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Identification of phospholipase D genes in *Brassica napus* and their transcription after phytohormone treatment and pathogen infection

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Abstract

Phospholipase D (PLD) and its product phosphatidic acid are now considered to be one of the key elements of numerous physiological processes in plants including the salicylic acid signalling pathway. The presented study investigates the transcriptional regulation of *Brassica napus PLD*s following treatments with defense-related stimuli. We cloned eight *B. napus* genes encoding members of PLDβ, γ, and δ isoforms and performed phylogenetic analysis with its ancestor species *Brassica rapa* and *Brassica oleracea*, and with the model plant *Arabidopsis thaliana*. Transcription of the identified genes was monitored after treatment with benzothiadiazole (BTH), methyl jasmonate (MeJA), bacterial elicitor flg22, wounding, and after infection with fungal pathogens *Sclerotinia sclerotiorum* and *Leptosphaeria maculans*. Most of the genes responded specifically to a particular treatment. Remarkably the genes encoding the PLDγ and PLDβ isoforms were up-regulated by stimuli associated with the salicylic acid signalling pathway. The generality of this finding was confirmed by the analysis of public transcriptional data from *Arabidopsis thaliana*.

Additional key words: benzothiadiazole, *Leptosphaeria maculans*, methyl jasmonate, microarray, oilseed rape, salicylic acid, *Sclerotinia sclerotiorum*, transcriptomics.

Introduction

Oilseed rape (*Brassica napus* L.), one of the most important oilseed crops, is an amphidiploid species derived from the recent hybridisation between *Brassica rapa* and *Brassica oleracea*. The genomes of both ancestors were recently sequenced (Wang *et al.* 2011, Yu *et al.* 2013). It is widely accepted that the *Brassica* species diverged from a common ancestor with the *Arabidopsis* lineage ~17 mya ago (Cheung *et al.* 2009).

Phospholipase D (PLD) is an enzyme which hydrolyses structural phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, producing a free head-group and phosphatidic acid (PA). PLD occurs in several isoforms in plants, 12 in *Arabidopsis thaliana* (Elias *et al.* 2002), 17 in *Oryza sativa* (Li *et al.* 2007), 18 in soybean and poplar (Liu *et al.* 2010, Zhao *et al.* 2012), and 11 in grape (Elias *et al.* 2002, Liu *et al.* 2010). The plant PLD family is more diverse than in any other eukaryotic organisms. Mammals contain only two PLD isoforms and yeast contain only one (Wang 2005). PLD share some structural similarities across the eukaryotic kingdom, like two highly conserved HKD [H(X)K(X)$_4$D] catalytic motif or PIP_2 binding domain (Wang 2002). On the other hand, plant PLDs have also unique structures. The majority of plant PLDs carries a C2 binding domain

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Abbreviations: BTH - benzothiadiazole; EST - expressed sequence tag; JA - jasmonic acid; MeJA - methyl jasmonate; PA - phosphatidic acid; PIP_2 - phosphatidylinositol-4,5-bisphosphate; PLD - phospholipase D; SA - salicylic acid.
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that is not present in animals. Based on the phylogenetic relationship and biochemical properties, plant PLDs are classified into six distinct classes, PLDα, ε, β, γ, δ, and ζ (Pleskot *et al.* 2012). These classes are diverse in different biochemical properties, such as dependence on Ca^{2+}, PIP_2, oleic acid, or by the preference for different substrates (*e.g.*, phosphatidylcholine). The isoforms from β, γ, δ, and ζ belong among PIP_2-dependent PLDs (Bargmann and Munnik 2006).

Plants actively defend themselves against pathogens and pests. Numerous receptors at the cell surface sense the presence of pathogen-derived molecules, such as bacterial flagellin or chitin from fungal cell walls (Greeff *et al.* 2012). The defense responses after an enemy recognition are regulated by diverse plant hormones where salicylic acid (SA) and jasmonic acid (JA) are recognised as major players (Pieterse *et al.* 2012). Phospholipase D has been repeatedly shown to participate in these signalling events as well (Janda *et al.* 2013). The infection with virulent and avirulent strains of bacteria induced the transcription of *PLD* genes in *A. thaliana* (De Torres Zabela *et al.* 2002). Using a nonspecific probe for PLD the increase in rice PLD in response to *Xanthomonas oryzae* infection was observed (Young *et al.* 1996). Several elicitors have been shown to modulate PLD activity. For example, phosphatidic acid (PA), a product of PLD hydrolytic activity, accumulates in tomato cells treated with the elicitors xylanase, chitosan, and flagellin (Van der Luit *et al.* 2000, Raho *et al.* 2011). The PLD also seems to be involved directly in hormone signalling pathways. Treatment with SA increased the PA content or PLD activity in *A. thaliana* and soybean (Kalachova *et al.* 2012, Rainteau *et al.* 2012). The indispensable role of PLD in SA signalling pathway has been shown by Krinke *et al.* (2009). Manipulation of PLD activity by *n*-butanol strongly affected transcriptomic changes in response to SA. Activation of PLD was also observed after treatment with methyl jasmonate in *Taxus cuspidata* and *Silybum marianum* (Yang *et al.* 2008, Madrid and Corchete 2010). The JA also plays a crucial role in mediating responses to wounding caused by herbivores. The rapid increase in the PA content in wounded leaves seems to be a common response in numerous plants (Lee *et al.* 1997). Wang *et al.* (2006) has shown that the majority of PA produced in response to wounding originates through PLDα1 activity. The remainder of PA is produced by PLDδ. However, the *pldα1/δ* knock-out plants are altered neither in transcriptional response to wounding nor in their resistance to feeding caterpillars (Bargmann *et al.* 2009).

