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Investigating the role of a putative endolysin-like candidate effector protein in *Verticillium longisporum* virulence

Vahideh Rafiei, Yasaman Najafi, Heriberto Vélëz, Georgios Tzelepis*

Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala Biocenter, Box 7026, SE-750 07, Uppsala, Sweden

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ABSTRACT

Verticillium is a genus of ascomycete fungi that encompasses several plant pathogenic species, and cause severe annual yield losses on many economically important crops worldwide. One of the most important species in this genus, is *V. longisporum*, which causes disease mainly on plants in the Brassicaceae family. Genome analysis of *V. longisporum* strain VL1 revealed a number of candidate effector genes that may be associated with fungal virulence. One of these candidate effector-genes encodes a putative endolysin-like protein. Endolysins are hydrolytic enzymes that are secreted by bacteriophages and recently, they have been identified in fungal genomes as well. In this study, the potential role of this gene has been investigated in *V. longisporum*. Our data showed that this gene was highly induced in the fungus during *Brassica napus* infection and its overexpression significantly increased *V. longisporum* virulence, indicating an involvement in the fungal infection process.

1. Introduction

Verticillium is a genus of ascomycete fungi, which encompasses several plant pathogenic species. The most important species of this genus are *Verticillium dahliae*, *V. albo-atrum* and *V. longisporum*, which are soilborne pathogens, causing severe annual yield losses on many economically important crops across the world [1]. The long-term survival of their resting structures in soil, their broad host range, and their fungicide resistance make them very difficult to be controled [2,3]. Symptoms of Verticillium disease are dependent on the host, the species, and the environmental conditions. Some *Verticillium* species cause wilting and browning in many plant species, while others may cause chlorosis and necrosis [1]. *Verticillium dahliae*, is the most important species in this genus, causing vascular wilt disease on more than 200 dicotyledonous plant species worldwide [1]. A new species of *Verticillium* species that showed a preference to Brassicaceae plants was first reported in southern Sweden, and it is the dominant one in the fields, where oilseed rape is cultivated [4–6]. It produced microsclerotia similar to *V. dahliae*, but its conidia were significantly longer and thus, it was named *Verticillium longisporum* [7,8]. Interestingly, it is the only non-haploid species in *Verticillium* genus, as a result of hybridization events between *V. dahliae* and another unknown haploid ancestor [9–11].

Plant cell walls are composed of different polysaccharides (e.g., cellulose, hemicellulose, and pectin). Therefore, fungi need to secret a plethora of molecules and hydrolytic enzymes to break down polysaccharides and carbohydrates to pass the plant cell wall and enter the host cells [12]. Most phytopathogenic fungi produce various types of cell wall degrading enzymes (CWDEs), which facilitate infection and colonization. Glycoside hydrolases (GHs) are one of the most important characterized enzymes which hydrolyse glycosidic bonds and consist of different protein family groups in the carbohydrate activity enzymes (CAZymes) database. Furthermore, some of the CWDEs that belong to different GH families act as pathogen-associated molecular patterns (PAMPs), which in turn trigger plant immune responses [13].

General molecules (i.e., microbe- or pathogen-associated molecular patterns, MAMPs or PAMPs, respectively) are sensed by plants through plant recognition receptors (PRRs) that trigger immediate defense responses known as PAMP triggered immunity (PTI). Well-known PAMPs are chitin and flagellin, which are components of fungal cell walls and bacterial cells respectively. PTI mechanisms aim to prevent further colonization of the host by the pathogen [14]. In order to overcome PTI, pathogens secreted specific molecules known as effectors, which can be recognized by the plant resistance (R) proteins, leading to the induction of effector triggered immunity (ETI) [14]. In recent years, significant progress

* Corresponding author.
E-mail address: Georgios.Tzelepis@slu.se (G. Tzelepis).

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has been made towards understanding the plant-fungal interactions. However, many key aspects about fungal virulence and defense response mechanisms in plants still remain to be elucidated.

Recently, putative endolysin-like proteins have been identified in fungal genomes. However, their role in the fungal infection process is completely unknown. In this study, the potential role of the *VL1_T00003720* (denominated as *Vl3720*) gene, encoding a putative endolysin-like protein, has been investigated in the soil-borne plant pathogen *V. longisporum,* using different molecular approaches, such as RT-qPCR, construction of overexpression mutants, and infection assays. Our results showed that this protein is potentially involved in fungal virulence, helping us to understand better the role of these lytic enzymes in fungal infection biology.

