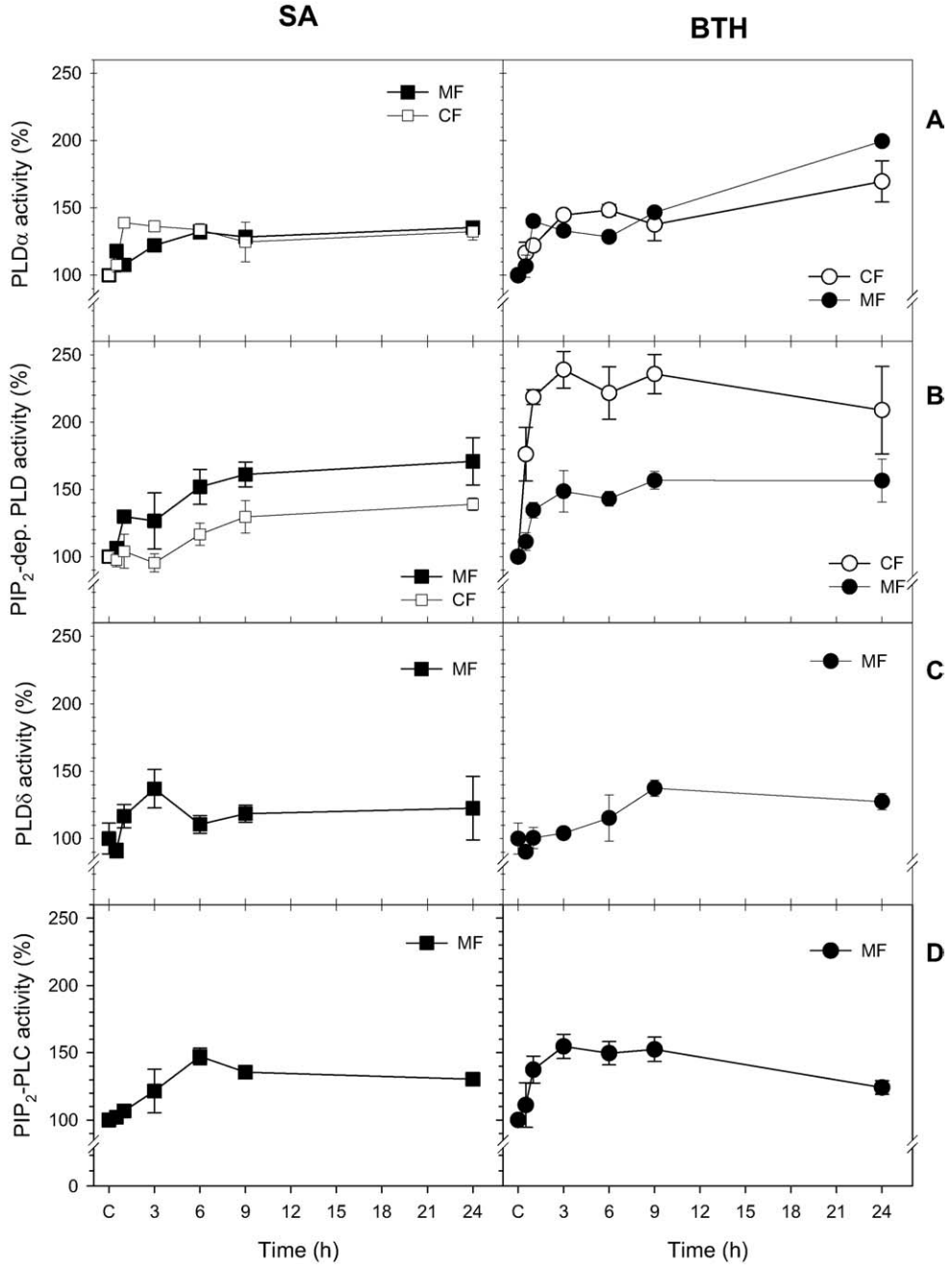

Fig. 2. Changes of phospholipase relative activities after treatment with SAR inducers. Enzyme activities of 3-week-old plants of *B. napus* treated with SAR inducers SA (1 mM) and BTH (0.3 mM) were determined in cytosolic and microsomal fractions prepared from fully expanded leaves. The activities of conventional PLDα (**A**), PIP_2-dependent PLD β/γ (**B**), oleate-stimulated PLD (**C**) and phosphoinositide-specific PLC (**D**) were followed in a time span of 24 h. Assay conditions for all enzymes were described in "Section 3". Values are mean ± S.E. of two experiments, each done in triplicate. Controls (C) were done for mock-treated plants and no significant activity changes were observed within 24 h.

