The phospholipase VlsPLA$_2$ from the plant pathogen *Verticillium longisporum* is a virulence factor targeting host nuclei and suppressing PTI-related hypersensitive response

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Abstract

Verticillium longisporum is an amphidiploid fungal species infecting plants mainly in Brassicaceae family. It is one of the most important pathogens in rapeseed (*Brassica napus*) cultivation, causing severe annual yield losses worldwide. Pathogens secrete a plethora of small proteins, termed effectors, to evade plant immunity and to establish a successful infection. Genome analysis of the *V. longisporum* stain VL1, isolated from Swedish infested soils where rapeseed has intensively been cultivated, showed that it contains more than 80 candidate effector genes. Among them, the *VlsPLA$_2$* gene was highly induced during *B. napus* infection. Protein expression in *E. coli* cells, showed that it is an active A$_2$ phospholipase. Further characterization showed that it has a catalytic dyad HD crucial for its enzymatic function, a signal peptide at the N-terminus, and two active NLS, one monopartite and one bipartite, with the latter one to be more crucial for its nuclear localization. Transient expression in *Nicotiana benthamiana* plants showed it to be localized to host nuclei 48hpi, while it is translocated to chloroplasts 72hpi. However, the enzymatically inactive VlsPLA$_2$ protein did not enter the nucleus or localized to chloroplasts even 48hpi. Our data from *B. napus* plants showed that *V. longisporum* mutants overexpressing *VlsPLA$_2$* were more virulent as compared to strain that expressed an enzymatically inactive one. Finally, this effector is a negative regulator of hypersensitive response (HR) triggered by the Cf4/Avr4 complex, but not by *Pseudomonas syringae* pv. *tomato* DC3000 (PsPto). Also, it was able to induce genes that encode pathogen related (PR) proteins in this host. In conclusion, these data show that VlsPLA$_2$ is a virulence factor targeting host nuclei and suppresses Pattern Triggered immunity (PTI)-related HR, possibly through interference in signal transduction pathways, altering gene expression.

Keywords: effector, nucleus, phospholipase, *Verticillium*

Introduction

The genus *Verticillium,* which belongs to division Ascomycota in the Plectosphaerellaceae family, includes species causing mainly vascular wilt disease in a plethora of economically important crops. The most important one is the haploid species, *V. dahliae,* which has a broad host range comprising a high number of dicotyledonous plants (e.g., tomato, cotton, olive) (Berlanger, 2000). In contrast, the *V. longisporum* species has a narrow host range, infecting mostly plants in Brassicaceae family (e.g., rape seed, cabbage, and broccoli) (Depotter et al., 2016). However, the increasing hectares of winter rape seed (*Brassica napus*) cultivation in Europe and in Canada, showed that *V. longisporum* is one of the most devastating pathogens in this crop (Dunker et al., 2008, Tzelepis et al., 2017). *V. longisporum* is the only non-haploid species in this genus and its genome, characterized as amphidiploid, is the result of hybridization of two species, *V. dahliae* and an unknown lineage (Fahleson et al., 2004, Inderbitzin et al., 2011, Inderbitzin et al., 2013). Progenitors were named A1 and D1, D2 and D3 leading to three *V. longisporum* lineage compositions A1/D1, A1/D2 and A1/D3. The origin of A1 and D1 progenitors is unknown, while D2 and D3 derived from two *V. dahliae* lineages (Inderbitzin et al., 2011; 2013).

One of the most important challenges in the control of this pathogen, is the formation of microsclerotia in soil and plant debris. These structures can survive for long periods under harsh environmental conditions (e.g., chemical treatments, drought, radiation, etc.). The disease cycle of this pathogen in not completely known, but it is thought that microsclerotia are stimulated by root exudates, such as monoterpenes, produced by the nearby host plants, and germinated to hyphae (Roos et al., 2015, Rafiei et al., 2022). Then hyphae invade roots, followed by colonization of the xylem elements.
Then pathogen can spread via the plant transpiration system. Since the most prominent symptom is stunting, the disease remains easily unnoticed before microsclerotia forms at the end of the disease cycle (Depotter et al., 2016).

Pathogens secrete a plethora of small proteins, termed effectors, in order to avoid plant immunity mechanisms and to establish a successful infection (Giraldo & Valent, 2013). These proteins can have diverse functions, for example, to induce necrosis; to suppress the hypersensitive response (HR); to protect fungal hyphae from host secreting enzymes, such as chitinases; to be involved in host energy production etc. (Lo Presti et al., 2015). Although the sequencing of fungal genomes has helped to identify and understand better the diverse roles of fungal effectors, there are still many aspects that remain to be elucidated. From the plant side, the host deploys defense mechanisms to counteract the pathogens attack and hence, signaling plays a prominent role in this process. As the first layer, the plasma membrane-localized pattern recognition receptors (PRRs) recognize the microbe molecular patterns (MAMPs) (e.g., chitin, flagellin, etc.) and this recognition can lead to a burst of reactive oxidate species (ROS), influx of calcium (Ca^{+}), the activation of mitogen-activated protein kinases (MAPK) and the induction of defense genes, resulting in pattern triggered immunity (PTI) (Couto and Zipfel, 2016). When pathogens overcome PTI, then hosts deploy Nod-like receptors (NLRs), able to recognize effectors leading to effector triggered immunity (ETI) (Boller and Felix, 2009). The outcome of ETI is usually development of HR, although PTI can also result in program cell death (Jones and Dangl, 2006).

Phospholipids are essential structural components of plasma membranes and are conserved in living organisms, from bacteria to plants and humans. They form a lipid bilayer with a hydrophobic and hydrophilic environment and are abundant in mitochondrial and nuclear envelopes, whereas they are scarce in the thylakoid membranes (Nakamura, 2017). Phospholipases are enzymes responsible to hydrolyze phospholipids and producing phosphatidic acid (PA), diacylglycerol (DAG), free fatty acids (FFAs), and lysophospholipids (LPLs). These products play important roles in different aspects of cell physiology and are involved in signal transduction, cytoskeletal dynamics, and protein secretion (Hong et al., 2016). They are also involved in plant defense mechanisms probably as early signaling molecules (Zhao, 2015).

Based on the site of glycerophospholipid hydrolysis, phospholipases are categorized in phospholipases A_1 (PLA_1s) and A_2 (PLA_2s); phospholipases C (PLCs); phospholipases D (PLDs), and they further subdivided in many families and subfamilies (Aloulou et al., 2012). Previous studies showed that secreted phospholipases are involved in the regulation of different processes in bacterial and fungal pathogens, but their precise roles in virulence remain widely unknown (Ghannoum, 2000, Sitkiewicz et al., 2006). In this study, we investigated the role of the secreted phospholipase $VlsPLA_2$ from the phytopathogen, *V. longisporum.* We found that the gene is highly induced during the infection of *B. napus* plants, and heterologous expression and purification of this protein showed it to be an active PLA_2. Furthermore, transient expression of $VlsPLA_2$ in *Nicotiana benthamiana* plants, showed that it localized to the host nucleus. In addition, $VlsPLA_2$ was able to suppress the PTI-related HR, induced by the Cf4/Avr4 complex. Lastly, overexpression of this gene increased *V. longisporum* virulence, indicating a crucial role in fungal infection biology. The preliminary results from the current study give us insights towards understanding the functional role of these enzymes in fungal infection biology.