2. Material and methods

2.1. Fungal isolate and inoculum preparation

The *Verticillium longisporum* strain VL1 was used for inoculation. This strain has been isolated from Swedish soils, where rapeseed has intensively been cultivated [9]. To produce conidia, the fungal mycelia were streaked on potato-dextrose agar (PDA) medium at 20 °C in darkness for one week. PDA plates were inoculated from stock cultures preserved in 10% (w/v) glycerol at −80 °C.

2.2. Protein structure and phylogeny

The domain structure of the Vl3720 candidate effector was investigated using the SMART prediction tool [15]. In order to predict the localization of this effector in host cells the ApoplastP and LOCALIZER software were used [16,17]. The phylogenetic analysis was inferred by using the Maximum Likelihood method and JTT matrix-based model [18]. In total, 74 amino acid sequences were involved, including putative endolysins derived from fungi, bacteria and bacteriophages were aligned with Clustal W [19]. Analysis was conducted using the MEGA X software [20]. Bootstrap analysis was performed using 1000 replicates.

2.3. Plant inoculation and gene expression analysis

For the gene expression analysis, the infection process as described previously was used [21]. Briefly, seeds of the rapeseed cultivar “Hannah” were surface sterilized in 70% Ethanol and 10% bleach and placed in ½ Murashige and Skoog medium (MS) supplemented with 1% sucrose. Seeds were grown for approximately one week in the growth chamber at 22 °C with a 16-hr photoperiod. The seedlings were removed and inoculated by dipping the roots in a 10^6 conidia suspension, while mock-inoculated plants were dipped in autoclaved deionized H_2O for 10 min, and then transferred to ½ MS media without sucrose. Inoculated roots were harvested at different time points (e.g., 2-, 4-, 6-, 8- and 10-days after inoculation), immediately frozen in liquid nitrogen and stored at −70 °C.

Frozen mycelia were homogenized using pestle and mortar with liquid nitrogen, and total RNA was extracted using the Plant Total RNA kit (Sigma-Aldrich), according to manufacturer's instructions. RNA concentration was determined spectrophotometrically using a NanoDrop (Thermo Scientific), while RNA integrity was analysed after DNase I treatment by electrophoresis on an Agilent Bioanalyzer, using the RNA 6000 Nano kit (Agilent Technologies). For cDNA synthesis, 1000 ng of total RNA, after DNase I treatment (Fermentas, St-Leon-Rot, Germany), was reversed transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using oligo(dT)18 and random hexamer primers in a total volume of 20 μl, followed by 10-fold dilution of the cDNA and stored in −20 °C. The following protocol was used for reverse transcription: 5 min at 25 °C, 30 min at 42 °C followed by enzyme inactivation at 85 °C for 5 min.

Transcript levels were quantified by reverse transcriptase qPCR (RT-qPCR) in an Thermal Cycler, CFX96 Dx Real-Time PCR detection system (Bio-Rad), using the following primers; (Vl3720_F: 5′-ACCGAATGTAAACGCCGCCA-3′ and Vl3720_R: 5′- CGGGATAGCGGGATGGGGTA-3′), while primer amplification efficacy was deducted from amplification of standard curves using serial dilutions of *V. longisporum* genomic DNA. Expression of the *Vl3720* gene was normalized by *VlGAPDH* [22], and relative expression values were calculated according to the $2^{-\Delta\Delta Ct}$ method [23]. This analysis was conducted in at least four biological replicates each based on two technical ones.

2.4. Construction of the Vl3720 overexpression vector, fungal transformation and mutants' validation

The overexpression vector was generated using GeneArt Seamless cloning technology (Invitrogen). For this purpose, the *Vl3720* gene (from the fungal cDNA) and the pRFHUE-eGFP vector [24], driven by the *gpdA* constitutively expressed promoter, were amplified using the following primers (Vl3720_Forward OE: 5′-CATCACCCATGGCCAGATTCAACTT-3′, Vl3720_Reverse OE: 5′-TCAGCCCCTCATGAGCACGCCGG-3′ and pRHUE_Forward: 5′-GCTCATGAGGGGCTGAGGACTTA-3′, pRHUE_Reverse: 5′-CTGGCCATGGGTGATGTCTGCTC-3′, respectively), designed by the GeneArt software (Invitrogen). PCR reactions were carried out using the Phusion Green Hot Start II High-Fidelity PCR master mix (Thermo Scientific), and the correctly assembled vectors were confirmed by restriction digestion analysis and by Sanger sequencing (Macrogen), followed by transformation to the AGL-1 competent *Agrobacterium tumefaciens* cells.