We have previously shown that SA and methyl jasmonate (MeJA) induce PLD activity in *B. napus* plants (Profotova *et al.* 2006). We were interested in identifying *B. napus* genes involved in SA and JA signalling pathways. We cloned eight *B. napus* phospholipase D genes and analysed their transcriptional response to hormone treatment and pathogen infection. The results are compared with the available microarray data from *A. thaliana,* so as to show similarities and differences between the model and the crop plant.

Materials and methods

Plants of *Brassica napus* L. var. *napus* cv. Columbus were grown at a 14-h photoperiod, an irradiance of 150 μmol m^{-2} s^{-1}, day/night temperatures of 24/19 °C, and a relative humidity of 65 %. Plants were cultivated either in a soil mixture with *Perlite* (*Plagron*, Ospel, the Netherlands) or in *Perlite* supplied with Steiner's nutrient solution (Steiner 1966).

Treatments: Leaves of 21-d-old plants cultivated in soil mixture were sprayed with 32 μM benzothiadiazole (BTH; *Bion 50 WG*; *Syngenta*, Basel, Switzerland) in 0.01 % (v/v) *Tween 20* and covered with a plastic bags to maintain high relative humidity. Control plants were treated only with 0.01 % *Tween 20*. Alternatively, plants were placed in a transparent airtight container (20 dm^3) together with cotton swabs containing either 0.002 cm^3 of MeJA (*Sigma-Aldrich*, St. Gallen, Switzerland) in 0.018 cm^3 of ethanol or only ethanol as a control. For flagellin treatment, cotyledons of 14-d-old plants grown in *Perlite* were infiltrated with 1 μM flagellin 22 (flg 22; QRLSTGSRINSAKDDAAGLQIA; *GenScript*, Piscataway, USA) solution using a 1 cm^3 plastic syringe. Control plants were treated with inactive peptide Δflg22 (QRLSTGSRINSAKADAAGLQIA; *GenScript*) under the same conditions. Wounding was performed on cotyledons of 14-d-old plants grown in *Perlite* by applying pressure with forceps having serrated jaws. Infection with *Leptosphaeria maculans* avirulent isolate JN3 was performed on plants grown in *Perlite* as described by (Sasek *et al.* 2012). *Sclerotinia sclerotiorum* infection with isolate Ss 05 was performed on leaves of 21-d-old plants according to Novakova *et al.* (2014).

Gene cloning and bioinformatic analysis: Primers for cloning of *B. napus PLD* genes were designed within the conserved regions of *A. thaliana PLD* genes determined by alignment with mRNA sequences of other plant species and if available also with *B. napus* ESTs. The following primers were used: *BnaPLDβ*, forward (F): 5′-CATTTTGTTGTTAARGACAGTGA-3′, reverse (R): 5′-CTCCCATTGCAATCTCTGTAT-3′; *BnaPLDγ*, F: 5′-ATTTGGGTKAAGGAAGCTAAA-3′, R: 5′-TCC ACTACCATACCTTTGGA-3′; *BnaPLDδ*, F: 5′-TGG GATGATAAGACTTCTCATGAT-3′, R: 5′-CAATGA TCTTACCACCAACATC-3′. The RNA was isolated from untreated cotyledons of *B. napus*. The template

cDNA was prepared using the same protocol as for the gene transcription analysis. The fragment was amplified with *Phusion*® high-fidelity DNA polymerase (*Finnzymes*, Espoo, Finland) using the following PCR programme: 98 °C for 30 s, followed by 35 cycles of 98 ºC for 8 s, 60 °C for 20 s, and 72 °C for 60 s, with final extension at 72 °C for 10 min. The 1.5 - 2 kb PCR products were purified using a *High Pure PCR Cleanup* micro kit (*Roche*, Basel, Switzerland), cloned into pJET1.2 vector supplied with the *CloneJET* PCR cloning kit (*Fermentas*, Waltham, USA) and then sequenced from both ends using *pJET1.2* sequencing primers and an additional primer used for *BnaPLDγ* (5′-TGGTACATT AGGGGATTGC-3′) necessary for filling the gap within the sequence. Seventeen clones of *BnaPLDγ* and *BnaPLDδ* each, and 27 clones of *BnaPLDβ* were sequenced at *Macrogen* (Seoul, South Korea). Multiple alignments were constructed with *TranslatorX* (Abascal *et al.* 2010) using the t-coffee algorithm at a default setting. Regions that were not spanned by all aligned sequences were removed. The maximum likelihood method using the *PhyML* programme (Guindon *et al.* 2010) was employed for phylogeny inference with the WAG substitution model. The public databases *arabidopsis.org* and *brassicadb.org* served as the source for genomic data (Cheng *et al.* 2011, Lamesch *et al.* 2012). For the studies of similarity of the genes sequences of PLDs from distinct plants, we used Basic local alignment search tool (*BLAST*) (Table 1) (Boratyn *et al.* 2013).

