Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources
Department of Plant Protection

THESIS SUMMARY

Identification and Molecular Characterization of the Putative Immunophilins (IMMs) in the Oilseed Rape Pathogens *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, and *Plasmodiophora brassicae*

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Defence of thesis will be taken place on 29th of August 2016 at……..
Faculty of Agrobiology, Food and Natural Resources
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1. Introduction

Brassica is one of the most economically important oil crops in the world. However, the crop has been challenged by several pathogens including fungi *Leptosphaeria maculans*, *L. biglobosa*, and protist *Plasmodiophora brassicae*. Phoma stem canker (blackleg) caused by fungus species complex *L. maculans* and *L. biglobosa* and "clubroot" caused by *P. brassicae* are diseases of concern throughout the world on oilseed rape, and cause serious losses for crops globally.

The Immunophilins (IMM) class of proteins are highly conserved in all known eukaryotes and prokaryotes and involved in a variety of cellular functions. The family comprises three unrelated sub-families: the cyclophilins (CYPs), the FK506-binding proteins (FKBPs), and the parvulin-like proteins (PARs). All three families bind to immunosuppressant molecule of fungal origin. The cyclophilins bind to cyclosporin A (CsA), the FK506-binding proteins bind macrolides FK506/rapamycin and parvulins bind to juglone.

Biochemical and sequence analyses following genome analysis have led to the identification of several members of IMMs in various organisms. However, in phytopathogens the number of IMM family is poorly understood due to unavailability of sequenced genomes. IMMs are present in all cellular compartments involved in processes including protein trafficking and maturation, apoptosis, receptor signaling, RNA processing, miRNA activity and RISC assembly.

In spite of the fact that IMMs are highly conserved in all organisms, the family is not well studied in phytopathogens. Recent studies in some phytopathogens including *Magnaporthe oryzae*, *Botrytis cinerea*, *Cryphonectria parasitica* and *Phellinus sulphurascens* demonstrated the role of cyclophilins as a pathogenicity factor. While in others, such as *Phytophthora* the high expression of cyclophilins was monitored during disease infection. However, it is still intriguing to decipher the perplexing functions of IMMs in other distinctive and emerging plant pathogens of economic importance.

Consequently, characterizing IMMs in phytopathogens may provide more insight into their roles and functions towards pathogenicity and the infection process of yet not completely understood host-pathogen interactions. Therefore, in the present study, we exploited the putative IMM proteins in *L. maculans*, *L. biglobosa* and *P. brassicae* with the following objectives described below.

2. Hypotheses, aims and objectives

Hypotheses

Immunophilins proteins including cycolphilin, FKBPs and parvulins are highly conserved in oilseed rape pathogens *Leptosphaeria maculans* and *L. biglobosa* and *Plasmodiophora brassicae*. Cyclophilins play important role in the pathogenicity of these phytopathogens.

Aims and objectives

Leptosphaeria maculans* and *L. biglobosa

A. Characterization of various *L. maculans* and *L. biglobosa* isolates in the Czech Republic

B. Detailed *in silico* (Bioinformatics) analysis of putative immunophilins (IMMs) proteins in the genome sequence of *L. maculans* and *L. biglobosa*.

C. Functional characterization of *L. maculans* and *L. biglobosa* cyclophilin A (*CYP4*)

Plasmodiophora brassicae

A. Detailed *in silico* (bioinformatics) analysis of putative IMMs proteins in the genome sequence of *P. brassicae.*

B. Deciphering the role of cyclophilin A (*PbCYP3*) in *P. brassicae* with the following objectives.

3. Materials and Methods

3.1.1 Fungal isolates

Isolate name	Species	Location of collection	Country of Origin	IBCN No.	*B. napus* cv.	References
LmT1/13	*L. maculans*	TURSKO	CZ	-	Unknown	Present study
LmVr3/13	"	VELVARY	"	-	"	"
LmV1/12	"	JIHLAVA	"	-	"	"
LmU7/13	"	UNĚTICE	"	-	"	"
LmU3/13	"	UNĚTICE	"	-	"	"
LmU1/13	"	UNĚTICE	"	-	"	"
LmS5/11	"	STANKOV	"	-	"	"
T12aD34	"	Waitzrodt, Hessia	DE	IBCN02	Liberator	Kuswinanti et al., 1999
T11aB04	"	Hebenshausen, Hessia	"	IBCN03	Libraska	"
T12aC25	"	Waitzrodt, Hessia	"	IBCN05	Lirajet	"
LbB3/12	*L. biglobosa*	BLINKA	CZ	-	Unknown	Present study
LbB7/12	"	BLINKA	"	-	"	"
Lbb8/12	"	BLINKA	"	-	"	"
LbB10/12	"	BLINKA	"	-	"	"
LbB18/12	"	BLINKA	"	-	"	"
LbHB6/12	"	HAVLÍČKŮV BROD	"	-	"	"
4BV6	"	Göttingen, Lower Saxony	DE		Lirajet	Beate Volke
LbD14	"	“	"	1BV1	Bristol	"
IIa2	"	Husum, Schleswig-Holstein	"	IBCN09	Unknown	"