Verticillium longisporum was transformed using the *Agrobacterium*-mediated protocol as described previously [25]. Briefly, *A. tumefaciens* colonies carrying the *Vl3720+_ pRFHUE* vector were used to inoculate 10 ml liquid LB medium and incubated at 28 °C until $OD_{600} = 0.5–0.9$. The bacteria were harvested by centrifugation and the pellet was washed twice with liquid IM (800 μl of 1.25 M potassium phosphate buffer, 20 ml MN buffer, 1 ml of 10 mg ml^{-1} $CaCl_2$, 1 ml of 1 mg ml^{-1} $FeSO_4$, 5 ml IM-salts, 2 ml of 200 mg ml^{-1} NH_4NO_3, 10 ml of 50% glycerol, 40 ml of 1 M MES and 1 ml (liquid medium) or 5 ml (solid medium) of 200 mg ml^{-1} glucose). The washed cell pellet was resuspended in liquid IM, and the OD_{600} was adjusted to 0.15–0.2. The bacterial culture was incubated until it reached an $OD_{600} = 0.3–0.4$, and then was mixed in equal amount of freshly harvested *V. longisporum* conidia (10^7 spores ml^{-1}). Then, bacterial/spore mixture was spread on sterilized cellophane membranes, on IM agar plates and incubated at 23 °C for 60 h, before the cellophane was transferred to PDA agar plates containing 400 μg/ml cefotaxime and 50 μg/ml hygromycin. Hygromycin-resistant fungal colonies were selected on PDA plates, containing 50 μg/ml hygromycin, for further analysis. The expression levels of the *Vl3720* gene were analysed in eight hygromycin-resistant fungal colonies, by RT-qPCR as described above. Single spore cultures were performed from the five colonies with the highest expression and used for the virulence assays.

2.5. Virulence assays

For the virulence assays, the model plant species *Arabidopsis thaliana* was used. Seeds were surface sterilized and grown in vitro on ½ MS for two weeks as described above. Then, plants were infected with *V. longisporum* WT and overexpression (*Vl3720*$^+$) strains as described above, while mock inoculation was conducted

by dipping the roots in distilled H_2O for 30 min. The plants were transferred to peat soil and grown in short-day conditions (8 h light, 16 h dark) at 23 °C/18 °C for four weeks and watered regularly. Plant symptoms were evaluated using a disease index scale ranging from 1 to 4; 1: healthy, 2: mild symptoms, 3: severe symptoms and 4: dead plants. The rosette diameter also was measured.

2.6. Statistical analysis

Analysis of variance (ANOVA, one way) was conducted on gene expression and phenotypic data using a General Linear Model implemented in SPSS version 28 (IBM). Pairwise comparisons were performed using the Student's T test at the 95% significance level.

3. Results and discussion

3.1. The Vl3720 gene encodes a putative endolysin-like protein

Genome analysis of the *V. longisporum* VL1 strain, showed that contained more than 80 genes, putatively encoded effector proteins [26]. Among them, the Vl3720 protein, which is composed of 187 amino acids, was analysed using the SMART tool application, and it showed that this protein contains an endoplasmic reticulum (ER) signal peptide that expands from amino acids one to 21 and a lysozyme-like domain from amino acids 53 to 169. This would suggest that the Vl3720 protein is targeted to the ER and is a putative endolysin protein. In order to predict the localization of this candidate effector in plant cells, the LOCALIZER and ApoplastP software were used. Our analysis did not show any peptide to indicate subcellular localization, while it was predicted to be putatively localized to the plant apoplast. Further, blast searches at the NCBI server to identified similar proteins, found homologues in the other *Verticillium* spp., as well as in other fungi, bacteria, and viruses. Similar to other endolysins, the catalytic residues of the Vl3720 protein are made up of amino acids Glu, Asp, and Thr and thus, the endolysin-like protein from *V. longisporum* most likely belongs to the GH family 24 or 25. Some of these sequences were included in a phylogenic analysis, which showed that all the endolysin-like proteins from the *Verticillium* spp. clustered together and with other fungi (Fig. 1). Some of the bacteriophage lysozyme sequences were also found close to the fungal cluster (Fig. 1, Fig. S1).