Gene transcription analysis in *Brassica napus*: The plant tissue was sampled at indicated times and ground in liquid nitrogen using a mortar and pestle. Total RNA was isolated from 100 mg of tissue powder using the *Spectrum* plant total RNA kit (*Sigma-Aldrich*) and treated with the DNA free kit (*Ambion*, Austin, TX, USA). Subsequently, 1 µg of RNA was converted into cDNA with *M-MLV RNase H⁻ Point Mutant* reverse transcriptase (*Promega*, Madison, USA) and an anchored oligo dT_{21} primer (*Metabion*, Martinried, Germany). An equivalent of 6.25 ng of RNA was loaded into a 0.01 cm^3 reaction mixture with the *DyNAmo Capillary SYBR Green* qPCR kit (*Finnzymes*). All reactions were performed in polycarbonate capillaries (*Genaxxon*, Ulm, Germany) and *LightCycler 1.5* (*Roche*). The following PCR programme was used: 95 ºC for 10 min, followed by 45 cycles of 95 ºC for 10 s, 55 ºC for 10 s, and 72 ºC for 10 s, followed by melting curve analysis. Relative transcription was calculated with an efficiency correction and normalisation to the *actin* gene (*Act*). The P values were calculated using the two-tailed Student's *t*-test. Primers (Table 1) were designed using *PerlPrimer v. 1.1.17* (Marshall 2004).

Analysis of public *Arabidopsis* microarray data: The normalised microarray data of gene expression in leaves of BTH (Wang *et al.* 2006) and MeJA (Pozo *et al.* 2008) treated *A. thaliana* plants were downloaded from the *NASCArrays* database (Table 2 Suppl.). The data of flg22 treated (Qutob *et al.* 2006) and wounded (Kilian *et al.* 2007) plants were downloaded from the gene expression *Omnibus* database (Table 2 Suppl.). All data were obtained from the *Affymetrix ATH1* genome array. The normalised signal values were converted to a linear scale. The *P* values were calculated using the two-tailed Student's *t*-test.

Table 1. *B. napus* genes and primers used.

Genes	Forward primer 5′-3′	Reverse primer 5′-3′
BnaX.PLDβ.a	GTGTTGGCATGATATGTTTGA	GTACATGCTGAGGCGG
BnaX.PLDβ.b	AAGTGCTGGCATGACAT	GAGTACATGCTGACGCA
BnaX.PLDβ.c	AAACCTTTCATTTGCCCTG	CTACAACTGGAATGCTCAC
BnaX.PLDγ.a	GAGCAGTTGTCTTCAGGG	GTAAAGCCTCATCCTTTCCAC
BnaX.PLDγ.b	GTGTTGGTAATGAGTGCGA	AATCAAGTTCTTTGCCTGTC
BnaX.PLDδ.a	AGCCGTTGCCAGAAGATA	GATGTTAGCAGATCCTATTAGCAC
BnaX.PLDδ.b	GTTCCCAGATGATAAGCCA	GCAGATCCCATTAGTACATACTC
BnaX.PLDδ.c	TGTTGGTAACAATCGCAAGA	CGTCTAGCCTACAGTGCAAA
Act	CTGGAATTGCTGACCGTATGAG′	TGTTGGAAAGTGCTGAGGGA

Results

Our previous study showed that the activity of PIP_2-dependent PLDs and PLDδ are strongly induced by BTH and MeJA, respectively (Profotova *et al.* 2006). Thus, we decided to clone members of the *PLDβ*, *PLDγ*, and *PLDδ* families. Using primers designed within the conserved regions of plant *PLD*s, we amplified cDNA fragments of sizes ranging between 1.5 to 2 kb from *B. napus* cotyledon tissue. Eight genes were identified. Three of them belonged to *PLDβ* family, two belonged to *PLDγ*, and three genes were annotated as *PLDδ*. They were

named according to *Brassica* gene nomenclature (Ostergaard and King 2008) and their characteristics and accession numbers are shown in Table 2. As mentioned above, *B. napus* originated from *B. rapa* and *B. oleracea*. We attempted to identify the ancestor of each gene within the *B. rapa* and *B. oleracea* genome. Although both species were sequenced, the gene annotation is still tentative in both genomes. For example, Bol040619 was annotated as an ion channel but this annotated gene included a PLD-specific sequence. Table 2 contains a list of the most similar putative genes from genomes of *B. napus* ancestors *B. rapa* and *B. oleracea*. All *B. rapa* genes have an amino acid similarity higher than 97 % with corresponding *B. napus* genes. *B. oleracea* genes have also a high similarity (higher than 92 %) except of *BnaX.PLDγ.b* (77 %).

Table 2. Characteristics of identified fragments of *B. napus PLD* genes, their proteins, and homology (identity percentage) to *B. rapa* and *B. oleracea* orthologs.

B. napus *PLD* genes	mRNA acc. No.	length [nt]	Protein acc. No.	[aa]	*B. rapa* genes	[%]	*B. oleracea* genes	[%]
BnaX.PLDβ.a	JF911419	1786	AEQ02410.1	595	*Bra033225*	98.5	*Bol004004*	94.8
BnaX.PLDβ.b	JF911420	1786	AEQ02411.1	595	*Bra033225*	97.1	*Bol004004*	96.4
BnaX.PLDβ.c	KF010155	1776	AGN52970.1	591	*Bra008515*	98.8	*Scaff000013*	98.1
BnaX.PLDγ.a	JF911421	1974	AEQ02412.1	658	*Bra014871*	97.5	*Bol008311*	99.9
BnaX.PLDγ.b	JF911422	1913	AEQ02413.1	637	*Bra001854*	92.8	*Bol008311*	79.4
BnaX.PLDδ.a	JF911423	1587	AEQ02414.1	529	*Bra017730*	95.5	*Bol018692*	91.4
BnaX.PLDδ.b	JF911424	1587	AEQ02415.1	529	*Bra017730*	91.4	*Bol018692*	95.2
BnaX.PLDδ.c	JF911425	1587	AEQ02416.1	529	*Bra011638*	96.0	*Bol018692*	86.7