Material and Methods

Fungal strains and growth conditions

In the current study, the *V. longisporum* isolate VL1 (CBS110220) was used (Steventon et al., 2002, Fogelqvist et al., 2018). The fungus was kept on potato dextrose agar (PDA, Difco) at 20°C in darkness and sporulation occurred within seven days on this medium.

Gene expression analysis

The transcription profiles of *V. longisporum* genes encoding candidate effector proteins, were investigated under conditions previously described (Roos et al., 2015). Briefly, *B. napus* seedlings were infected with *V. longisporum* by dipping in 10^6 conidia/ml suspension for 15min. Plants were harvested at 2, 4-, 6-, 8- and 10-days post infection (dpi) and frozen in liquid nitrogen, while mycelia grown in PDB were used as a control. RNA was extracted using the Spectrum Plant Total RNA kit (Sigma or Thermo Fisher) according to their protocol and treated with DNase I (Thermo Fisher Scientific). For qPCR analysis, one µg of total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). RT-qPCR analysis was conducted as described previously (Tzelepis et al., 2012). Primers sequences are listed in Table S1, and gene

expression was normalized using the reference gene, glyceraldehyde phosphate dehydrogenase (*GAPDH*) (Rafiei et al., 2022). Relative expression values were calculated from the threshold cycle (Ct) values according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Protein sequence analysis, structure prediction, and phylogeny

The amino acid sequence of $VlsPLA_2$ was retrieved from the *V. longisporum* VL1 genome (Fogelqvist et al., 2018). Analysis of conserved domains was performed using the SMART and InterProScan protein tools (Quevillon et al., 2005; Letunic et al., 2009). To investigate whether $VlsPLA_2$ is an apoplastic or a cytoplasmic effector, the ApoplastP prediction software was used (Sperschneider et al., 2018), and its subcellular localization was predicted using the LOCALIZER and the cNLS Mapper software (Sperschneider et al., 2017, Kosugi et al., 2009).

For the phylogenetic analysis, homologs to $VlsPLA_2$ were retrieved from other fungal species from the FungaDB database (Amos et al., 2022). The amino acid sequences were aligned with CLUSTAL X (Thompson et al., 1997) and phylogenetic analysis was conducted using maximum likelihood implemented in MEGA X (Kumar et al., 2018), using the JTT amino acid substitution model (Whelan and Goldman, 2001). Statistical support for branches was supported by 1000 bootstraps.

Heterologous protein expression and phospholipase activity assay

The cloning, expression and purification of the $VlsPLA_2$ protein was done at the Protein Expertise Platform (PEP) at Umeå University. Briefly, Origami2 (DE3) cells were grown in LB media supplemented with 50 µg/mL kanamycin at 37°C with agitation. The culture was incubated until an OD_{600} ~ 0.6–0.7 was achieved. Protein expression was induced by the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated overnight at 20°C. Cells were harvested by centrifugation 4000 x g for 20min. The target protein was purified using His-Tag Purification Resin (Roche) according to the manufacturer. The His-Trx-tag was cleaved off using Prescission3C protease. Finally, the protein was run on a Superdex200 (Cytiva), 20mM NaP 8.0, 200mM NaCl, 0.5mM b-me. For the phospholipase A2 enzymatic activity, the EnzChek Phospholipase A_2 activity kit was used (ThermoFisher), according to manufacturer instructions. In all assays, 10µM of pure active ($VlsPLA_2^{WT}$) and inactive ($VlsPLA_2^{H98A/D99A}$) protein was used, previously constructed by Integrated DNA Technologies (idtDNA).

Confocal microscopy, hypersensitive response and ROS burst suppression assays

Primers Vl14035F_pDONOR and Vl14035R_pDONOR (Table S1), were used to amplify the *$VlsPLA_2$* gene from *V. longisporum* cDNA and cloned into the Gateway entry vector, pDONOR/Zeo (Thermo Fisher Scientific), followed by an LR reaction using the Gateway binary vector, pGWB605 (Nakamura et al., 2010). Cloning into this destination vector resulted in a protein tagged with GFP at the C-terminus. Overnight cultures of *A. tumefaciens* (C58C1) harboring the 35S:*$VlsPLA_2$*:GFP construct were infiltrated on leaves of 4-week old *Nicotiana benthamiana* plants (grown under 18h

light/6h dark at 23°C). The subcellular localization of the phospholipase was monitored by using the Zeiss LSM 800 confocal microscope at 48 and 72 hpi.

For the HR suppression assay, the *VlsPLA$_2$:pDONOR/Zeo* entry vector was cloned into the pGWB602 binary vector (Nakamura et al., 2010) and transiently expressed by in *N. benthamiana* plants harboring the Cf-4 receptor protein from tomato plants OD_{600} = 0.5 as previously described. The HR was triggered 24hrs after *VlsPLA$_2$* Agro-infiltration with the *Cladosporium fulvum* Avr4 effector protein at OD_{600} = 0.03 (Joosten et al., 1997). The ability of VlsPLA$_2$ to suppress ETI-triggered HR was investigated on *N. benthamiana* wild type plants upon infection with *Pseudomonas syringae* pv. *tomato* DC3000 as described previously (Gimenez-Ibanez et al., 2018). Agro-infiltration with empty vector, only induction buffer (mock), and the enzymatically inactive VlsPLA$_2$ (VlsPLA$_2^{H98A/D99A}$) were used as controls.

For suppression of chitin-induced oxidative burst of reactive oxygen species (ROS), a luminol-based protocol was used (Albert et al., 2015). Briefly, leaf discs from *N. benthamiana* plants, treated with chitin (100 µl/ml), luminol (200µM) (Sigma) and 10 µg/ml horseradish peroxidase (Sigma). The suppression of ROS was analyzed by using 10 µM VlsPLA$_2^{WT}$ and VlsPLA$_2^{H98A/D99A}$ proteins and measuring the chemiluminescence levels in the BMG LABTECH microplate reader.

Construction of overexpression strains, phenotypic analysis and virulence assays

For construction of the overexpression strains (*VlsPLA$_2$*+^{WT} and *VlsPLA$_2$*+$^{H98A/D99A}$), the *VlsPLA$_2$* gene from *V. longisporum* cDNA was amplified using the primers listed in Table S1. The gene fragment was ligated to the pRFHUE-eGFP vector, driven by the gpdA constitutively expressed promoter (Crespo-Sempere et al., 2011), using the GenArt Seamless cloning technology (Thermo Fisher Scientific) and transformed into *V. longisporum* using the *Agrobacterium*-mediated protocol (Utermark & Karlovsky, 2008). The expression levels of the *VlsPLA$_2$* gene in overexpression strains was investigated by RT-qPCR techniques as described above.