It is tempting to speculate that fungi acquired these through horizontal gene transfer (HGT). For example, when the apple-canker causing fungus, *Valsa mali*, was investigated for HGT events, the phylogenetic analyses revealed 32 HGT events in *V. mali,* most of which were from bacteria. Some of these HGTs putatively played roles in competition with actinomycetes and in nitrogen uptake. Moreover, it was reported that 10 HGTs were potentially involved in pathogenicity, since they were related to known virulence factors (e.g., cell wall-degrading enzymes and candidate effector proteins), suggesting that HGTs has a significant role in evolution and virulence of *V. mali* [27].

Lysins consist of different domains including a cell-wall binding domain and a catalytic domain, and are usually greater than 25 kDa in size [28,29]. In bacteriophages, endolysins are hydrolytic enzymes produced at the latest stages of their replication cycle that degrade the bacterial cell walls from within and thus, play a crucial role in the release of the newly formed bacteriophages. Bacteria produce lysins to grow and reshape the peptidoglycan layer as part of their normal growth processes. However, some bacteria also produce “exo” lysins, which are used to degrade the cell wall of other bacteria that occupy the same niche [28]. For this reason, endolysins have been considered as potential antibacterial drugs and could be potentially used as biocontrol agents [28,30]. Some endolysin-like proteins have been reported in different fungal species, some which are known to be pathogenic, but their function in biology and pathogenicity of fungal species are unknown. However, evidence is emerging where epiphytic basidiomycete yeasts produce lysins against other microorganisms that occupy the phyllosphere in *A. thaliana* [31].

3.2. The Vl3720 gene is highly induced upon host infection

To further characterize the *Vl3720* gene, transcription analysis was conducted, upon host infection. Rapeseed plants were used and seedlings were grown for approximately one week before

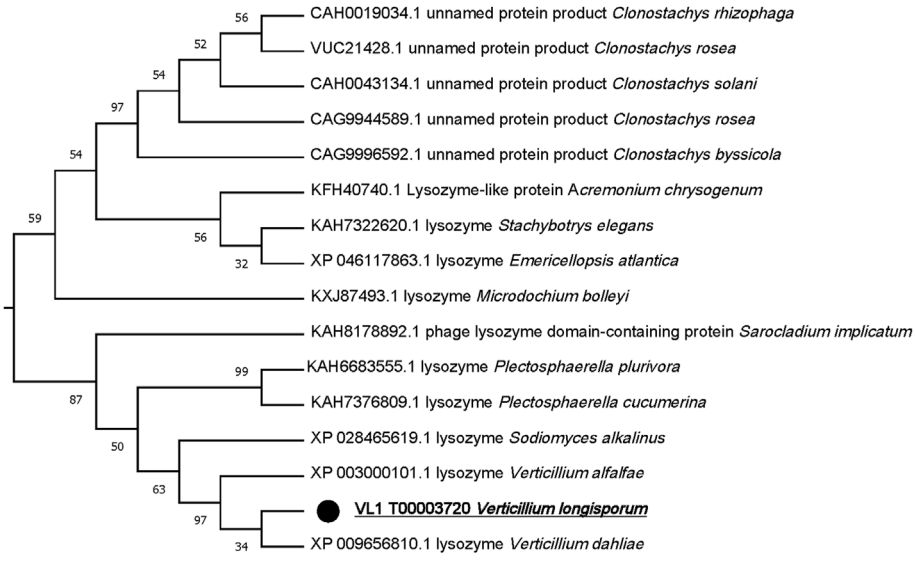

Fig. 1. Phylogenetic analysis of putative endolysin proteins from different fungal species. Analysis was conducted using the maximum likelihood based on amino acid sequences and 1000 bootstraps using the JTT matrix-based model. Predicted amino acid sequences were aligned using the Clustal W algorithm and phylogeny was constructed in the MEGA X software.

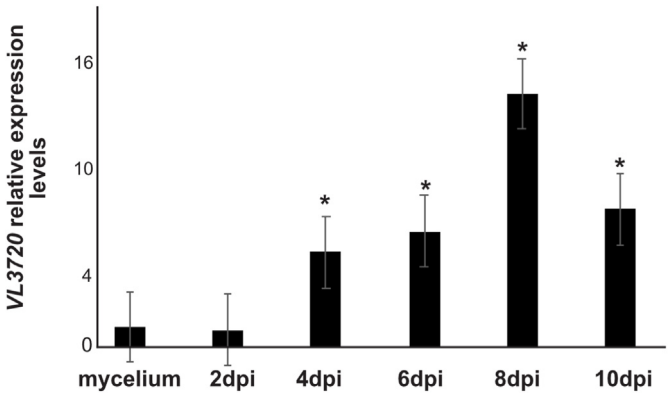

Fig. 2. Transcription analysis of the *Verticillium longisporum VL3720* gene during interaction with *Brassica napus* seedlings. Mycelia of *V. longisporum* grown on PDB media were used as control. *V. longisporum GAPDH* was used as a reference gene. Error bars show standard errors (SE). Asterisks (*) indicate statistically significant differences ($p < 0.05$), between mycelium and infection stages, according to the Student's T test.