Table 1. Properties of the *Leptosphaeria maculans* and *Leptosphaeria biglobosa* isolates used in the study: The isolates were collected from the Czech Republic (CZ) and Germany (DE).

3.1.2 Growth conditions of fungal isolates

The cultures were maintained on solid 10 % V8 juice agar media, at 25-26 °C in the dark conditions for 10-12 days. Fresh mycelium was grown for 3 weeks on V8 juice media supplemented with streptomycin (100 µg/ml) in dark conditions. Thereafter, mycelium was inoculated into 100 ml of fresh liquid media in 250 ml Erlenmeyer flasks. Mycelia and spores were harvested after growing for 7 days in the dark at 27 °C.

3.1.3 Detection of *L. maculans,* and *L. biglobosa*

The fungi were detected using primers from *LmacF*, *LmacR* and *LbigR* (Liu et al., 2006). The list of the primers is in Table 4. PCRs were performed in 25 µL volumes, each containing 2.5 µL 10 x reaction buffer, 2·5 mM MgCl2, 10 mM dNTP, 10 µM each primer, 1 U Taq DNA polymerase (Thermo Scientific, USA) and 50-100 ng template DNA. PCR condition was: an initial denaturation period of 95°C for 2 min, followed by 35 cycles of 95 °C for 15 s, annealing

at 70 °C for *LmacF*, *LmacR* and *LbigR* for 30 s and 72 °C for 1 min, followed by final extension at 72 °C for 10 min.

3.1.4 Plant material and fungal inoculation in oilseed rape

Cotyledon inoculation

Various *B. napus* cultivars were grown from untreated seeds in plastic trays (20 cm x 30 cm) filled with potting soil under controlled conditions at 22°C and a day/night photoperiod of 16h and 8h. For the first three days, trays were covered by a glass plate. After 5 days seedlings were transferred into multiport plates and seven days old seedlings were inoculated. Before inoculation, each cotyledon was wounded using sterile needle. Thereafter, 10 µL of fungal spore suspension (10^6 spores/ml) was placed on each lobe of cotyledon. True leaves were removed in order to delay senescence of cotyledons. Additionally, to the cultivar-isolate combination a water control (Mock) was used. Infected plants were incubated for 14 days. Disease severity was then assessed on cotyledons.

Petiole inoculation

B. napus cv. Westar was grown at 22°C and 16 h/8 h photoperiod. For the first three days, trays were covered by a glass plate. After 5 days seedlings were transferred into 9 x 9 cm plastic pots filled with a mixture of potting soil, steamed compost and sand (8:4:1). Plants were grown under controlled conditions at 22°C and 16 h/8 h photoperiod in climate chambers (Rumed, Rubarth Apparate GmbH, Laatzen, Germany) for 4-6 weeks. Thereafter, three petioles per plant were inoculated by wounding the petiole with a sterile needle and 100 µl of fungal spore suspension (106 spores/ml) were then supplemented. The spore suspension contained 2% of methycellulose (Sigma Aldrich, USA) so as to provide thickness. Plants mock inoculated with water served as controls. Following inoculation, plants were incubated for 3 days at 94% relative humidity to foster infection. Subsequently, relative humidity was kept at 70%. Petiole tissue was harvested 5-7 days post inoculation (dpi).

3.1.5 Quantification of fungal DNA

Quantification of *L. maculans* and *L. biglobosa* DNA was used to determine differences of growth of the pathogen. The amount of *CYP4* DNA in each *L. maculans* and *L. biglobosa* inoculated petiole was quantified using LmCYP4F/R and LbCYP4F/R primers using qPCR. Nuclease-free water was used as the no-template control. In each qPCR run, standards with an amount of 0.1 pg, 1 pg, 10 pg, 100 pg, and 1 ng DNA of *L. maculans* isolate C40 and *L. biglobosa* isolate Na21 was included to produce a standard curve. The amount of *L. maculans* and *L. biglobosa* DNA for each unknown sample was extrapolated after normalizing the values

with the standards. The reaction conditions were the same as described above for expression analyses.