treated with the elicitor xylanase [14]. The results of Northern blots obtained for *A. thaliana* plants treated with SA showed significantly increased expression of *PLDγ1* within 4 hours after treatment compared to a very slight increase of the *PLDβ* form [15,16]. Previous results all originated from expression data and nothing is known about the enzyme activity of PLD proteins after treatment with inducers of plant resistance. We can conclude that both PIP_2-independent and PIP_2-dependent PLD are involved in the process elicited by SAR inducers, but obviously the effect of BTH is much more pronounced. Though BTH and SA have structural similarities, BTH probably binds more effectively than SA to a receptor and acts with different kinetics. This was shown in tobacco where BTH bound 15-fold more efficiently to an SA-binding protein than SA [34].

Membrane-bound oleate-activated PLD was only very slightly increased after SA and BTH treatment. A 10–30% activation was observed within 3–9 hours after exposure (Fig. 2C) and no changes were observed in the cytosolic fractions. As the basal activities (Table 1) of this isoform are much lower then those of PIP_2-dependent PLD, the contribution of this form is not clear.

The PI-PLC activity in the microsomal fractions of 3-week-old plants of *B. napus* is elevated by about 50% within a very short interval after application of both chemical SAR inducers (SA, BTH) (Fig. 2D) followed by slow decrease almost to the control level. No significant changes in protein levels (immunoblots with anti-NtPI-PLC obtained from Dr. Pical) were observed (data not shown). According to our knowledge, only one study on characterization of a *PI-PLC* gene activated in SAR has been published [11]. Many results suggest that PI-PLC participates in plant defense responses induced by elicitors [35–37]. Song and Goodman [11] cloned rice *PI-PLC1* and tested various inducers of disease resistance. BTH as well as SA induced *OsPI-PLC1* gene expression very rapidly. The level of induction was different for SAR inducers BTH, SA but also for JA, MeJA, INA and wounding. They showed that PLC1 mediated signal transduction may play a role in pathways leading to SAR. Our results confirm, on the activity level, that PI-PLC is obviously involved in the process triggered by SAR inducers, its exact role should be defined by more detailed studies.

2.4. Early response to the ISR inducer (MeJA treatment)

Here we show that the activation pattern of phospholipases involved in the MeJA pathway differs significantly from that found for SA/BTH treatment.

Significantly different mode of activation of all three PLD isoforms investigated was observed in plants treated with the ISR inducer MeJA compared to SAR inducers. Exogenous application of MeJA causes substantial activation of cytosolic PLDα without any indication of activation of the microsomal form (Fig. 3A). We did not find any sign of translocation of the soluble enzyme towards membranes as was observed after wounding of *Arabidopsis* leaves [15].