The growth rate of *V. longisporum* overexpression strains were evaluated on PDA plates. For rapeseed infection assays, the ''Hannah'' cultivar was used. Plants were grown in greenhouse conditions in a photoperiod of 16 hours light and 8 hours darkness with temperatures between 18°C and 23°C on commercial peat soil. Two-week-old plants were uprooted carefully and dipped in a suspension containing 10^6 conidia/ml, derived from *V. longisporum*, overexpression *VlsPLA$_2$*+^{WT} and *VlsPLA$_2$*+$^{H98A/D99A}$ strains, while mock inoculation was conducted dipping the roots in only dH_20 for 30 min. At least three biological replicates were used, with each replicate consisting of eight plants. Fresh and dry plant biomass was measured 25dpi.

Results and Discussion

The *VlsPLA2* gene is highly induced upon *Brassica napus* infection

Effectors are small proteins, secreted by pathogens, in order to evade plant immunity and to establish a successful infection. In general, the criteria to categorize a protein

as a candidate effector is to be secreted, to contain a cysteine rich motif at the C-terminus and to be relatively small (< 400 aa) (Lo Presti et al., 2015). Furthermore, up-regulation of the genes, upon host infection could also be a strong indication that the encoded protein is an effector. According to previous studies the *V. longisporum* VL1 isolate contains more than 80 genes, encode candidate effectors (Fogelqvist et al., 2018). Among them, we prioritized 13 predicted candidate effector genes from the VL1 genome to study their transcription patterns. Our analysis showed the *VL1_T00014035* gene, coding for a putative phospholipases A_2; the *VL1_T00003220* gene, coding for a putative peptidase inhibitor in I78 family; and the *VL1_T00003720,* coding for a putative endolysin-like protein, were highly induced during *B. napus* infection as compared to the mycelium grown in PDB (**Figure 1**). Up-regulation of a secreted *PLA$_2$* gene was observed during nitrogen and carbon starvation conditions, and during establishment of symbiosis in mycorrhizal species *Tuber borchii.* It was also activated during autoproteolytic activity and promoted mycorrhiza development with a specific host plant, indicating involvement in fungal-host interactions (Soragni et al., 2001, Miozzi et al., 2005, Cavazzini et al., 2013). Regarding the rest of the genes studied, no up regulation was observed (**Figure 1**), while no expression was seen, for the *VL1_T00006681*, *VL1_T0001013*, *VL1_T00009155* and *VL1_T00008255* genes, under our tested conditions. Since the *VL1_T00014035* gene (denominated as *VlsPLA$_2$*) showed the highest up regulation as compared to the others, and the role of A_2 phospholipases in fungal virulence against plants is not well understood, this gene was selected for further studies. Interestingly, previous genome analysis in the parental species *V. dahliae* showed that this gene derived through horizontal gene transfer from a prokaryotic ancestor (Shi-Kunne et al., 2019).

VlsPLA$_2$ is an active phospholipase A2 enzyme

In order to investigate the domain structure of VlsPLA$_2$, the SMART and InterProScan tools were used. Analysis showed that this protein contains a conserved phospholipase A_2 domain (IPR015141), present in fungal and bacterial phospholipase A_2 enzymes, that are able to hydrolyze the 2-ester bond of 1,2-diacyl-3-sn-phosphoglycerides releasing fatty acids and lysophospholipid (Matoba et al., 2002). Furthermore, sequence analysis of homologs to VlsPLA$_2$, derived from fungal and bacterial species, showed a conserved CxRHDFGYxN domain and the catalytic dyad (HD) also to be also present in this protein (**Figure 2A**).

Further analysis with multiple prediction tools, showed that VlsPLA$_2$ is a putative cytoplasmic effector, while the presence of a chloroplast transit peptide (cTP) and two nuclear localization signals (NLS; one monopartite KDPKRCRWDSDGC, and one bipartite DDSIVKRAETEEAEEEFEYFDANDVDPDIEG) were predicted, indicating a nuclear and chloroplast localization of this effector (**Figure 2A**). Phylogenetic analysis also was conducted on putative PLA$_2$ homologs derived from different fungal species. Our analysis showed these proteins were grouped in two major groups, and VlsPLA$_2$ was closely related to *V. dahliae* PLA$_2$ and enzymes derived from *Aspergillus* and entomopathogenic species, such as *Metarhizium* and *Cordyceps* (**Figure 2B**).