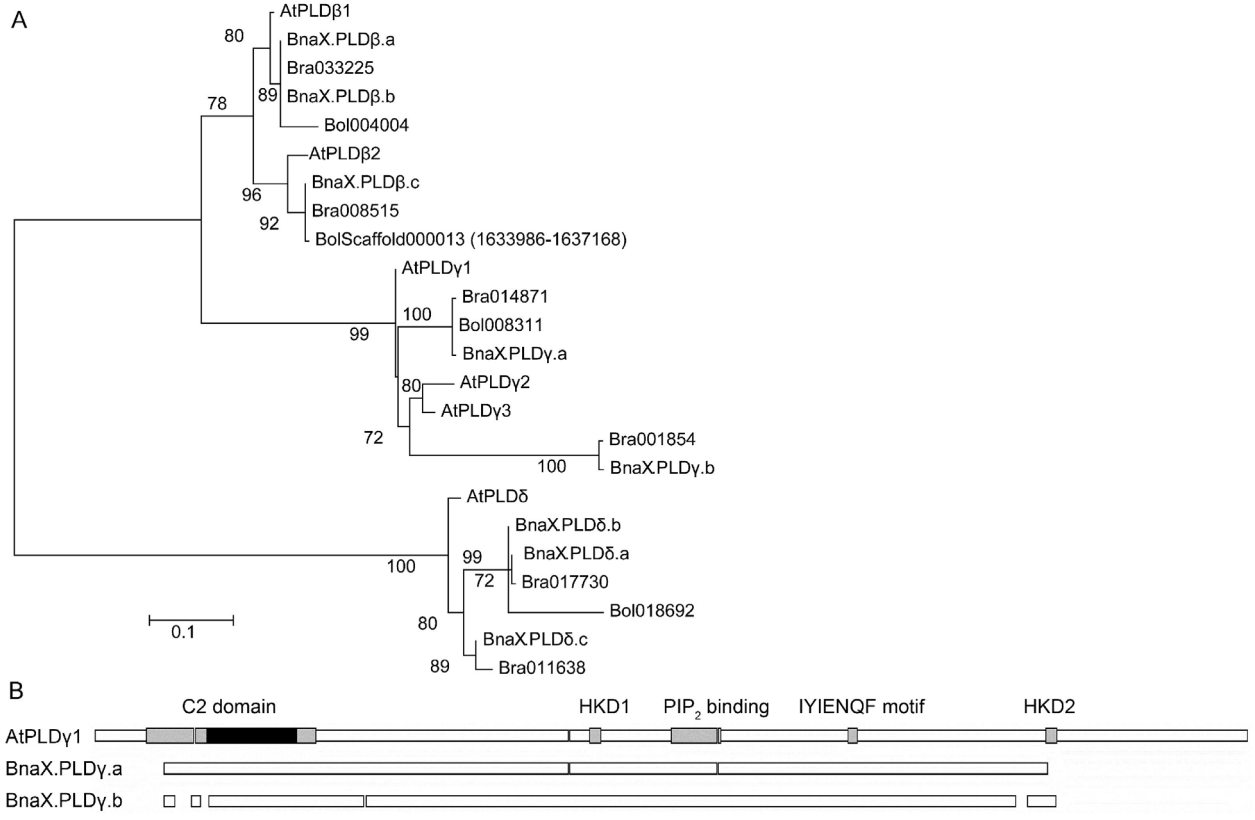

Fig. 1. Phylogenetic analysis of *PLD* genes: *A* - tree representing the protein maximum likelihood (ML) phylogeny of particular genes. An aligned region of 296 amino acids covered by all sequences was analysed. Numbers at nodes correspond to the approximate likelihood ratio test with the Shimodaira-Hasegawa (SH)-like support. *Scale bar* indicates the rate of substitutions/site. *B* - schematic protein alignment of *A. thaliana* PLDγ and the fragment of *B. napus* PLDγ isolated in this study. Domains are shown *in grey* and are annotated according to Qin and Wang (2002). A *black box* within the C2 domain marks a calcium-binding region.

Whereas *BnaX.PLDβ.a* and *BnaX.PLDβ.b* genes showed high similarity of nucleotide sequences (97.3 %) with *BnaX.PLDβ.c*, the amino acid sequence showed only 88 % amino acid identity (Table 1 Suppl.). Both genes, *BnaX.PLDβ.a* and *BnaX.PLDβ.b,* were more similar to *A. thaliana AtPLDβ1* than to *AtPLDβ2*, whereas *BnaX.PLDβ.c* was more similar to *AtPLDβ2* (Fig. 1*A*, Table 1 Suppl.). In contrast to the single isoform of *PLDδ* in *A. thaliana,* we identified three different *PLDδ* genes in *B. napus.* All of them shared more than 90 % identity with *AtPLDδ* at the protein level. The two *B. napus PLDγ* identified in this study were clearly more divergent in their sequence than we observed for the *PLDβ* and *PLDδ* genes. They shared only 74.5 % amino acid identity with each other (Table 1 Suppl.). Whereas *BnaX.PLDγ.a* clustered together with *A. thaliana PLDγ's, BnaX.PLDγ.b* formed a phylogenetically distinct clade together with the *B. rapa* gene *Bra001854* but without any *B. oleracea* gene. We wanted to know if the most divergent regions were located in any of the important PLD domains. Schematic protein alignment of AtPLDγ1 shows that BnaX.PLDγ.b had two large gaps in the N-terminal of C2 domain. However, these gaps were located outside of the calcium-binding region.