infecting with *V. longisporum* conidia. Inoculated roots were harvested at different time points (e.g., 2-, 4-, 6-, 8- and 10-days post inoculation), and the transcription patterns of this gene were investigated using RT-qPCR techniques. Our data showed that the expression levels of *Vl3720* was induced after four days-post inoculation ($p = 0.0049$), as compared to the mycelia used as a control, and the highest expression level was observed eight days post inoculation, indicating an involvement of this gene in fungal virulence (Fig. 2).

3.3. Overexpression of the Vl370 gene increases V. longisporum virulence

As it was mentioned above, the *V. longisporum* genome is the only diploid one in this genus, and it is not amenable to gene deletion. Thus, overexpression of the target gene is an appropriate method to study its potential function. To create an overexpression mutant, the *VL3720* gene was amplified from *V. longisporum* cDNA, and ligated to the pRFHUE-eGFP vector and transformed into *V. longisporum.* For validation of the overexpression mutants, eight hygromycin-resistant colonies were selected and RT-qPCR was conducted. As shown in Fig. 3a, five hygromycin-resistant colonies showed significantly high expression of the *VL3720* gene as compared with WT mycelia, confirming that the overexpression vector was successfully transformed into *V. longisporum.* Although, the morphology of overexpression mutants was further investigated on PDA, no significant macroscopic differences in morphology between wild type-VL1 (WT-VL1) and the VL3720^{+} mutants were observed (data not shown).

The *VL3720*$^{+}$ mutants were used to conduct virulence assays using the plant species *A. thaliana*. Seeds were grown on ½ MS for two weeks, and then, plants were infected with *V. longisporum* WT and *VL1370*$^{+}$, derived from single spore colonies (mutant 2 and 7) (Fig. 3b), as described above, while mock inoculation was conducted by dipping the roots in distilled H_2O. After four weeks, plant symptoms were evaluated using and the rosette diameter was also measured. Our results showed the infected plants displayed clear chlorotic and stunting symptoms as compared to the mock-inoculated ones (Fig. 4a). In addition, the plants infected with the *VL3720*$^{+}$ mutants displayed more severe symptoms, as compared to the *V. longisporum* WT-infected ones ($p = 0.021$) (Fig. 4a and b),

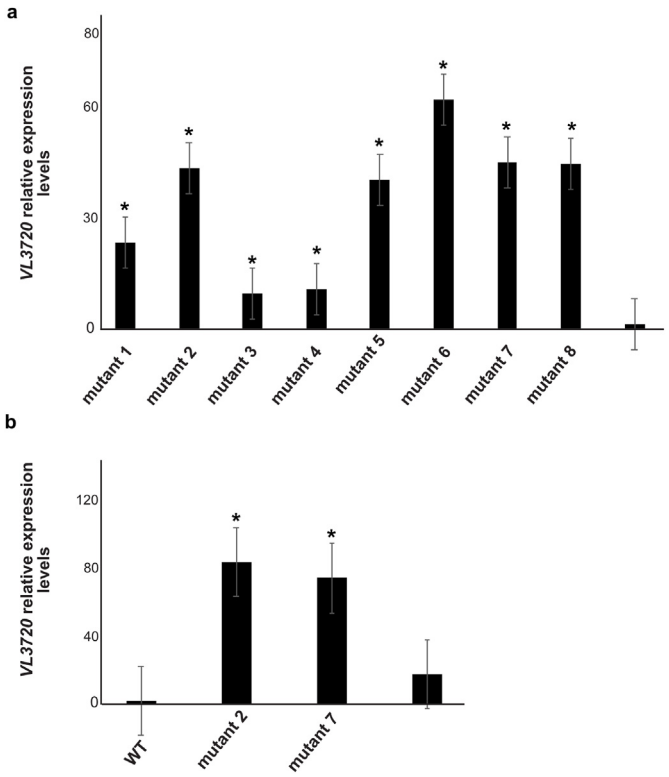

Fig. 3. Transcription analysis of the *VL3720 V. longisporum* overexpression mutant strains. a) Independent overexpression mutant strains and b) single spore overexpression strains derived from mutant 6. *V. longisporum* reference gene *GAPDH* was used as a reference gene. Mycelia of *V. longisporum* WT were used as control. Asterisks (*) indicate statistically significant differences ($p < 0.05$), between mycelium and infection stages, according to the Student's T test ($p < 0.05$). Error bars show standard errors (SE).

indicating that this protein has a prominent role in *V. longisporum* virulence. Finally, the rosette growth was measured, and no statistically significant differences were observed between WT and *VL3720*$^{+}$ inoculated plants (Fig. 4c).