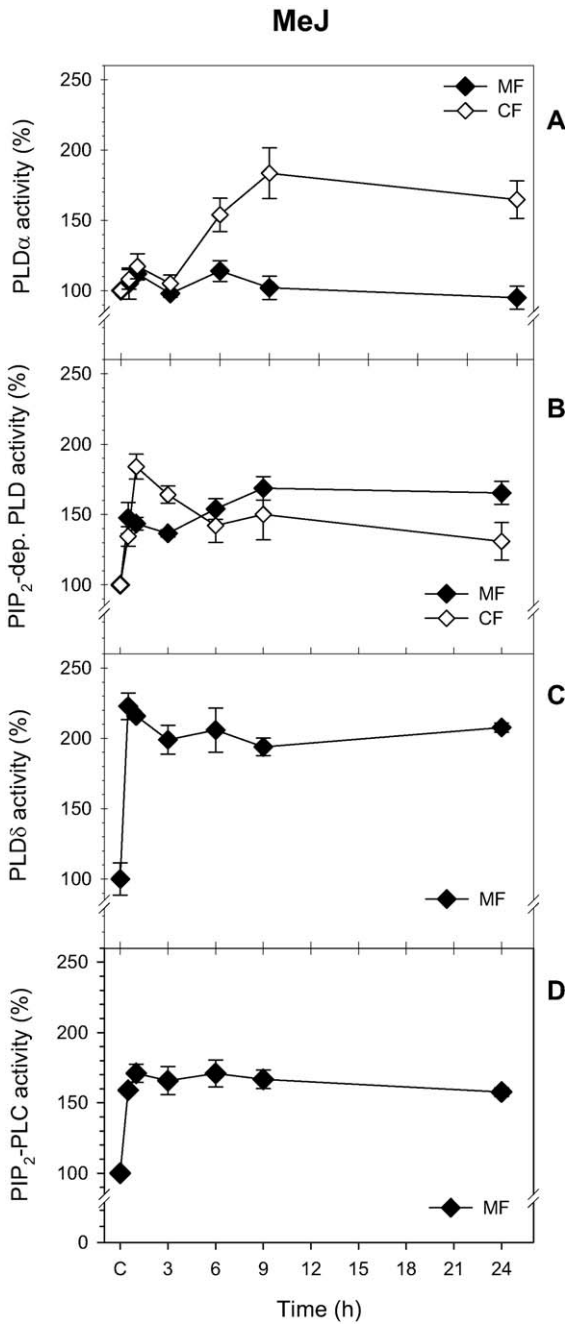

Fig. 3. Changes of phospholipase relative activities after MeJA treatment. Activities of 3-week-old plants of *B. napus* sprayed with ISR inducer MeJA (1 mM) were determined in cytosolic and microsomal fractions prepared from fully expanded leaves. The activities of conventional PLDα (**A**), PIP_2-dependent PLD β/γ (**B**), oleate-stimulated PLD (**C**) and PI-PLC (**D**) were followed for a time span of 24 h. Assay conditions for all enzymes were described in "Section 3". Values are mean ± S.E. of two experiments, each done in triplicate. Controls (C) were done for mock-treated plants and no significant activity changes were observed within 24 h.

PIP_2-dependent PLD (β and/or γ) activities increased more rapidly within 1 hour (Fig. 3B) compared to SAR inducers, but the increase was transient. The subsequent decrease in activity was accompanied by an increase of the activity associated with membranes, which implicates the possibility of translocation of

Table 2
Identification of PLDδ in *B. napus* by MALDI-TOF mass spectrometry
The microsomal fraction containing PLD protein was separated by native PAGE in an 8% gel (see Fig. 4). The band of interest was excised and cut into 1 mm-cubes and in-gel digestion of proteins with trypsin was used. A tryptic digest extracted from the gel was vacuum dried and purified on $ZipTip_{C18}$ column (Millipore). One microliter of the tryptic digest was mixed with 3 μl of matrix solution prepared as follows: 10 mg of DHB (2-hydroxy-5-methoxybenzoic acid, Sigma) was dissolved in 1 ml of acetonitrile/0.1% TFA (1:2, v/v). The mixture was spotted on a MALDI target plate. The peptide mixture for external calibration was purchased from Bruker, Germany. A mass spectrometer BIFLEX IV (Bruker, Germany) with a reflector was used for analyses. The spectra of the peptide mixtures were recorded in the reflector mode at an accelerating voltage of 19 kV at the laser wavelength 337 nm. Peptide mass maps were searched (Biotools software) against theoretically derived maps of AtPLDα, β1, β2, γ1, γ2, γ3, and δ. Search parameters: 0.3 Da, 1 missed cleavage, Cys residues modified with iodacetamide

Mass in the spectrum M + H^+ (Da)	Theoretical mass M + H^+ (Da)	Number of missed cleavages	Sequence of fragment	Position of fragment in sequence
842.56	842.41	0	GQVYGYR	758–764
966.56	966.49	1	YKSQEGVR	288–295
1529.94	1529.68	0	DDDVFGAQIIGTAK	125–138
1653.99	1653.77	1	CVLVDTQAVGNNRK	365–378
1772.95	1772.68	0	YEDEAEAQHLECAK	536–549
2211.30	2211.05	0	AVQSDAHPLDYLNFYCLGK	659–677

the enzyme towards the membranes. Wang et al. [15] showed at the mRNA level that expression of both *PLD β* and *γ* remained unchanged within 1 hour in wounded *Arabidopsis* plants. Thus we can assume that the immediate increase in activity is due to the regulation of PLD protein. A similar very rapid response was observed for PI-PLC (Fig. 3D).