3.1.6 Microscopy

Microscopic analyses were performed with a Leica DM2700 M fluorescent microscope (Leica, Mannheim, Germany) equipped with red shifted TRITC filter. Fluorescent images were acquired by scanning 545 nm for excitation, 620 nm for emission for DsRed tagged isolates both to screen colonies of transformants and for fungal colonization in plant tissues. Images were taken by digital camera (Leica DFC 300FX) operated with IM50 software (Leica DC Twain, v. 4.1.5.0).

3.1.7 *Plasmodiophora brassicae* pathotypes

P. brassicae single spore isolate e3 and pathotypes P1, P3 and P5 used in this study were provided by Prof. Christina Dixelius, Swedish University of Agriculture, Sweden.

3.2 Bioinformatic analyses

3.2.1 Searching protein database, domain analyses and subcellular localization

The HMM profiles unique to cyclophilin (PF00160) and FKBP (PF00254) and parvulin-like proteins (PF00639) from Pfam database (http://pfam.sanger.ac.uk/) were retrieved and thereafter searched against corresponding genome browser database of *L. maculans, L. biglobosa* and *P. brassicae* using HMMER 3.0 software (http://hmmer.janelia.org/). The identified proteins were then analyzed for the presence of respective catalytic domain using Pfam database (http://pfam.xfam.org/) and SMART database (http://smart.embl-heidelberg.de/). Subcellular localization of the putative IMMs was predicted using PSORT (http://wolfpsort.org/). Signal peptide and mitochondrial target peptide (mTP) were predicted using SignalP 1.1 (http://www.cbs.dtu.dk/) and TargetP 1.1 sever (http://www.cbs.dtu.dk/). Nuclear localization signals (NLS) were predicted using NLS mapper http://nls-mapper.iab.keio.ac.jp/) and Nucpred (https://www.sbc.su.se/) and trans-membrane domain was predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/).

3.2.2 Gene Structure determination of IMMs

Gene structure for IMM genes in *L. maculans* was identified from contigs and supercontigs (SCs). The contigs and SCs were aligned and subjected to FGENESH software (http://linux1.softberry.com/) using *Laptosphaeria* genome as a query with default parameters. The genomic sequences and coding sequences were thus mapped using Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/) and crosschecked by using spidey (http://www.ncbi.nlm.nih.gov/spidey/). The final figures were drawn using GSDS. In the case of *P. brassicae* IMMs, the exon-intron architecture was obtained from European Nucleotide

Archive (ENA) as described in Schwelm et al. (2015). Thereafter the scaled position of putative IMMs was drawn using R software.

3.2.3 Multiple Sequence alignment and Phylogenetic analyses

Multiple sequence alignment of amino acid sequences were performed using ClustalX (version 2.0) (http://www.ebi.ac.uk/) with default parameters and built in Jalview software version 2.7 (http://www.jalview.org/). The phylogenetic trees were generated using ClustalX and MEGA 6.0 (http://www.megasoftware.net/) implementing neighbor-joining (NJ) method and maximum-likelihood (ML). Bootstrap values were calculated at 1, 000 iterations. The final phylogenetic tree was then edited using ITOL (http://itol.embl.de/).

3.3 Molecular analyses

3.3.1 Isolation of fungal and plant genomic DNA

DNA was extracted from freeze-dried mycelia and from Lm and Lb inoculated *B. napus* cv. Westar petiole using a CTAB method modified according to Brandfass and Karlovsky (2008). First a master mix was prepared containing 1 ml CTAB buffer, 2 µl mercaptoethanol and 1 µl proteinase K per sample. A volume of 1 ml master mix was added to each sample and subsequently mixed with a vortexer before treatment with ultrasonics for 5 sec. Then samples were incubated at 42 °C and afterwards at 65 °C for 10 min either. During temperature incubation samples were shaken six times. Thereafter 800 µl chloroform isoamyl alcohol was added and samples were mixed with a vortexer before incubation on ice for 10 min. Following the sample was centrifuged at 10397g at room temperature. An amount of 600 µl of the upper clear phase was transferred to a new Eppendorf tube and reacted with 193.6 µl of a 30% PEG 6000 solution and 100 µl of 5 M sodium chloride. Samples were mixed with a vortexer and centrifuged for 15 min at 18078g at room temperature. The precipitated DNA pellet was washed with 500 µl of 70% ethanol. After centrifugation the DNA pellet was dried at room temperature. To dissolve the DNA 50 µl TE buffer was added and samples were incubated for 1 hour before freezing DNA samples at -20 °C until use.