We were interested in the transcriptional regulation of *B. napus PLD* genes in response to hormone treatment and biotic stresses. We analysed the expression of all *B. napus* genes identified in this study and gene-specific primers were designed for all genes (Table 1). Because *BnaX.PLDβ.a* and *BnaX.PLDβ.b* share high sequence homology, the specificity of the designed primers was verified using a plasmid containing *BnaX.PLDβ.a* or *BnaX.PLDβ.b* sequence as a template. Each primer differed by two nucleotides from the non-target sequence. The difference in Ct values of qPCR between the target and non-target template was 12.9 and 15.1 for *BnaX.PLDβ.a* and *BnaX.PLDβ.b* primers (data not shown). These results clearly confirmed the specificity of *BnaX.PLDβ.a* and *BnaX.PLDβ.b* primers.

Among the hormones regulating plant defence responses to pathogens and pests, SA and JA play a major role. We sprayed leaves of *B. napus* with 32 μM BTH, a functional analogue of SA. Alternatively, the plants were treated with MeJA, a compound that is metabolised to JA in plants. The treatment of plants with BTH induced both *BnaX.PLDγ* genes and also

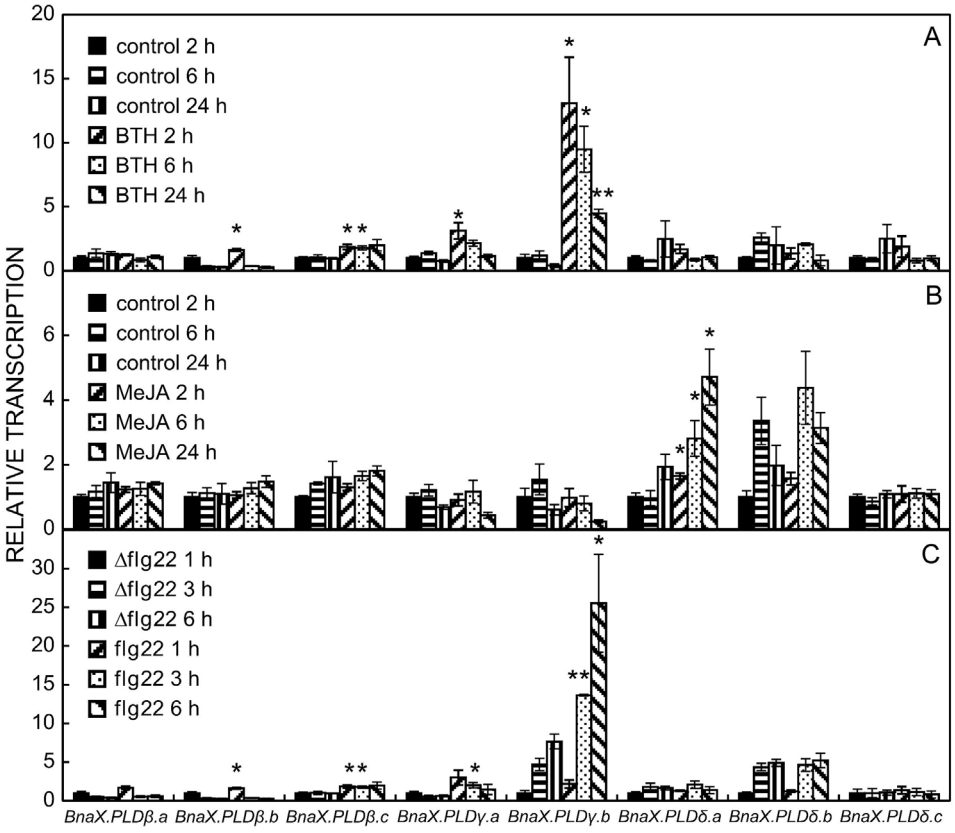

Fig. 2. Transcription analyses of *B. napus PLD* genes in plants treated with benzothiadiazole (BTH) (*A*), methyl jasmonate (MeJA) (*B*), and flagellin derived peptide flg22 (*C*). Plants 21-d-old were sprayed with water, or 32 μM BTH or plants were incubated in an airtight container in an atmosphere with MeJA. Plants 14-d-old were infiltrated with 1 μM flg22 or its inactive derivate Δflg22. RT-qPCR analyses were performed at indicated times after induction. Means ± SE of three independent experiments. *Asterisks* indicate statistically significant differences compared to the corresponding control (* - $P < 0.05$, ** - $P < 0.01$, Student's *t*-test).

BnaX.PLDβ.b and *BnaX.PLDβ.c* (Fig. 2*A*). The *BnaX.PLDβ.b, BnaX.PLDβ.c*, and *BnaX.PLDγ.a* responded only slightly at 2 h after treatment. Strikingly, *BnaX.PLDγ.b* was induced much more strongly. After 2 h, the gene was induced 13 times relative to control plants. The transcription dropped after a longer period of time, but the gene was still significantly induced 24 h after treatment. The only gene induced by MeJA treatment was *BnaX.PLDδ.a* (Fig. 2*B*). Its transcription increased already at 2 h after treatment and rose continuously to 24 h when it was almost five times higher compared to the control plants. We investigated further the response of *PLD* genes to the treatment with a synthetic elicitor derived from bacterial flagella. This 22 amino acid peptide termed flg22 is recognised by plants and triggers various defence responses (Gomez-Gomez and Boller 2002). The expression pattern after flg22 treatment was similar to that after BTH. Treatment with flg22 induced *BnaX.PLDβ.b*, *BnaX.PLDβ.c*, and both *B. napus PLDγ* genes, especially *BnaX.PLDγ.b* (its highest expression was observed 6 h after treatment; Fig. 2*C*).