4. Conclusions

In conclusion, a candidate effector protein and its role in *V. longisporum*, was investigated during this research. The Vl3720 protein belongs to glycoside hydrolase family 24 or family 25, and shows a high similarity to lysozymes encoded by bacteriophages, bacteria, and fungi. Although the role of Vl3720 as an endolysin-like protein in *V. longisporum* is not clear, pathogenicity results in this study showed that inoculation of overexpression mutants in *A. thaliana*-seedlings, could result in significant disease symptoms, as compared to the mock and WT-inoculated seedlings, indicating that this gene could have a role in *V. longisporum* virulence. To our best of our knowledge this is the first study showed the role of endolysins in fungal infection biology. The results from this study could contribute towards understanding the role of these lytic enzymes in host-microbe interactions.

Declaration of competing interest

The authors declare that they have no conflict of interest.

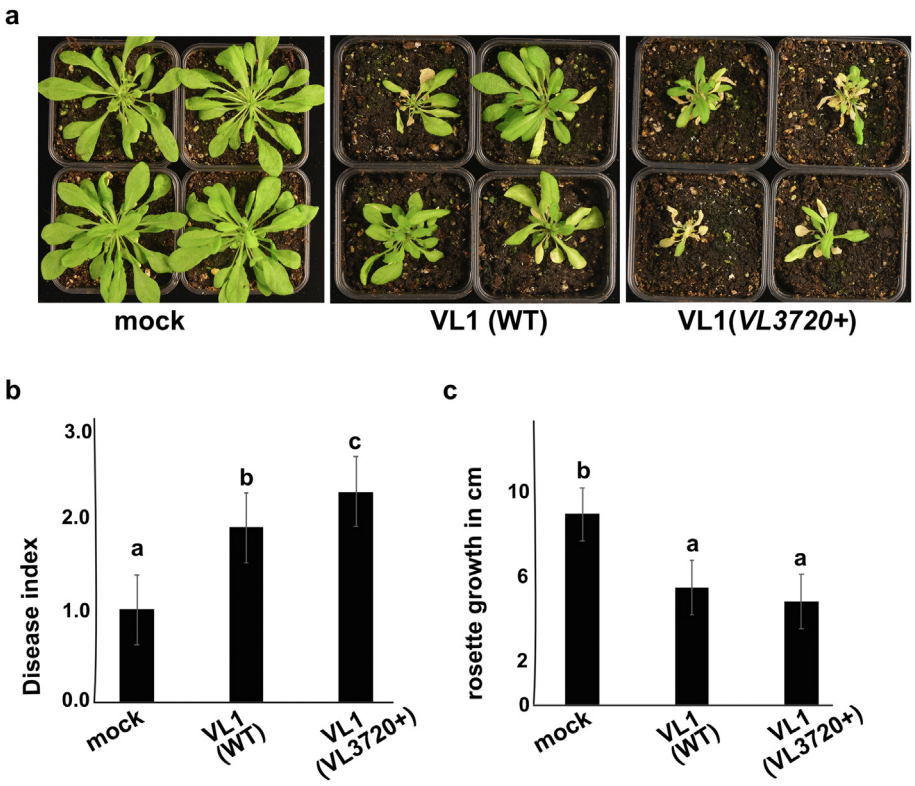

Fig. 4. Virulence assays in *Arabidopsis thaliana* plants. a) symptoms in representative plants infected with *V. longisporum* VL1 wild type (WT) and VL3720 overexpression (*VL3720*+) strains four weeks after inoculation. b) Disease index in plants infected with *V. longisporum* WT and *VL3720*+ strains. Disease index was estimated using a scale between 1 and 4 where 1: healthy plants and 4: dead plants. c) Rosette growth in cm in plants infected with *V. longisporum* WT and *VL3720*+ strains. Mock inoculated plants were used as a negative control. In total 18 plants were used per treatment. Error bars represent standard error (SE). Different letters (a, b, and c) represent statistically significant differences according to the Students T test ($p < 0.05$).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.08.086.

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