3.3.2 Isolation of total RNA

Total RNA was extracted from freeze-dried mycelia and from *L. maculans* and *L. biglobosa* inoculated *B. napus* cv. Westar petioles using TRIzol reagent® (Life Technologies, USA) following the manufacturer's protocol. 100 mg of each sample was taken in a mortar and homogenized in liquid nitrogen to obtain very fine powder. 1 ml TRIzol® was added and homogenized. Samples were allowed to stand at room temperature for 5 min. 200 µl of chloroform was added to each microcentrifuge tube and mixed vigorously for 15 sec. This was left for 2-3 min at room temperature and then centrifuged at 15805g, 4°C for 15 min. The upper

aqueous supernatant was transferred to pre-sterilized fresh microcentrifuge tubes and 500 µl of 100% iso-propanol was added to each tube containing supernatant. This was mixed gently and incubated at room temperature for 10 min. The mixture was then centrifuged at 15805g, 4°C for 10 min. Supernatant was discarded and a white glassy pellet of total RNA was obtained at the bottom of tube. The pellet was washed with 75% ethanol at 4°C, centrifuged at 15805g for 10 min and then air dried inside the hood for 5-10 min. The pellet was dissolved in appropriate volume of DEPC treated water for further use.

3.3.3 Synthesis of cDNA

First strand cDNA was synthesized using first strand cDNA synthesis kit (Fermentas). A reaction mix was prepared in a PCR tube containing 1 µg RNA and 1 µg oligo $(dT)_{18}$. The reaction mixture was incubated at 65°C for 5 min and chilled on ice for 5 min. Then, following components were added in the given order, 5X reaction buffer 5 µl, RiboLocK™ RNase Inhibitor of concentration 20 U/µl, 10 mM dNTP mix. The mixture was incubated at 37°C for 5 min. 200 U/µl ReverseAid™ H minus reverse transcriptase enzyme was added to make final reaction volume equivalent to 40 µl. The mixture was incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and subsequent chilling on ice.

3.3.4 Real-Time PCR

The real-time PCR mixture comprised 4 µl of cDNA (10 times diluted), 10 µl of 2X SYBR Green PCR Master Mix (Applied Biosystems, USA), and 100 nM of each gene-specific primers in a final volume of 20 µl. qRT-PCR was performed employing CFX Connect™ Real-Time PCR System and software (Bio-Rad, USA). The reaction conditions were 95°C (3 min), and 35 cycles of 10 sec at 95°C and 20 sec at 59°C. The specificity of amplification was tested by dissociation curve analysis. Three technical replicates were analyzed for each sample. Relative expression was calculated using comparative C_T value method (Schmittgen and Livak, 2008), using actin as the internal control. Housekeeping gene actin was taken as the reference gene for the analysis. Experiments were repeated thrice (three biological replicates). Statistical significance was tested using two-tailed Student's t-test, $p < 0.05$.

3.3.5 Polymerase Chain Reaction

DNA/cDNA/Plasmid PCR

PCR amplification was carried out in a microcentrifuge tube containing 50-100 ng template DNA, 10 µM each of forward and reverse primers (Sigma-Aldrich, Czech Republic) and 2X DreamTaq Green PCR Master Mix (Thermo Scientific, USA). The programming for the PCR amplification was as follows: First cycle consisted of initial denaturation for 3 min at 95°C. Then, 35 cycles were carried out with each cycle having denaturation at 95°C for 30 sec,

annealing depending on the primers and extension at 72°C for 30 sec to 3 min. Last cycle was run for a final extension at 72°C for 10 min.

3.3.6 DNA ligation

For the ligation of DNA fragments different vector into insert ratios were tried and finally 3:1 (vector: insert) ratio was selected for ligation. The ligation reaction was set up in a water bath at 22°C with 1U T4 DNA ligase (Thermo Scientific, USA), 10X T4 DNA ligase buffer in 20 µL reaction volume for 15 h.