Plants are also capable of responding to mechanical damage caused by herbivores. We analysed transcription of *B. napus PLD* genes in cotyledons wounded with forceps having serrated jaws. Transcriptional responses of plants to wounding are known to be very fast and transient (Walley *et al.* 2007). So as to track only the primary response to wounding, we analysed gene transcription within the first 90 min after wounding. Among the eight *PLD* genes, *BnaX.PLDβ.a, BnaX.PLDβ.b, BnaX.PLDγ.a, BnaX.PLDδ.a*, and *BnaX.PLDδ.b* were significantly induced by wounding (Fig. 3*A*). However, only *BnaX.PLDβ.a* and *BnaX.PLDβ.b* were already induced 30 min after wounding and also their transcriptions were higher than of other genes. Interestingly, the third *BnaX.PLDβ.c* isoform was not noticed (Fig. 3*A*).

Finally, we analysed gene transcription in plants infected by two fungal pathogens, a hemibiotroph *L. maculans* and a necrotroph *S. sclerotiorum*. Infection with *L. maculans* increased transcription of both *B. napus PLDγ* genes. As with BTH and flg22, *BnaX.PLDγ.b* responded more intensively than *BnaX.PLDγ.a* (Fig 3*B,C*). At 7 d after infection, the *BnaX.PLDγ.b* transcription was 20 times higher than in control plants.

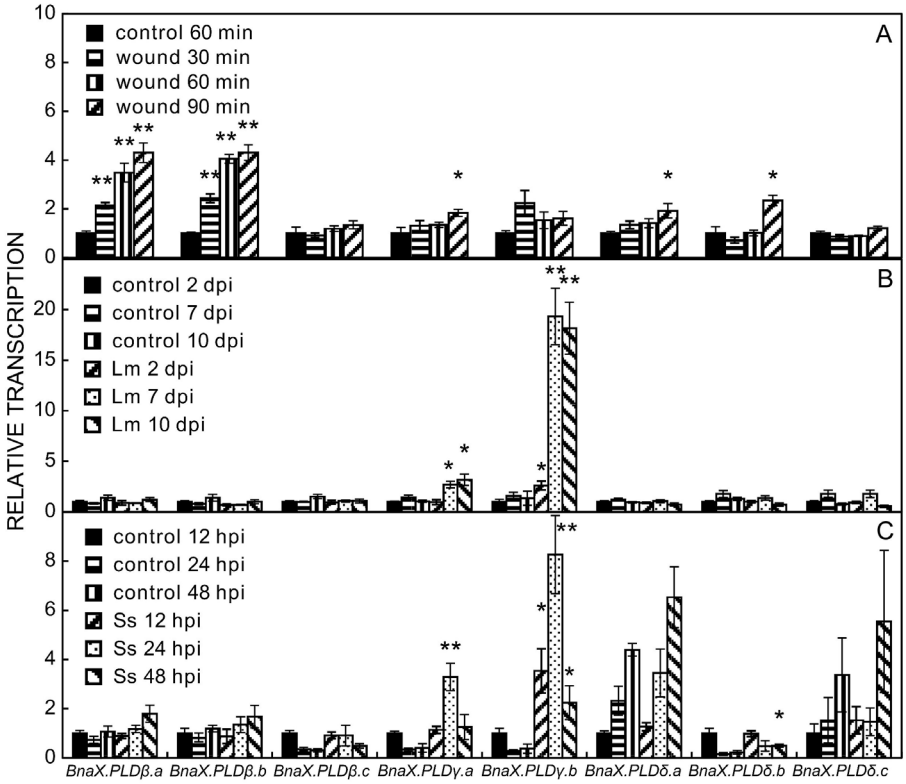

Fig. 3. Transcription analyses of *B. napus PLD* genes in plants wounded with forceps (wound) (*A*), infected by an avirulent strain of hemibiotrophic fungus *Leptosphaeria maculans* (Lm) (*B*), or infected by necrotrophic fungus *Sclerotinia sclerotiorum* (Ss) (*C*). Plants 14-d-old were wounded by applying pressure with forceps having serrated teeth. Plants 11-d-old were inoculated by infiltration of a spore suspension of *L. maculans* JN3 into the intercellular space (10^5 cm^{-3}). Control plants were infiltrated with water. Plants 21-d-old were inoculated with a 5 mm agar plugs covered with *S. sclerotiorum* mycelium. RT-qPCR analyses were performed at indicated times after wounding/inoculation. Means ± SE of at least three independent experiments. *Asterisks* indicate statistically significant differences compared to the corresponding control (* - $P < 0.05$, ** - $P < 0.01$, Student's *t*-test).

The infection by *S. sclerotiorum* affected transcription of *PLD* genes similarly as *L. maculans* and the both *PLDγ* genes were up regulated (Fig. 3*B,C*). The maximum transcription of both genes was observed 24 h after infection. We also observed a rise in the transcription of *BnaX.PLDδ.a* and *BnaX.PLDδ.c* during the course of experiment but the rise was similar in the infected and control plants. These changes could be explained by the change of the environment when plants were transferred to the dark and in nearly 100 % relative humidity to facilitate the infection process.