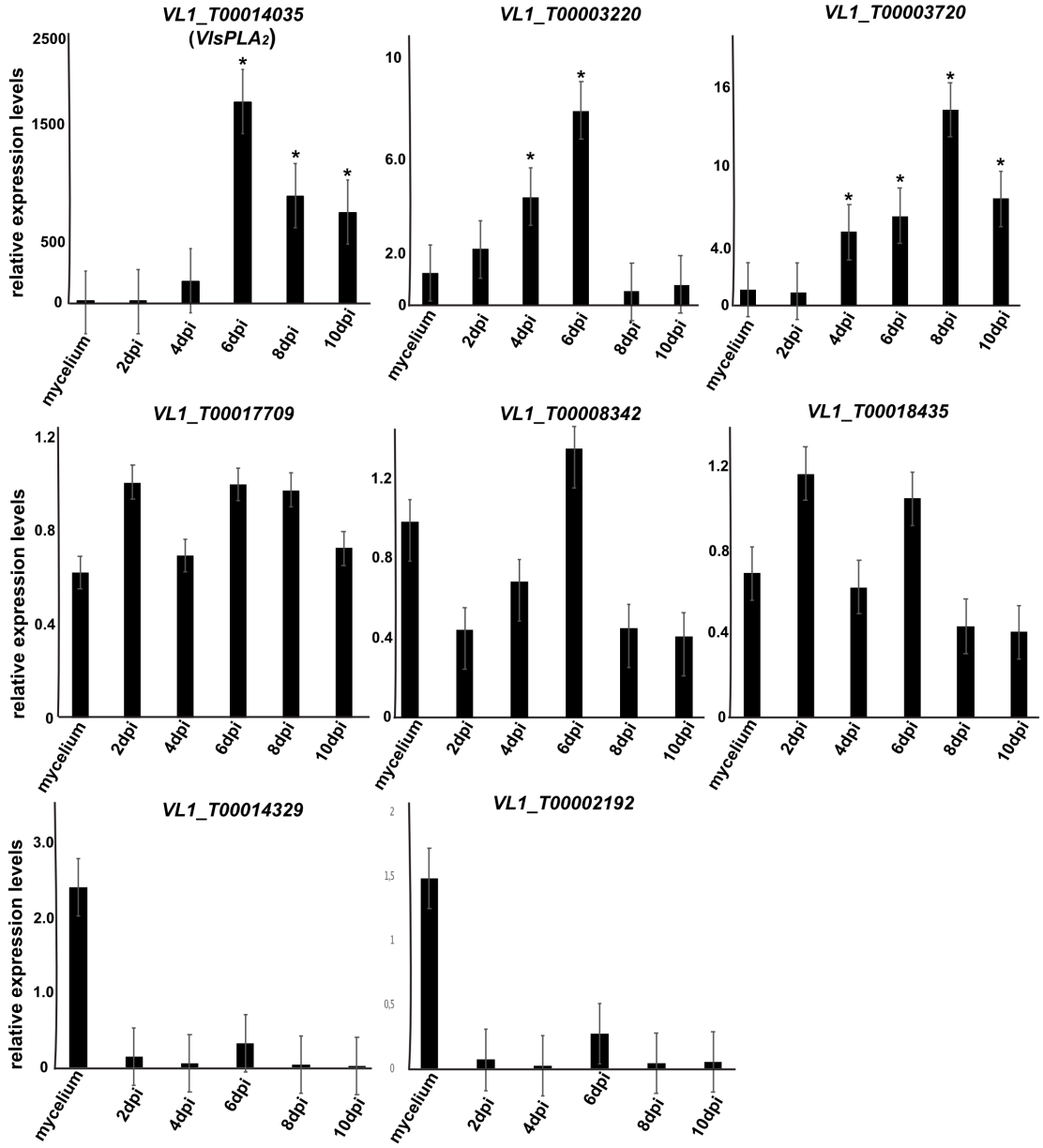

Figure 1. *Gene expression analysis of candidate effector genes from* Verticillium longisporum *during* Brassica napus *infection.* Verticillium longisporum *mycelia grown on PDB was used as control for comparison. The* GAPDH *gene was used as an internal control during the RT-qPCR analysis. Asterisks (*) indicate statistically significant difference as compared to mycelia control according to Student's T test (p>0.05). For the analysis, four biological replicates were used. Error bars show standard error (SE).*

Analysis of the predicted 3D protein structure revealed 54% identity with the PLA_2 from the ascomycete *Tuber borchii* and 48% similarity with the PLA_2 from the bacterial species *Streptomyces violaceoruber* (Soragni et al., 2001, Burke et al., 2008) (**Figure 2C**). In order to investigate whether $VlsPLA_2$ is an active phospholipase A_2, protein was heterologously expressed in *E. coli* cells, tagged with 6xHis epitope and purify though a His-tag purification resin. Our analysis showed that $VlsPLA_2$ is an active phospholipase A_2, able to degrade the PLA_2 selective substrate similar to the PLA_2, derived from the venom of *Apis mellifera* and used a positive control (**Figure 2D**). To

investigate whether the HD catalytic dyad is crucial for its enzymatic activity (Murakami and Kudo, 2004), both amino acids were exchanged with Alanine and it was heterologously expressed to *E. coli* as well. Our results showed that the truncated version of $VlsPLA_2$ was not able to degrade the selective substrate, indicating that this dyad is important for its enzymatic function, similar to the other PLA_2 previously described by fungal and bacterial species (**Figure 2D**).

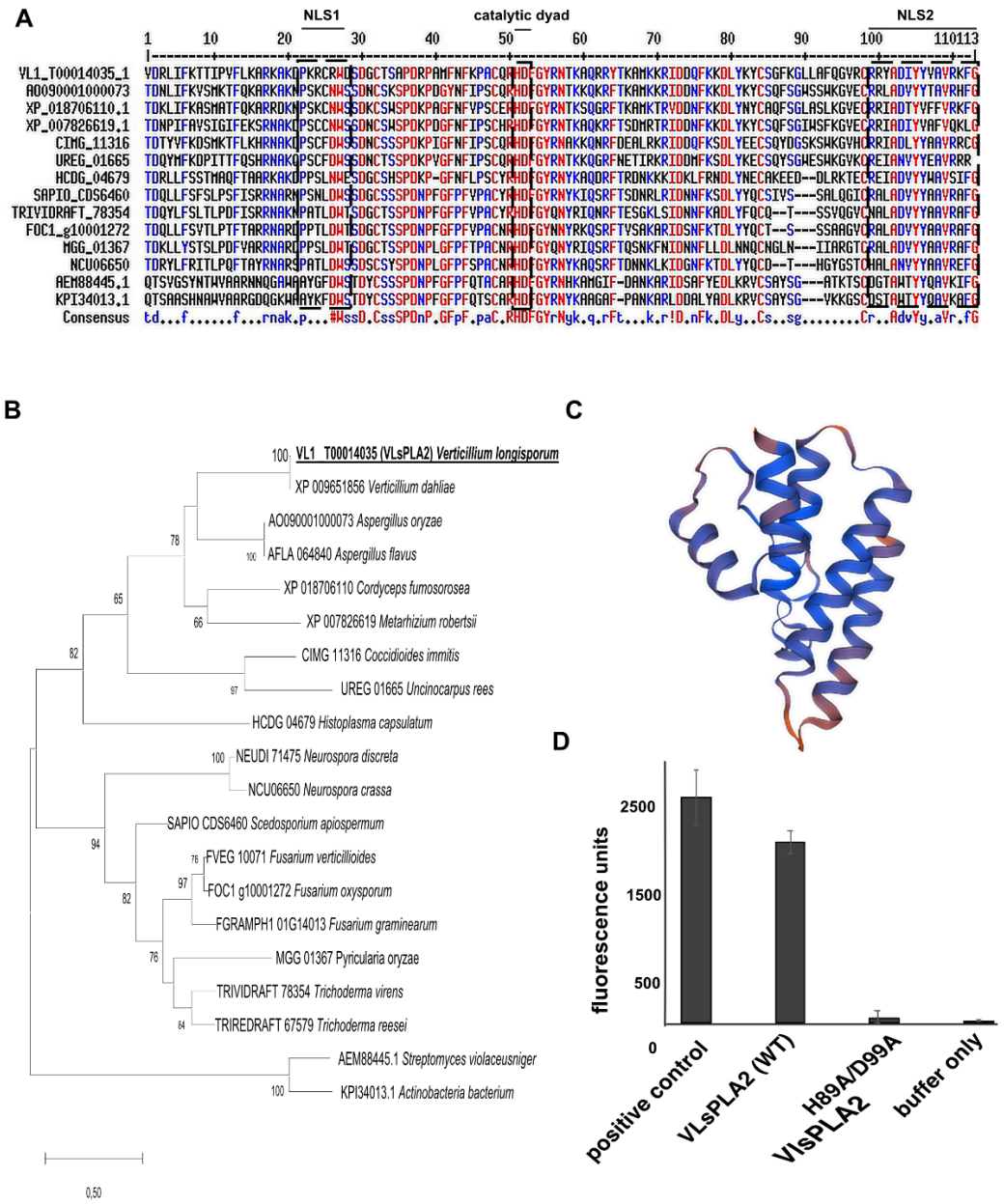

Figure 2. *Analysis of the $VlsPLA_2$ protein. (**A**) Sequence alignment of PLA_2 homologs derived from fungal and bacteria species. (**B**) Phylogeny of $VlsPLA_2$ and homologs derived from different fungal species. (**C**) Predicted 3D structure of the $VlsPLA_2$ protein. (**D**) Enzymatic activity of the WT and the truncated version (H89A/D99A) of the $VlsPLA_2$ protein. The protein was heterologously expressed in* E. coli *cells and used for enzymatic assays with the selective PLA_2 substance. PLA_2 derived from* Apis mellifera *venom was used as a positive control. For each treatment, eight technical replicates were used. Error bars show SE.*

$VlsPLA_2$ is initially localized to the host nuclei

To confirm the predicted subcellular localization of $VlsPLA_2$, a construct was made tagging the protein with GFP at the C-terminal, transiently expressed in *N. benthamiana* leaves, and monitored under confocal microscope 48 and 72hpi. Our observations showed that the protein was clearly localized to host nuclei 48hpi, while it was translocated to plant chloroplasts and cell periphery at 72 hpi (**Figure 3**). The nuclear localization of PLA_2 was previously observed in human endothelial cells, and in *Arabidopsis thaliana* plants (Grewal et al., 2002; Froidure et al., 2010). However, the exact role of PLA_2 nuclear localization still remains unknown. Peters-Golden et al., (1996) showed that the nuclear translocation of a cytosolic PLA_2 elicits localized hydrolysis of phospholipids in rat cells. We could speculate that $VlsPLA_2$ targets phospholipids in nuclear envelope and the products from this enzymatic function possibly are involved in signal transduction pathways.