However, the most considerable changes of the PLD activity in microsomal fraction of MeJA-treated leaves were observed for oleate-stimulated PLD(δ). The activity increased more than twofold within 30 min and remained high during the whole interval investigated (Fig. 3C). Concomitantly a significant increase in the amount of protein was observed after native PAGE on immunoblot with anti-PLDγ prepared against the C-terminal 13 AA peptide of AtPLDγ (Fig. 4A) [38]. Because it is now clear that this peptide is homologous with the corresponding C-terminal sequence of PLDδ and β, these isoforms could be expected in the detected band as well. This assumption was proved by the oleate-dependent PLD activity measured in the corresponding gel-cut (R_f 0.134) after native PAGE (Fig. 4B). The presence of PIP_2-dependent activity in this band was shown previously [38]. MALDI-TOF analysis of the in-gel tryptic digest of this band revealed six peptides corresponding to AtPLDδ (Table 2) and, as expected, a few peptides of PLDγ and β were also identified (maximum four peptides for PLDγ3 isoform). Thus we can conclude that both PIP_2 and oleate-dependent isoforms could be involved in MeJA pathway in a different way than in the process triggered by SAR inducers. These differences were observed on the activity level and protein level as no corresponding changes on immunoblots after SA and BTH treatment were observed (Fig. 4A).

As a novelty it should be taken into an account, that very likely oleate-dependent PLD is involved in the early events of induced plant resistance.

A massive increase of protein amount starting 6 hours after MeJA treatment was not accompanied by corresponding increase of in vitro activities. Interpreting such an inconsistency is not easy. Both types of PLD (β/γ and) are present in the corresponding immunoblot band. However, we can reasonably speculate that in the MeJA-signaling pathway the isoform PLDδ is the key enzyme. Very rapid activation of PLDδ can be caused by fatty acid released by phospholipase A, an enzyme obviously involved in wound-induced JA synthesis [39].

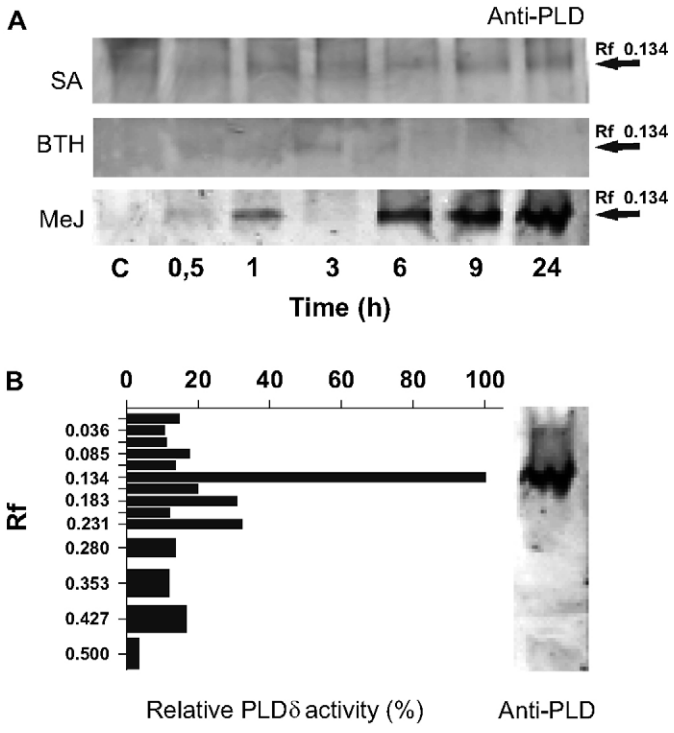

Fig. 4. Immunoblot analysis of microsomal fractions prepared from leaves of 3-week-old plants of *B. napus* treated with 1 mM MeJA in a time interval from 0.5 to 24 h in comparison with SA (1 mM) and BTH (0.3 mM) treatment. (**A**) After native PAGE and transfer to a NC membrane, the antibody raised against a 13 AA peptide from the C-terminal of AtPLDγ1 was used for detection. MALDI-TOF analysis showed presence of PLD β and δ isoforms in this band as well. (**B**) Alternatively the NC membrane (lane with proteins from microsomal fractions after 24 h MeJA treatment) was sliced into strips of 2, 5 and 10 mm widths (each bar represents one strip) and oleate-dependent PLD δ activity was found on the strip corresponding to the immunoreactive band with R_f 0.134.