Next, we compared our results with the *A. thaliana* transcriptomic data deposited in public microarray databases. We selected experiments with at least partially similar experimental conditions to those used in our study. For each stimulus, we compared the transcription of selected marker genes in our *B. napus* samples and *A. thaliana* microarray data (data not shown). Transcriptional data of *A. thaliana PLD* genes in plants treated with BTH, MeJA, and flg22 or in wounded plants are shown in Fig. 4. BTH significantly induced *AtPLDγ1, AtPLDγ3* and to some extent also *AtPLDδ.* Among the genes that were most induced by MeJA were *AtPLDβ2* and *AtPLDγ1*. Nevertheless, the experiment with MeJA treatment cannot be statistically evaluated since no replicates were carried out. The only gene whose transcription was strongly affected by the flg22 treatment was *AtPLDγ1*. This increase was apparent at 1 h but not at 4 h after treatment (Fig. 4*C*). Wounding had no effect on transcription of *A. thaliana PLD* analysed in this study except for very weak induction of *AtPLDγ1*.

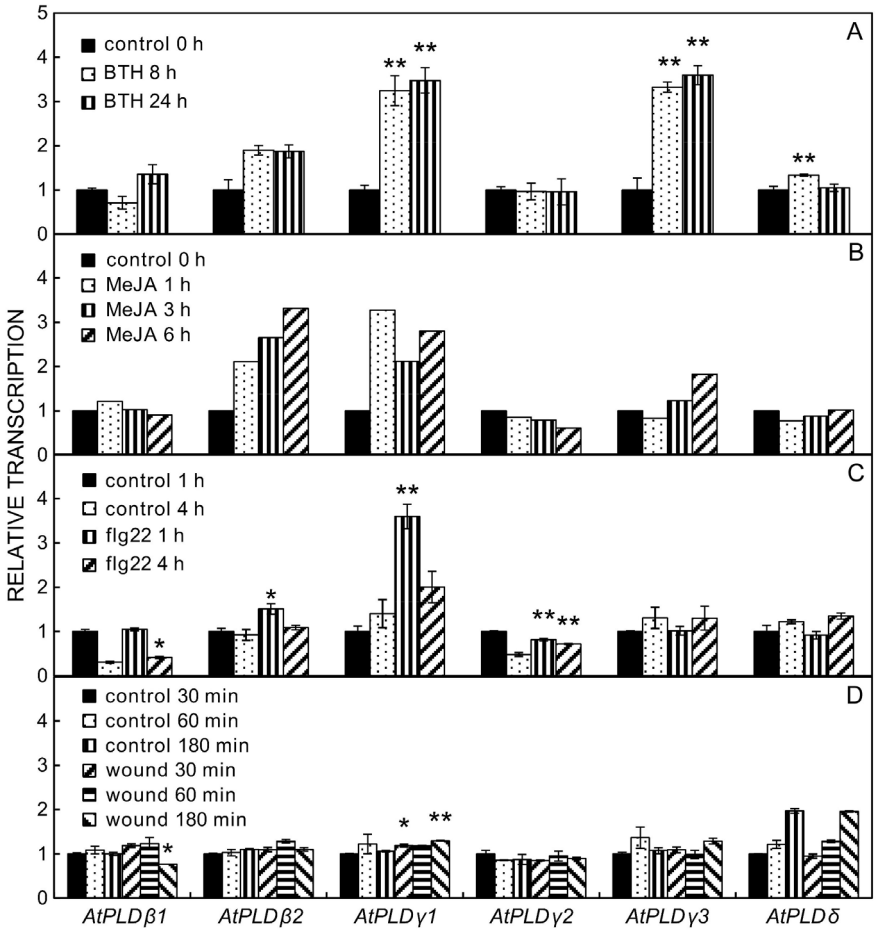

Fig. 4. Transcription analyses of *A. thaliana PLD* genes in plants treated with BTH (*A*), MeJA (*B*), flg22 (*C*), or wounded with needles (*D*). All data originate from microarray experiments deposited in public microarray databases. Plants 4-week-old were sprayed with 60 μM BTH. Plants 5-week-old were dipped in 50 μM MeJA. Plants 5-week-old were infiltrated with 1 μM flg22. Seedlings 16-d-old were wounded by puncturing with needles. Means ± SE of three (BTH and flg22) or two (wounding) experiments. *Asterisks* indicate statistically significant differences compared to the corresponding control (* - $P < 0.05$, ** - $P < 0.01$, Student's *t*-test). Data from MeJA experiment represent results of a single analysis therefore no error bars and statistical analysis are displayed.