3.3.7 Transformation of *E. coli*

For high efficiency transformation DH5α (Stratagene, Texas, USA) competent cells were used. These cells were thawed on ice and aliquots of 100 µl were transferred to pre-chilled new 1.5 ml tubes. About 20-40 ng of DNA was mixed with the 100 µl of competent cells and the suspension was incubated on ice for 30 min. The cells were then heated for 30 sec in 42°C in water bath and transferred on ice for 2 min. Then 500 µl SOC medium without antibiotic were added and bacterial cells were incubated for 1h at 37°C with shaking at 200 rpm. Transformants were screened on LB agar plates containing ampicillin (50 µg/ml). Plates were incubated overnight at 37°C. The observed colonies on the plates were screened for the cloned insert by colony PCR.

3.3.8 Transformation of *Agrobacterium*

Agrobacterium electro competent cells AGL1 (Göttingen, DE) were used for the transformation. About 10-100 ng of DNA was mixed with 40 µl of AGL1 electro competent cells and transferred to the cuvette. The mix was electroporated by using the following conditions: 1.80 kV voltage, 25 µF (capacitance), 200Ω resistance/pulse controller (Bio-Rad, USA). Then SOC Medium (800 µl) was added and cells were incubated at 28°C for 3 hr at 250 rpm. Bacterial cells (100 µl) were plated onto LB medium containing appropriate antibiotics and plates were incubated overnight at 28°C. The observed colonies on the plates were screened for the cloned insert by colony PCR.

3.3.9 Cyclophilin construct preparation

For the construction of pCB1532:*PbCYP3* construct, ORF was PCR amplified using primer pairs *PbCYP3*BamHI-F and *PbCYP3KpnI*-R using phusion taq polymerase. The gel purified PCR product was digested using *BamHI* and *KpnI* and ligated into pCB1532 vector overnight at 16°C and transformed into *E. coli* (DH5α). The positive clones were digested and sequenced for the confirmation.

3.3.10 Transformation of *Magnaporthe oryzae*

Erlenmeyer flasks containing 50 ml of complete medium were inoculated with fresh *M. oryzae* mycelium (1 sq. m). The medium was incubated at 28°C, with shaking at 150 rpm for 48 hr. Cultures were harvested by filtration through sterile miracloth and mycelium was washed in sterile water. Mycelium was then transferred to a 50 ml conical flask and subsequently 40 ml of OM buffer was added containing filter sterile 50 mg Novozym 234 (Novozyme, Copenhagen, Denmark). The mixture was then shaken gently to disperse hyphal clumps and incubated at 28°C with gentle shaking (75 rpm), for 1 hr. Protoplasts/OM buffer was transferred to 2 sterile polycarbonate or Falcon tubes (approx. 20 ml in each) and overlaid gently with an equal volume cold ST buffer. Protoplasts were centrifuged at 3000g for 10 min at 4°C. Protoplasts were recovered from the OM/ST interface using a 1 ml Pasteur-pipette. The protoplasts were kept on ice until transformation. Protoplasts were transferred to 2-3 Falcon tubes and filled with STC buffer. Protoplasts were then pellet down at 3000g for 10 min at 4°C. Protoplasts were resuspended in 1.0 ml of STC, count microscopically, and adjusted to a concentration of 2-5 X 10^8 protoplasts/ml. Protoplasts (concentration 10^6 to 10^7) were then mixed with 4-8 µg of construct pCB1532: *PbCYP3* in a total volume of 150 µl of STC buffer and incubated at room temperature for 15 to 25 min. PTC buffer was then added in 2 to 3 aliquots, mixed gently by inversion followed by incubation at room temperature for 15 to 20 min. Finally, the protoplasts were added to 150 ml molten (45°C) BDC medium. The plates were incubated for at least 16 h at 24 °C and overlaid with approx. 15 ml top agar (BDC medium (without sucrose), 1% agar) containing 600 µg/ml chlorimuron ethyl (Sigma Aldrich, USA). Transformants were selected onto BDCM (without sucrose) containing 150µg/ml chlorimuron ethyl (Sigma Aldrich, USA).

3.3.11 *In planta* quantification of *Magnaporthe* DNA using qPCR

Rice cultivar Kitaake was grown in growth chamber at 28 °C for 14 h light and 10 h dark. Four weeks old plants were artificially inoculated with wild type Guy11, *Δcyp1* mutant and overexpressed line Pb6. One leaf was inoculated per plant (three replicates) using agar plugs (4 cm diameter) derived from 2 weeks old cultures. Mock samples were inoculated with only agar. The disease symptoms on leaves were monitored after two weeks. DNA was extracted using CTAB method. DNA quantification (qPCR) of *M. oryzae* was performed using actin primer from *M. oryzae* (MgActF/R) and elongation factor primer from *Oryza sativa* (OsElfF/R) (listed in table 4).