PLD proteins themselves could be also functioning. Indirect support for this assumption can perhaps be found in Wang and

Wang's [18] experiments. The two orders of magnitude difference published for the specific activities of PLDδ measured in the extracts of plant tissue and those of recombinant protein, may mean that the activity of the protein in plant tissues is suppressed by an unknown mechanism. One possible mechanism is the protein–protein interaction in the process of microtubule rearrangement after fungal attack which leads to the focus of microtubules at the infection site [40]. As was recently shown, PLD protein was identified as a bridge [41] between microtubules of cytoskeleton and plasma membrane.

Activity changes of PI-PLC caused by MeJA (Fig. 3D) were comparable to SA and BTH treatment.

2.5. Conclusions

Both phospholipases PLC and PLD are involved in processes triggered by inducers of systemic resistance in plants. The pattern of involvement of biochemically distinguished isoforms of PLD in the SA and MeJA pathway, respectively, differs significantly. PLDδ is predominantly activated by the ISR inducer MeJA. One of the relevant questions which is often posed by researchers in this field refers to the high diversity of plant PLDs compared to those of other eukaryotes. One of the possible answers evoked by the results presented in this paper could lie in a fine regulation of the activities of each isoform creating an overall rapid response of the cell.

3. Materials and methods

3.1. Plant material and treatments with inducers of disease resistance

B. napus L. cv. Lirajet plants were grown in perlit and watered with a half-strength Steiner [42] nutrient solution. The plants were grown for 3 weeks in a growth chamber, set as follows: day temperature 24 °C; night temperature 18 °C; 16 h photoperiod (photon flux density 60 μmol m^{-2} s^{-1}).

Leaves of 3-week-old plants were sprayed with solutions of 0.3 mM BTH (Bion®, Syngenta AG, Basel, Switzerland, containing 50% of active compound), 1 mM SA in distilled water or 1 mM MeJA in 3% ethanol, control plants were treated with distilled water or 3% ethanol. These concentrations were chosen according to our preliminary experiments. In all experiments, MeJA and control treated plants were maintained in separate containers. The leaves were used either for preparation of microsomal and cytosolic fractions or extracellular fluid (EF) extraction.

3.2. EF and immunoblot analysis of PR proteins

EF was prepared by vacuum infiltration of *B. napus* leaves as described by Pierpoint et al. [43] 5 days after the chemical treatment. Infiltrated leaves were gently packed into the barrel of a 25 ml syringe and placed in a centrifuge tube. The buffer, which infiltrated into the leaf extracellular space, was recovered by low speed centrifuging (1000 × *g*, 4 °C, 10 min).

PR proteins in EF were separated by 10% SDS-PAGE according to Laemmli [44] and transferred onto nitrocellulose (NC) membranes. The NC membranes were developed using antibodies against basic β-1,3-glucanase (Glu2) and basic chitinase class IV (Ch4) (Danisco, Denmark). Bands were visualized by staining alkaline phosphatase activity conjugated to a second antibody with a Bio-Rad immunoblotting kit.

3.3. Microsomal and cytosolic fractions and enzyme assays

Plant material was homogenized at 4 °C with a mortar and pestle in the buffer (1:4, w/v) containing 70 mM Tris-MES (pH 8.0), 0.25 M sucrose, 3 mM EDTA, 0.2% BSA, 5 mM DTT, and protease inhibitors (0.23 mM PMSF, 0.83 mM benzamidine, 0.7 μM pepstatin, 1.1 μM leupeptin, and 77 nM aprotinin). The homogenate was filtered through nylon cloth and centrifuged at 6000 × *g* for 15 min at 4 °C. The supernatant was filtered through Miracloth (Calbiochem, Switzerland) and diluted (in the ratio 1:3, v/v) with suspension buffer containing 1.1 M glycerol, 10 mM Tris-Mes (pH 8.0) and protease inhibitors. After centrifugation at 100,000 × *g* for 60 min at 4 °C, the soluble cytosolic fraction was obtained and the pelleted microsomal fraction was resuspended in suspension buffer. Protein concentration was determined according to Bradford [45]. Bovine serum albumin was used as a standard.