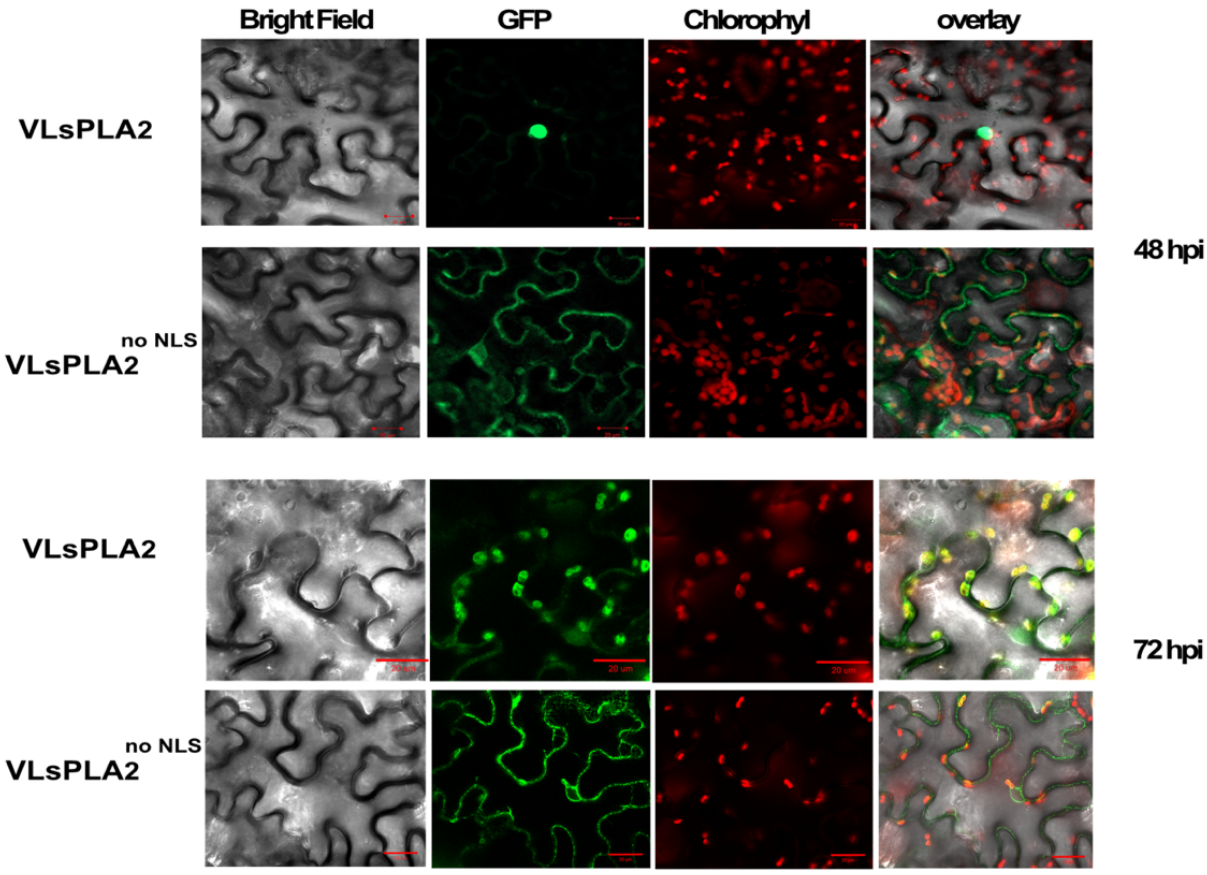

Figure 3. *Subcellular localization of C-terminal GFP-tagged* $VlsPLA_2$*, wild type, and NLS truncated versions at 48 and 72hpi. The localization was monitored with a laser-scanning confocal microscope with a sequential scanning mode. The GFP and the chlorophyll were excited at 488 nm. GFP (green) and chlorophyll (red) fluorescent signals were collected at 505– 525 and 680–700 nm, respectively.*

In order to investigate whether the predicted NLS were functional, a truncated version of $VlsPLA_2$ was constructed, where both NLS were disrupted. Confocal microscopy showed that this truncated version localized to cell periphery 48 hpi and translocated to chloroplasts at 72hpi, but no nuclear localization was observed in both time points

(**Figure 3**). Furthermore, we investigated whether both NLS are critical for nuclear localization and for that reason, truncated versions were constructed where either NLS1 or NLS2 were disrupted. Our analysis showed that both NLS were functional, while the bipartite NLS2 seemed to be more critical for nuclear localization (**Figure 4**). We also studied whether deletion of the signal peptide could affect $VlsPLA_2$ localization, thus a version lacking this signal was constructed. No changes in nuclear localization were observed between WT and non-SP version (**Figure 4**). We also investigated the localization of the non-enzymatically active $VlsPLA_2$ at 48hpi. Interestingly, we observed that $VlsPLA_2^{H98A/D99A}$ was not able to enter the nucleus and localized to the chloroplasts, although both NLS were intact, indicating that additional factors could be involved in its nuclear localization (**Figure 4**).