3.3.12 Primer used

Primer name	Sequence (5´- 3´)	Reference
LmacF	CTTGCCCACCAATTGGATCCCCTA	Liu et al., 2006
LmacR	GCAAAATGTGCTGCGCTCCAGG	"
LbigF	ATCAGGGGATTGGTGTCAGCAGTTGA	"
LbigR	GCAAAATGTGCTGCGCTCCAGG	"
TC2F	AAACAACGAGTCAGCTTGAATGCTAGTGTG	Cao et al., 2007
TCR2	CTTTAGTTGTGTTTCGGCTAGGATGGTTCG	"
PbqPCR-Fw	GGAATGCGTACCATGACCTG	Present study
PbqPCR-Re	GTCAGTCGATCGCGATAGTC	"
LmCYP4F	ATGTCCAACCCCCGTGTCT	"
LmCYP4R	TTACAATTGACCGCAGTTTGC	"
LbCYP4F	ATGTCCAACCCCCGTGTCTTC	"
LbCYP4R	CTACAACTGGCCGGAGTTGGCG	"
LmLbActinF	GAGCAGGAGATCCAGACTGC	"
LmLbActinR	GAGATCCACATCTGCTGGAAG	"
PbCYP3F	ATGTCCAACCCCCGTGTCTTC	"
PbCYP3R	CTACAACTGGCCGGAGTTGGCG	"
LmCYP4C_Fw	ATAT***GGATCC***ATGTCCAACCCCCGTGTCT	"
LmCYP4C_Rv	ATAT***CCATGG***TTACAATTGACCGCAGTTTGC	"
PbCYP3C_Fw	ATAT***GGATCC***ATGTCCAACCCCCGTGTCTTC	"
PbCYP3C_Rv	ATAT***CCATGG***CTACAACTGGCCGGAGTTGGCG	"
PbCYP3C_RT	ATTTCACGAACCACAACGGCACTG	"
PbCYP3C_RT	TGGACACGGTGCACACGAAGAAC	"
MgActF	ATGTGCAAGGCCGGTTTCGC	"
MgActR	TACGAGTCCTTCTGGCCCAT	"
OsElfF	TTGTGCTGGATGAAGCTGATG	"
OsElfR	GGAAGGAGCTGGAAGATATCATAGA	"

Table 4. List of primers used in the current study. Restriction site *Bam*HI (***GGATCC***) and *Kpn*I (***CCATGG***).

4. Results

4.1 Detection and characterization of *Leptosphaeria* in the Czech Republic

Leptosphaeria maculans and *L. biglobosa* isolates described in the Table 1 were investigated and characterized based on following methods.

Molecular detection

A

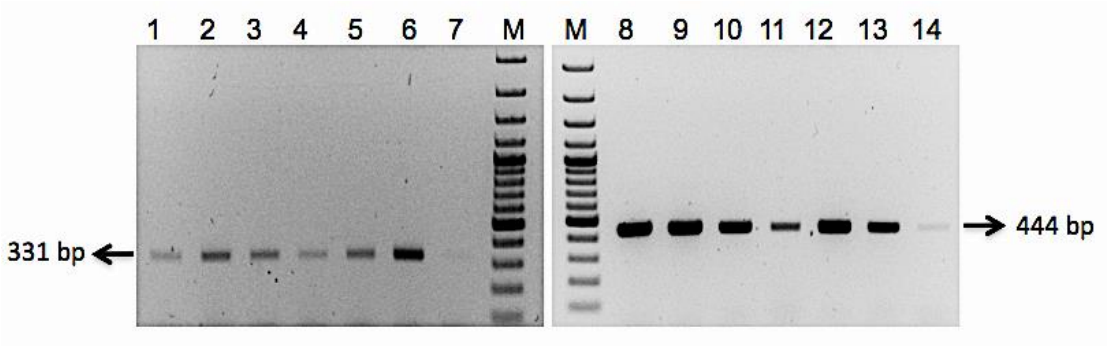

Fig 1. Ethidium bromide-stained 1% agarose gel of polymerase chain reaction (PCR) products amplified with species-specific primers from Liu et al., 2006. Lane 1-7: *L. maculans* and Lane 8-14: *L. biglobosa*. M: DNA ladder (100 bp plus GeneRuler^{TM} DNA ladder, Fermentas).