PIP_2-independent PLD(α) activity was measured spectrophotometrically using choline oxidase/peroxidase system for determination of released choline according to the procedure described earlier by Dyer et al. [32]. Enzymatic activity was determined for PC as a substrate in the presence of 120 mM Ca^{2+} and 10 mM SDS, the conditions found previously for *B. napus* PLDα [46]. PIP_2-stimulated PLD(β/γ) activity was measured as described by Xu et al. [33] using radiolabeled substrate [methyl-^{3}H]PC, with phosphatidyl ethanolamine dipalmitoyl (PE) and PIP_2 in the presence of 100 μM Ca^{2+}. Activity of oleate-stimulated PLD (PLDδ) was conducted as described earlier by Wang and Wang [18] using radiolabeled substrate [methyl-^{3}H]PC in the presence of 0.6 mM oleate and 100 μM Ca^{2+}.

PI-specific PLC activity was estimated using radiolabeled substrate [^{3}H]PIP_2 followed by biphasic extraction of the reaction product inositol 1,4,5-trisphosphate as described earlier by Crespi et al. [47].

3.4. Native PAGE of cytosolic and microsomal proteins and immunoblot analysis

The microsomal and cytosolic proteins were separated by 8% non-denaturing PAGE. To release membrane-bound proteins, microsomal fractions were incubated with 1% (w/v) Triton X-100 for 30 min at 4 °C and then centrifuged at 100,000 × *g* for 30 min at 4 °C. Thirty micrograms of protein was applied to the gel. Gels were run at constant current 15 mA per gel at 4 °C. For Western blot analysis proteins were transferred onto NC membranes and blotted with antibody raised against the 13 amino acid peptide from the *Arabidopsis* PLDγ1 C terminus. Antibodies were visualized by staining al-

kaline phosphatase activity conjugated to a second antibody with a Bio-Rad immunoblotting kit.

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References

[1] J. Kuc, Induced immunity to plant disease, Bioscience 32 (1982) 854–886.

[2] J.A. Ryals, U.H. Neunschwander, M.G. Willits, A. Molina, H.-Y. Steiner, M.D. Hunt, Systemic acquired resistance, Plant Cell 8 (1996) 1809–1819.

[3] R. Hammerschmidt, Induced disease resistance: how do induced plants stop pathogens?, Physiol. Mol. Plant Pathol. 55 (1999) 77–84.

[4] L.C. van Loon, P.A.H.M. Bakker, C.M.J. Pieterse, Systemic resistance induced by rhizosphere bacteria, Annu. Rev. Phytopathol. 36 (1998) 453–483.

[5] C.M.J. Pieterse, S.C.M. Van Wees, J.A. Van Pelt, M. Knoester, R. Laan, H. Gerrits, P.J. Weisbeek, L.C. Van Loon, A novel signaling pathway controlling induced resistance in *Arabidopsis*, Plant Cell 10 (1998) 1571–1580.

[6] J.-P. Métraux, P. Ahl Goy, T. Staus, J. Speich, A. Steinemann, J. Ryals, E. Ward, Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid pathogens, in: H. Hennecke, D.P.S. Verma (Eds.), Advances in Molecular Genetics of Plant–Microbe Interaction, vol. 1, Kluwer Academic Publishers, Dordrecht, 1991, pp. 432–439.

[7] L. Friedrich, K.A. Lawton, W. Ruess, P. Masner, N. Specker, H.G. Rella, B. Meier, S. Dincher, T. Staub, E. Ward, H. Kessmann, J. Ryals, A benzothiadiazole derivate induces SAR in tobacco, Plant J. 10 (1996) 61–70.

[8] L.C. van Loon, E.A. van Strien, The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins, Physiol. Mol. Plant Pathol. 55 (1999) 85–97.

[9] M. Elias, M. Potocky, F. Cvrckova, V. Zarsky, Molecular diversity of phospholipase D in angiosperms, BMC Genomics 3 (2) (2002).

[10] C.B. Qin, X.M. Wang, The *Arabidopsis* phospholipase D family. Characterization of a calcium-independent and phosphatidylcholine-selective PLDζ1 with distinct regulatory domains, Plant Physiol. 128 (2002) 1057–1068.