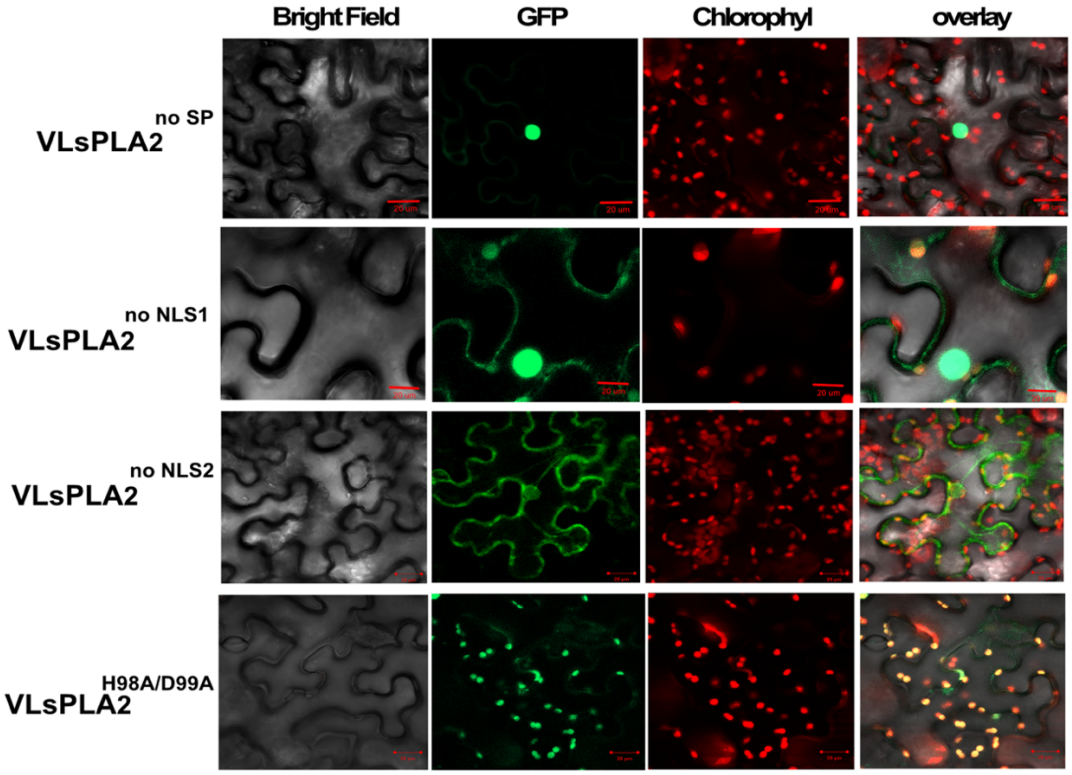

Figure 4. *Live cell imaging of C-terminal GFP-tagged truncated $VlsPLA_2$ protein without SP, disrupted NLS1, NLS2 or non-active PLA_2 catalytic domain at 48hpi. The localization was monitored with a laser-scanning confocal microscope with a sequential scanning mode. The GFP and the chlorophyll were excited at 488 nm. GFP (green) and chlorophyll (red) fluorescent signals were collected at 505– 525 and 680–700 nm, respectively.*

$VlsPLA_2$ suppresses PTI-related hypersensitive response

It is known that pathogens, especially necrotrophs, secrete a plethora of necrosis-inducing effectors (Lo Presti et al., 2015). Thus, we investigated whether $VlsPLA_2$ could have the same function. For that reason, $VlsPLA_2$ was transiently expressed in *N. benthamiana* plants and symptoms were monitored daily. No necrosis was observed even seven days after Agro-infiltration (data not shown). Moreover, many pathogens rely on an initial biotrophic stage to establish a successful infection by secreting HR suppressing effectors (Stergiopoulos and de Wit, 2009). Hence, the ability of $VlsPLA_2$ to suppress HR was investigated in the Cf4/Avr4 complex. The Cf4 is a Pattern Recognized Receptor (PRR) from tomato plants, recognized by the Avr4 effector produced by fungal pathogen *Cladosporium fulvum*, leading to PTI-related HR

(Joosten et al., 1997, van den Burg et al., 2006). Our results showed that in the area, where the VlsPLA$_2$ was previously Agro-infiltrated, a significant reduction of HR was observed, as compared to the mock inoculated area and the area where the empty-vector control was infiltrated (**Figure 5A**). In contrast, the enzymatically inactive version of VlsPLA$_2$ was not able to suppress HR (**Figure 5A).**

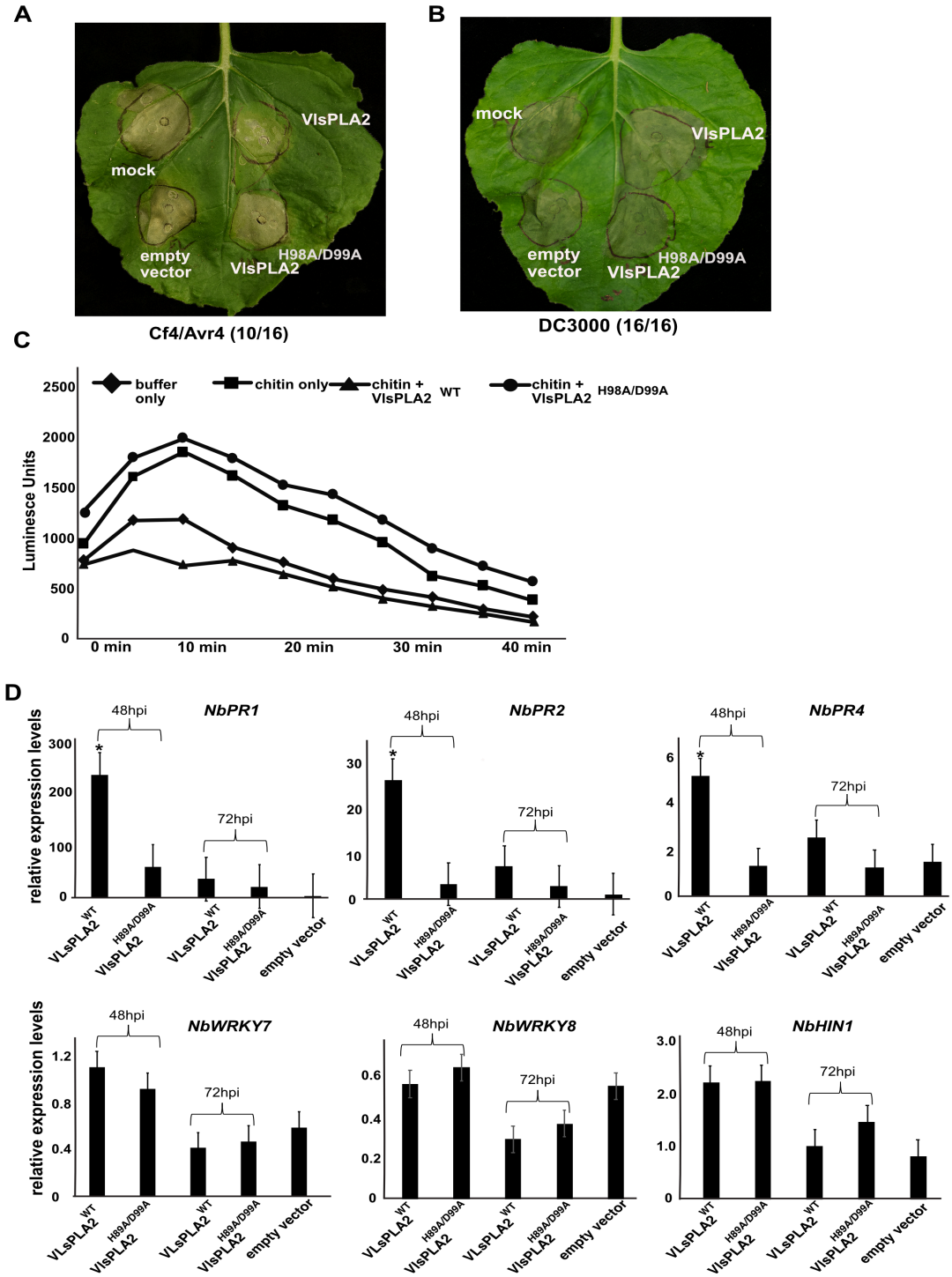

Figure 5. *HR suppression assays of the VlsPLA$_2$ effector. (**A**) Symptoms on transgenic N. benthamiana leaves expressing the tomato Cf-4 receptor. Leaves were Agro-infiltrated first with the VlsPLA$_2$ effector driven by the 35S promoter and HR challenged 24hpi with the Avr4 effector. (**B**) Symptoms on transgenic* N. benthamiana *leaves infected with Pseudomonas syringae pv tomato DC3000. Empty-vector, mock inoculation, and infiltration with the enzymatically inactive VlsPLA$_2$ (VlsPLA$_2^{H98A/D99A}$) were used as controls. Images taken 3dpi. (**C**) Chitin-induced oxidative*