Host-pathogen interaction analyses (differential set)

Cotyledon inoculation

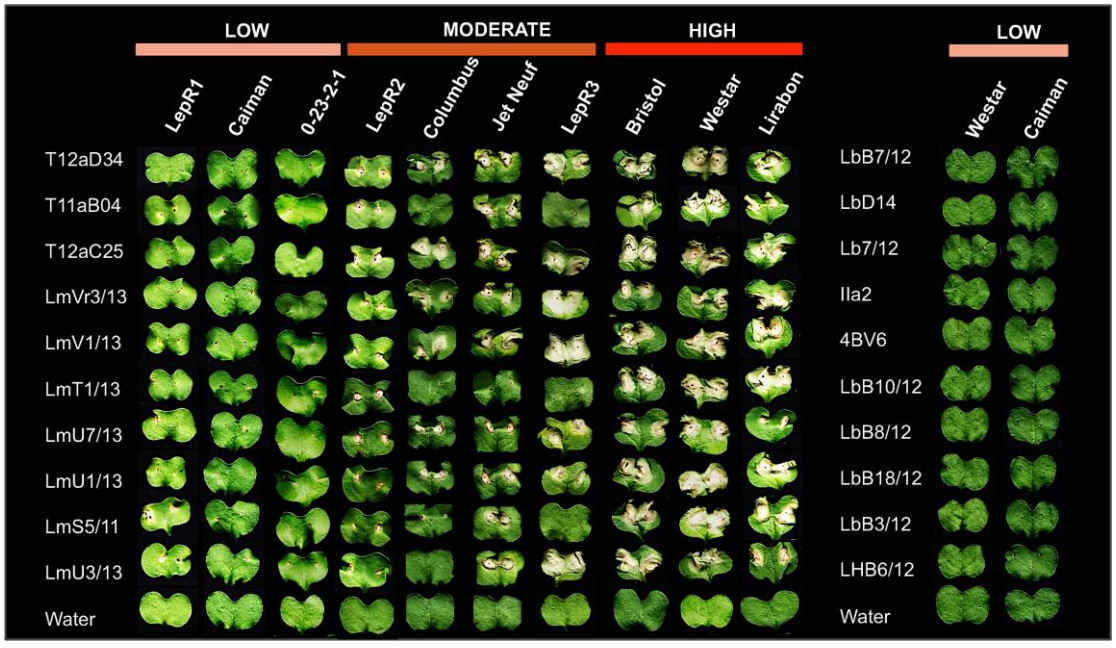

Fig 2. Host-pathogen interaction of *L. maculans* and *L. biglobosa* isolates in various *Brassica* cultivars (horizontally). Each cotyledon pair (4 lobes) was artificially inoculated and supplemented with 10 µL of fungal spore suspension (10^6 spores/ml). Water inoculated plants served as control. Disease symptoms were assessed 14 days post inoculation (dpi) using

IMASCORE (0-6 scale). *L. maculans* isolates (mostly named by prefixing 'Lm') and *L. biglobosa* isolates (named by prefixing 'Lb').

Petiole inoculation

Fig 3. Host-pathogen interaction of *L. maculans* and *L. biglobosa* isolates in asusceptible cv. Westar of *Brassica*. Petioles were artificially inoculated and supplemented with suspension of spores and 2% methycellulose (10^6 spores/ml). Water inoculated plants served as control. Disease symptoms were assessed 5 days post inoculation (dpi). *L. maculans* isolates; 1:T12aD34, 2:LmT1/13, 3:LmU1/12 and 4:LmS5/12 and *L. biglobosa* isolates; 5:LbB18/12, 6:LbD14, 7:LbHB6/12, and 8:LbB10/12.