[11] F. Song, R.M. Goodman, Molecular cloning and characterization of a rice phosphoinositide-specific phospholipase C gene, Os PLC1, that is activated in systemic acquired resistance, Physiol. Mol. Plant Pathol. 61 (2002) 31–40.

[12] T. Munnik, J.A.J. van Himbergen, B. ter Riet, F.-J. Braun, R.F. Irvine, H. van den Ende, A. Musgrave, Detailed analysis of the turnover of polyp hosphoinositides and phosphatidic acid upon activation of phospholipases C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan, Planta 207 (1998) 133–145.

[13] A.H. van der Luit, T. Piatti, A. van Doorn, A. Musgrave, G. Felix, T. Boller, T. Munnik, Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate, Plant Physiol. 123 (2000) 1507–1515.

[14] A.M. Laxalt, B. Riet, J.C. Verdonk, L. Parigi, W.I.L. Tameling, J. Vossen, M. Haring, A. Musgrave, T. Munnik, Characterization of five tomato phospholipase D cDNAs: rapid and specific expression of *LePLD 1* upon elicitation with xylanase, Plant J. 26 (2001) 237–240.

[15] C. Wang, C.A. Zhien, M. Afitlhile, R. Welti, D.F. Hildebrand, X. Wang, Involvement of phospholipase D in wound-induced accumulation of jasmonic acid in *Arabidopsis*, Plant Cell 12 (2000) 2237–2246.

[16] M.T. Zabela, I. Fernandez-Delmond, T. Niittyla, P. Sanchez, M. Grant, Differential expression of genes encoding *Arabidopsis* phospholipases after challenge with virulent or avirulent *Pseudomonas* isolates, Mol. Plant Microbe Interact. 15 (2002) 808–816.

[17] T. Katagiri, S. Takahashi, K. Shinozaki, Involvement of a novel *Arabidopsis* phospholipase D, PLDδ, in dehydration-inducible accumulation of phosphatidic acid in stress signalling, Plant J. 26 (2001) 595–605.

[18] C. Wang, X. Wang, A novel phospholipase D of *Arabidopsis* that is activated by oleic acid and associated with the plasma membrane, Plant Physiol. 127 (2001) 1102–1112.

[19] J.D. McGee, J.L. Roe, T.A. Sweat, X.M. Wang, J.A. Guikema, J.E. Leach, Rice phospholipase D isoforms show differential cellular location and gene induction, Plant Cell Physiol. 44 (2003) 1013–1026.

[20] W. Zhang, C. Wang, C. Qin, T. Wood, G. Olafsdottir, R. Welti, X. Wang, The oleate-stimulated phospholipase D, PLDδ, and phosphatidic acid decrease H_2O_2-induced cell death in *Arabidopsis*, Plant Cell 15 (2003) 2285–2295.

[21] T. Yamaguchi, S. Tanabe, E. Minami, N. Shibuya, Activation of phospholipase D induced by hydrogen peroxide in suspension-cultured rice cells, Plant Cell Physiol. 45 (2004) 1261–1270.

[22] K.A. Lawton, L. Friedrich, M. Hunt, K. Weymann, T. Delaney, H. Kessmann, T. Staub, J. Ryals, Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway, Plant J. 10 (1996) 71–82.

[23] A. Kohler, S. Schwindling, U. Conrath, Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the *NPR1/NIM1* gene in *Arabidopsis*, Plant Physiol. 128 (2002) 1046–1056.

[24] U. Rasmussen, H. Giese, J.D. Mikkelsen, Induction and purification of chitinase in *Brassica napus* L. ssp. *oleifera* infected with *Phoma lingam*, Planta 187 (1992) 328–334.

[25] H.-S. Chu, T.-J. Cho, Isolation of salicylic acid-induced genes in *Brassica napus* by subtractive hybridization, Mol. Cells 6 (1996) 766–771.

[26] C. Dixelius, Presence of the pathogenesis-related proteins 2, Q and S in stressed *Brassica napus* and *B. nigra* plantlets, Physiol. Mol. Plant Pathol. 44 (1994) 1–8.

[27] E.E. Farmer, C.A. Ryan, Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves, Proc. Natl. Acad. Sci. USA 87 (1990) 7713–7716.

[28] L. Rossato, J.H. MacDuff, P. Laine, E. Le Deunff, A. Ourry, Nitrogen storage and remobilization in *Brassica napus* L. during the growth cycle: effects of methyl jasmonate on nitrate uptake, senescence, growth, and VSP accumulation, J. Exp. Bot. 53 (2002) 1131–1141.