(ROS) burst assay in N. benthamiana *leaves. Production of ROS was determined using luminol-dependent chemiluminescence. Leaf discs were treated with chitin, 10 μM VlsPLA$_2^{WT}$ and enzymatically inactive VlsPLA$_2$ (VlsPLA$_2^{H98A/D99A}$) protein, while only buffer was used as a negative control. Eight biological replicates were run in both assays. (**D**) Transcription analysis of N. benthamiana PR genes at 48 and 72 hpi after Agro-infiltration with VlsPLA$_2^{WT}$ and enzymatically inactive VlsPLA$_2$ (VlsPLA$_2^{H98A/D99A}$). Agro-infiltration with empty-vector was used as a negative control. The Actin (act) gene was used as an internal control. Asterisks (*) indicate statistically significant difference according to Student's T test as compared to empty-vector (p>0.05). For analysis, four biological replicates were used. Error bars show standard error (SE).*

In addition, we investigated whether VlsPLA$_2$ was able to suppress ETI-induced HR caused by *P. syringae* pv *tomato* DC3000. This bacterial strain secretes effectors to the host through type III secretion system recognized by the *N. benthamiana* R proteins, leading to a strong HR (Collmer et al., 2002). Our results showed that VlsPLA$_2$ was not able to suppress ETI-related HR, indicating that this effector is involved only to PTI responses (**Figure 5B**). It is known that ROS signaling plays a key role in HR (Zurbriggen et al., 2010). Thus, in order to investigate whether VlsPLA$_2$, could suppress ROS burst, a luminol-based protocol was used on *N. benthamiana* leaves. ROS burst was induced by chitin and production was significantly reduced on leaves which previously had been treated with the VlsPLA$_2^{WT}$ protein compared to non-treated leaves (**Figure 5C**). In contrast, no ROS suppression was observed on leaves treated with the enzymatically inactive VlsPLA$_2$ protein (VlsPLA$_2^{H98A/D99A}$), suggesting that suppression of HR is possibly attributed to the reduction of ROS burst (**Figure 5C**). Previously, it has been shown that a nuclear-localized PLA$_2$ in *A. thaliana,* interacts with the AtMYB30 transcription factor and negatively regulates HR (Froidure et al., 2010). Whether VlsPLA$_2$ also interacts with host transcription factors, leading to repression of basal immunity, remains to be elucidated.
Finally, in order to study whether VlsPLA$_2$ can induce defense responses, RT-qPCR was carried out and the expression levels of immunity marker genes. We found that the WT version of VlsPLA$_2$ could induce genes encoding PR proteins at 48hpi, while no induction was observed when plants were Agro-infiltrated with the enzymatically inactive VlsPLA$_2$ (**Figure 5C**). However, no induction was observed at 72hpi, suggesting that VlsPLA$_2$ is involved in initial stages of infection. In contrast, no upregulation of *WRKY* transcription factor genes was observed in all tested conditions (**Figure 5C**)).

Overexpression of VlsPLA$_2$ increases *V. longisporum* virulence in *B. napus plants*

In order to evaluate the potential involvement of VlsPLA$_2$ in *V. longisporum* virulence, we tried to construct a deletion strain. Interestingly, all attempts to delete this gene, either in *V. longisporum* or in *V. dahliae*, resulted in mutants carrying both, the deletion cassette and the WT gene (data not shown). Attempts to segregate the mutated nucleus via multiple single spore isolations, continuously failed. Instead, *VlsPLA$_2$* overexpression strains were constructed, driven by the constitutively expressed *gpdA* promoter. The transcription analysis of six single spore isolates, three overexpressing the WT (*VlsPLA$_2$+^{WT})* and three the enzymatically inactive gene (*VlsPLA$_2$+$^{H98A/H99A}$*), showed a significantly increase of *VlsPLA$_2$* transcription levels in comparison to WT (**Figure 6A**). Then, isolate number 3 from *VlsPLA$_2$+^{WT}* and isolate number 1 from *VlsPLA$_2$+$^{H98A/H99A}$* were chosen from further analysis, since they showed similar expression levels (**Figure 6A**). First, the mycelial growth of overexpression strains was investigated on PDA plates. We found that the *VlsPLA$_2$+^{WT}* strain showed a slower mycelial growth as compared to the *VlsPLA$_2$+$^{H98A/H99A}$* one (**Figure 6B**). These results

are in agreement with previous studies in *Aspergillus oryzae*, where the overexpression strains showed a slightly slower growth rate, and a reduced condition rate, indicating a role of this enzyme in fungal physiology (Nakahama et al., 2010).

Furthermore, the virulence of the *VlsPLA*$_2$ overexpression strains was investigated on *B. napus* plants under greenhouse conditions. Plants were inoculated with *V. longisporum* overexpression strains and plant growth was monitored 25 dpi. We observed that plants infected with the *VlsPLA*$_2$+^{WT} strain showed to be more stunted as compared to plants infected with the *VlsPLA*$_2$+$^{H98A/H99A}$ one (**Figure 6C**). Similarly, biomass (fresh and dry) of *VlsPLA*$_2$+^{WT} infected plants was lower as compared to the *VlsPLA*$_2$+$^{H98A/H99A}$ infected ones (**Figure 6D).** Similar results were observed in *A. thaliana* plants (data not shown), indicating that this protein is a virulence factor. Previously, the role of these enzymes has been described in bacterial pathogenicity, either directly by degradation of the phospholipid components, and interference in signal transduction, or indirectly such as promotion of inflammation (Nilius & Malfertheiner, 1996, Schmiel et al., 1998, Flieger et al., 2000). Possibly, VlsPLA$_2$ has similar function during interaction with plant hosts.