Discussion

We identified and annotated eight coding sequences belonging to three distinct classes of *PLD* family in *B. napus*. Whereas the identified members of *PLDβ* and *PLDδ* classes are all paralogs of known *A. thaliana PLD* genes, the *BnaX.PLDγ.b* gene is distinct from *AtPLDγ*'s. The phylogenetic analysis (Fig. 1*A*) provided us knowledge about identified *PLD* genes in *B. napus* and their relation to ancestors *B. rapa* or *B. oleracea* (Fig. 1*A*). Five of them are related to *B. rapa* (*BnaX.PLDδ.a, BnaX.PLDδ.c, BnaX.PLDγ.b, BnaX.PLDβ.b,* and *BnaX.PLDβ.a*), one to *B. oleracea* (*BnaX.PLDδ.b*) and for two (*BnaX.PLDγ.a* and *BnaX.PLDβ.c*) we are not able to suggest the ancestor. The *BnaX.PLDγ.b* gene, which is phylogenetically distant from *BnaX.PLDγ.a* and *A. thaliana PLDγ*'s, probably originate from *B. rapa* (Fig. 1), gave the strongest transcriptional response to applied stimuli among all eight *B. napus PLD*'s. It was induced by treatment with BTH, flg22, and infection with *L. maculans* or *S. sclerotiorum* (Figs. 2*A,C* and 3*B,C*). The transcriptional profile of *BnaX.PLDγ.a* was very similar but the induction was always lower. BTH and flg22 also induced the expression of *PLDγ* genes in *A. thaliana* (Fig. 4*A,C*). All four stimuli that induced *BnaX.PLDγ*'s are linked to SA. BTH is a functional analogue of SA. Flg22 is known to increase SA content and induce SA-mediated resistance to bacterial pathogens (Tsuda *et al.* 2008). *L. maculans* has an initial biotrophic phase when *B. napus* plants respond to infection by activation of SA and ethylene signalling pathways (Sasek *et al.* 2012). Although *S. sclerotiorum* is a necrotrophic pathogen, infection of *B. napus* is also accompanied by elevated content of SA (Novakova *et al.* 2014). Therefore, it is possible that an increased transcription of *B. napus PLDγ*'s is mediated by SA. This may explain the observation that the increase in transcription of *BnaX.PLDγ.b* after flg22 treatment appeared later compared to the BTH treatment. In addition to *B. napus PLDγ*'s, *BnaX.PLDβ.b* and *BnaX.PLDβ.c* were also induced by BTH and flg22 (Fig. 2). The PLDβ and PLDγ classes belong to PIP_2-dependent PLD's whose activity increases after SA and BTH treatment in *B. napus* (Profotova *et al*. 2006). Yamaguchi *et al.* (2009) showed that the knockdown line of rice *PLDβ1* has constitutively activated defence responses, namely the production of reactive oxygen species and phytoalexins. More recently, Zhao *et al.* (2013) investigated the role of *AtPLDβ1* in defence responses to fungal and bacterial pathogens. *PLDβ1*-deficient plants were less susceptible to *Pseudomonas syringae* and the expression of SA responsive genes in infected plants was higher compared to wild-type infected plants. On the other hand, *PLDβ1*-deficient plants were more susceptible to *Botrytis cinerea* and showed weaker expression of JA responsive genes. These two studies support a view of PLDβ as a negative regulator of the SA signalling pathway. On the contrary, the *A. thaliana* responses to SA are partially blocked by the PLD inhibitor *n*-butanol (Krinke *et al.* 2009). Therefore, there might be another PLD isoform having an opposite role to *AtPLDβ1* in the SA signalling pathway. According to the results presented in this study PLDγ is an interesting candidate for further investigation.

Five of the eight *B. napus PLD* genes investigated in this study responded to wounding (Fig. 3*A*). Similar results were observed by Wang *et al.* (2000), who showed the induction of *PLD*α, *β*, and *γ* in *A. thaliana*. In contrast, no such effect was observed in microarray analysis of wounded *A. thaliana*. The discrepancy can be explained by the different experimental conditions. Wang *et al.* (2000) used plants grown in soil and caused wounding with a hemostat whereas plants used for the microarray experiment were grown in MS agar and were wounded with needles.

B. napus shares an 85 % coding sequence identity on average with *A. thaliana* (Yu *et al*. 2010). Hence, findings done in *A. thaliana* are often generalised for *Brassica* species. However, there are only a few reports directly comparing the responses to a particular stimulus between the two plants. In this study, we observed the similarities as well as the differences. Whereas BTH and flg22 had very similar effects on *PLD* gene transcription in *B. napus* and *A. thaliana* (Figs. 2*A*,*C* and 4*A,C*), almost no overlap was observed for MeJA treatment or wounding. Das *et al.* (2010) made a transcriptomic comparison between *A. thaliana* and *B. napus* in response to various herbicides. The authors concluded that the responses of both species were similar. Obermeier *et al.* (2009) compared the transcriptomic data of developing *B. napus* seeds with a publically available microarray analysis of *A. thaliana*. The number of genes whose transcription was similarly affected in both species appeared to be high. Yang *et al.* (2009) characterised the response of *B. napus WRKY* genes to phytohormone treatments. They observed both similarities and differences. For example, *WRKY45* and *WRKY75* were both induced by ethylene in *B. napus* as well as in *A. thaliana,* whereas *WRKY28* was down-regulated in *A. thaliana* but not in *B. napus* (Yang *et al.* 2009). In more complex events, such as infection by a pathogen where interactions of hundreds of molecules take place, the response of different plants can even be opposite. In our previous study with infection of *B. napus* by *L. maculans*, we observed positive contributions to resistance by Sa and ET signalling pathways that have been previously shown to negatively affect *A. thaliana* resistance to this fungus (Sasek *et al.* 2012).

In conclusion, our study describes the transcriptional profiles of eight novel *B. napus PLD* genes in response to phytohormones and biotic stresses. We have shown that the gene expression of PIP_2-dependent PLD isoforms

(*PLDβ*'s and *PLDγ*'s) is induced by stimuli related to SA. This is another line of evidence suggesting that PIP_2-dependent PLD's might be connected with the SA signalling pathway. Comparison of the results between *B. napus* and *Arabidopsis* showed many similarities but also some differences, which support the need for studies conducted on non-model plants.

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