[29] Z. Novotná, J. Martinec, B. Profotová, Š. Žďárová, J.-C. Kader, O. Valentová, In vitro distribution and characterization of membrane-associated PLD and PI-PLC in *Brassica napus*, J. Exp. Bot. 54 (2003) 691–698.

[30] J. Sajdok, J. Jandus, O. Valentová, Z. Novotná, J. Káš, J. Daussant, A microplate technique for phospholipase D activity determination, Anal. Chim. Acta 315 (1995) 109–112.

[31] K. Pappan, S. Zheng, X. Wang, Identification and characterization of a novel plant phospholipase D that requires polyphosphoinositides and submicromolar calcium for activity in *Arabidopsis*, J. Biol. Chem. 272 (1997) 7048–7054.

[32] J. Dyer, S. Ryu, X. Wang, Multiple forms of phospholipase D following germination and during leaf development of castor bean, Plant Physiol. 105 (1994) 715–724.

[33] L. Xu, A.Q. Paulsen, S.B. Ryu, X. Wang, Intracellular localization of phospholipase D in leaves and seedling tissues of castor bean, Plant Physiol. 111 (1996) 101–107.

[34] H. Du, D.F. Klessig, Identification of a soluble, high-affinity salicylic acid-binding protein in tobacco, Plant Physiol. 13 (1997) 1319–1327.

[35] C.F. de Jong, A.M. Laxalt, B.O.R. Bargmann, P.J.G.M. de Wit, M.H.A. J. Joosten, T. Munnik, Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction, Plant J. 39 (2004) 1–12.

[36] T. Yamaguchi, E. Minami, N. Shibuya, Activation of phospholipases by *N*-acetylchitooligosaccharide elicitor in suspension-cultured rice cells

mediates reactive oxygen generation, Physiol. Plant. 118 (2003) 361–370.
[37] J. Zhao, Y.Q. Guo, A. Kosaihira, K. Sakai, Rapid accumulation and metabolism of polyphosphoinositol and its possible role in phytoalexin biosynthesis in yeast elicitor-treated *Cupressus lusitanica* cell cultures, Planta 219 (2004) 121–131.
[38] Z. Novotná, J. Linek, R. Hynek, J. Martinec, M. Potocký, O. Valentová, Plant PIP_2-dependent phospholipase D activity is regulated by phosphorylation, FEBS Lett. 554 (2003) 50–54.
[39] J.G. Turner, C. Ellis, A. Devoto, The jasmonate signal pathway, Plant Cell 14 (2002) S153–S164.
[40] I. Kobayashi, Y. Kobayashi, A.R. Hardham, Dynamic reorganization of microtubules and microfilaments in flax cells during the resistance response to flax rust infection, Planta 195 (1994) 237–247.
[41] J.C. Gardiner, J.D.I. Harper, N.D. Weerakoon, D.A. Collings, S. Ritchie, S. Gilroy, R.J. Cyr, J. Marc, A 90-kDa phospholipase D from tobacco binds to microtubules and the plasma membrane, Plant Cell 13 (2001) 2143–2158.
[42] A.A. Steiner, The universal nutrient solution, in: Proc. Sixth International Congress on Soils Culture, Lunteren, International Society for Soils Culture, Pudoc, Wagenigen, 1984, pp. 633–650.
[43] W.S. Pierpoint, A.S. Tatham, D.J.C. Pappin, Identification of virus induced protein of tobacco leaves that resemble the sweet-tasting protein thaumatin, Physiol. Mol. Plant Pathol. 31 (1987) 291–298.
[44] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[45] M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein binding, Anal. Biochem. 72 (1976) 248–254.
[46] Z. Novotná, J. Káš, J. Daussant, J. Sajdok, O. Valentová, Purification and characterisation of rape seed phospholipase D, Plant Physiol. Biochem. 37 (1999) 531–537.
[47] P. Crespi, J. Martinec, I. Machackova, H. Greppin, Characterization of Ca^{2+}-stimulated polyphosphoinositide-phospholipase C in isolated plasma membranes from *Spinacia oleracea* and *Chemopodium rubrum* leaves. Arch, Sci. Geneve 46 (1993) 335–346.