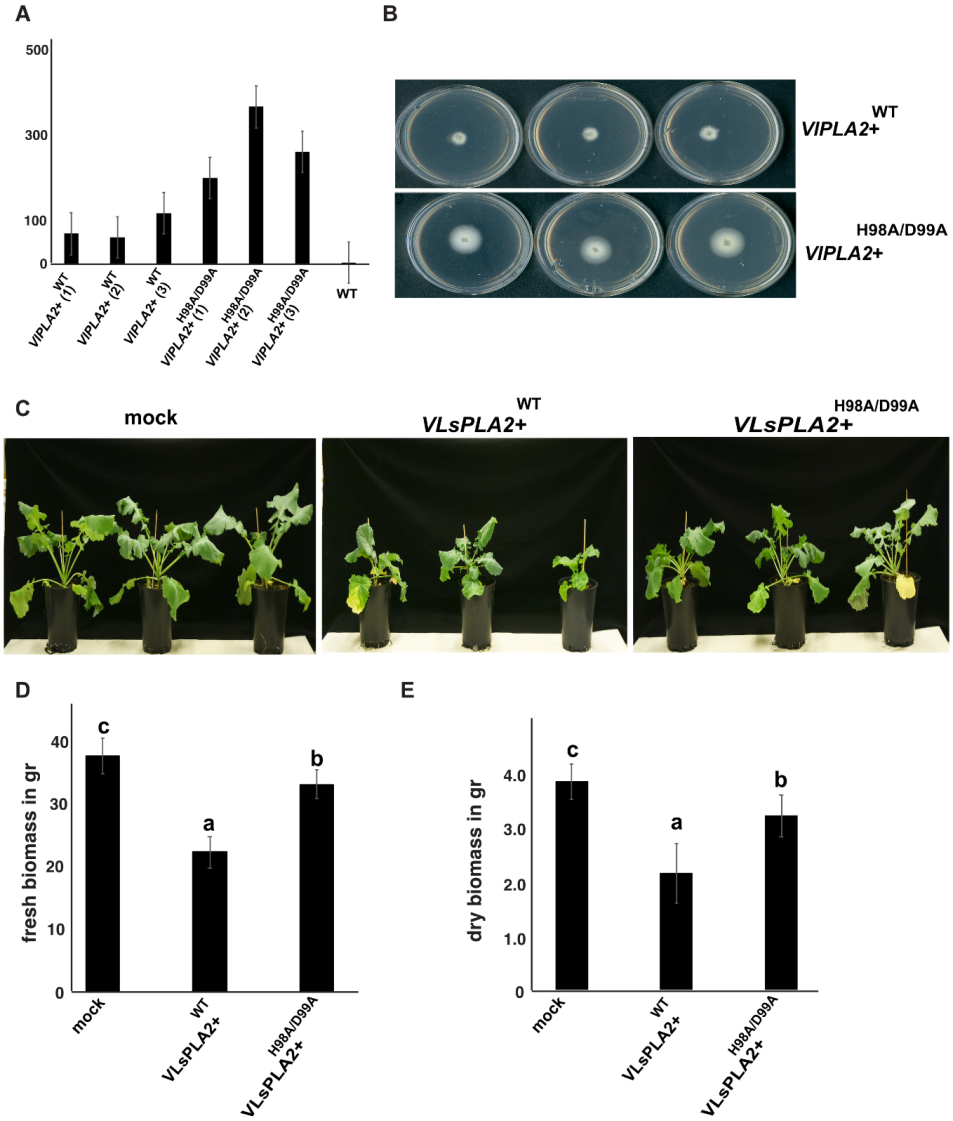

Figure 6. *Overexpression of* VlsPLA$_2$ *promotes* V. longisporum *virulence. (**A**) Overexpression levels of the* V. longisporum *WT (VlsPLA*$_2$+^{WT}*) and non-enzymatically-active version*

(VlsPLA$_2$+$^{H98A/H99A}$) of the VlsPLA$_2$ gene in single spore isolates as compared to WT control. The GAPDH *gene was used as the internal control for the analysis of gene expression. Error bars represent SE based on three technical replicates.* ***(B)*** *Mycelial growth of overexpression* VlsPLA$_2$+^{WT} *and* VlsPLA$_2$+$^{H98A/H99A}$ *strains on PDA medium five dpi.* ***(C)*** *Symptoms of representative plants infected with* VlsPLA$_2$+^{WT} *or* VlsPLA$_2$+$^{H98A/H99A}$*, while mock-inoculated plants were used as a negative control 25dpi.* ***(D)*** *Fresh and dry biomass of B. napus plants infected with* VlsPLA2+^{WT} *and* VlsPLA$_2$+$^{H98A/H99A}$*, while mock-inoculated plants were used as a control. Error bars represent SE. Data shown are the average of four biological replicates and each one includes eight plants. Different letters (a, b and c) show statistically significant differences according to Student's T test (p > 0.05).*

Conclusions

Phospholipids are the main components of cell membranes and phospholipases are the enzymes responsible for phospholipids degradation by cleaving the ester bonds. Their enzymatic function seems to play a crucial role in host penetration and signaling (Barrettbee et al., 1985, Cox et al., 2001). The current study is the first report of a PLA$_2$ enzyme, being involved in fungal virulence against plant host. Although further studies are needed to elucidate the precise role of *VlsPLA$_2$* in *V. longisporum* infection biology, we propose that this effector targets host nuclei. Then, by degrading phospholipids in the nuclear envelope and releasing arachidonic and lysophosphatidic acid, or by interaction with transcription factors, it induces signal transduction, altering the gene expression involved in initial plant immunity such as PTI-related HR **(Figure 7**).

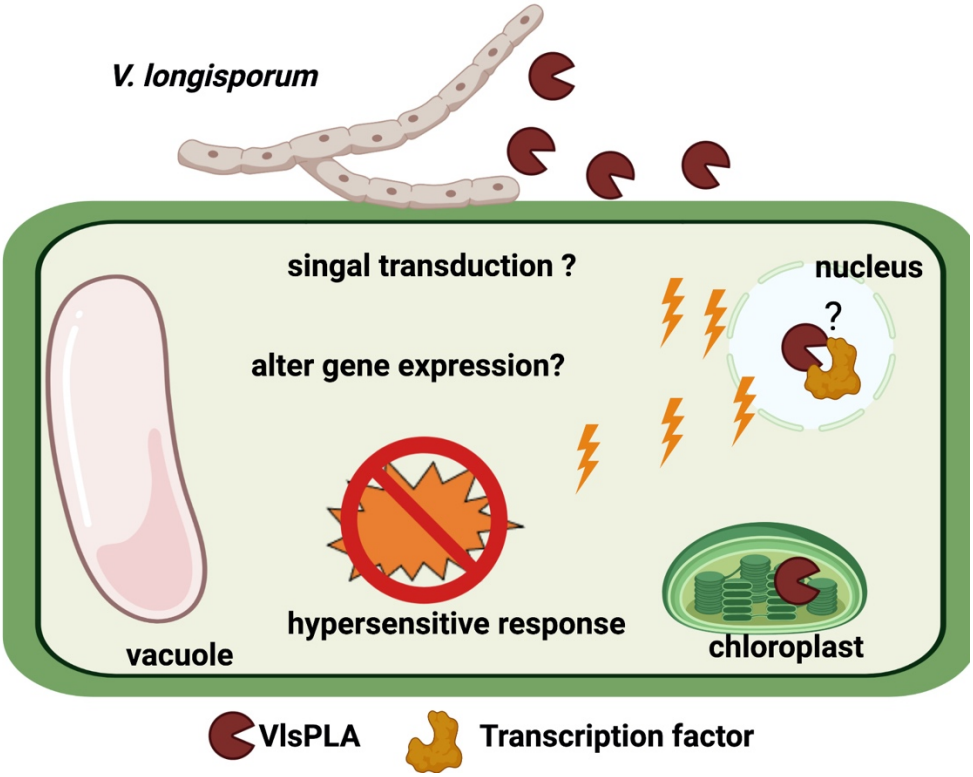

Figure 7. *Proposed function of the VlsPLA$_2$ effector in* V. longisporum *virulence. VlsPLA$_2$ is localized initially in host nuclei and then translocated mainly to the chloroplasts. VlsPLA$_2$ induces signal transduction and possibly alter the gene expression in host cells, either through degradation phospholipids in nuclear envelope, or through interaction with transcription factors.*

Conflict of interest

The authors declare no conflict of interest.

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