Validation of *Agrobacterium tumefaciens*-mediated transformation for discriminating *Leptosphaeria* spp. complex

L. maculans* grows slower compared to *L. biglobosa

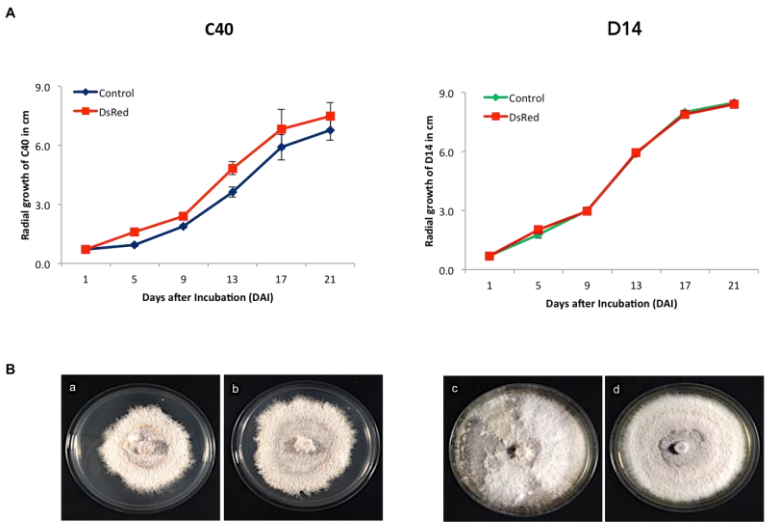

Fig. 4. A) Radial growth (cm) of *L. maculans* and *L. biglobosa* wild type and transformant at various time point. DsRed showed similar growth pattern like wild type. B). Growth shown in Czapek-Dox agar media. Plates were incubated in darkness at 24°C. Mean of five inoculated plates were taken.

DsRed expression in L. maculans and *L. biglobosa*

Overall, 30-35 transgenic L. maculans and 22-25 *L. biglobosa* isolates were obtained per 10^7 spores. 60 % percent of the hygromycin B-resistant L. maculans and 75 % of the obtained L. maculans isolates expressed the DsRed, which was a success rate similar to reports either on *Agrobacterium*-mediated transformation (Eckert et al., 2005).

DsRed expression was generally high and uniform in spores and hyphae (Fig. 5(I & J)). DsRed expression remained stable after successive transfers (5 generations) on Czapek Dox medium with and without hygromycin B. However, it was observed that the older mycelium occasionally included segments of hyphae with reduced expression (Fig. 16(K)) or without expression (Fig. 5(L & M)).

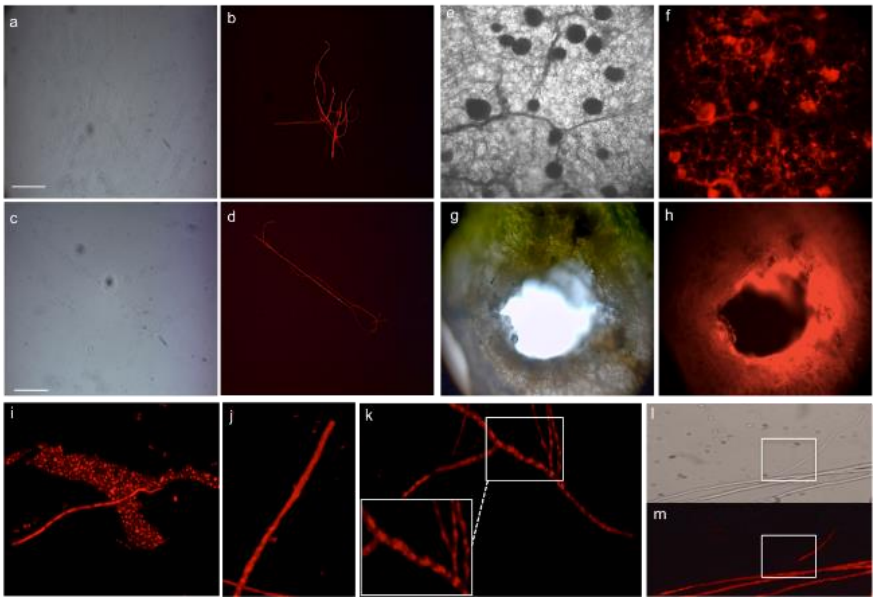

Fig. 5. Fluorescence micrographs of *DsRed* transformants of *L. maculans* C40 and *L. biglobosa* D14. Branching pattern of *L. maculans* (A & B) and *L. biglobosa* (C & D) in bright and fluroscence field. Pycnidia formation on the cotyledons by *L. maculans* DsRed transformants (E & F) and dark lesion formed *by L. biglobosa* DsRed transformant (G & H). Spores and fresh hyphae are shown in I & J. Old mycelium showing segments of hyphae with reduced expression or without expression (K-M). Leica fluorescence microscope was used for the visulization using DsRed filter.

Disease assessment on susceptible hosts

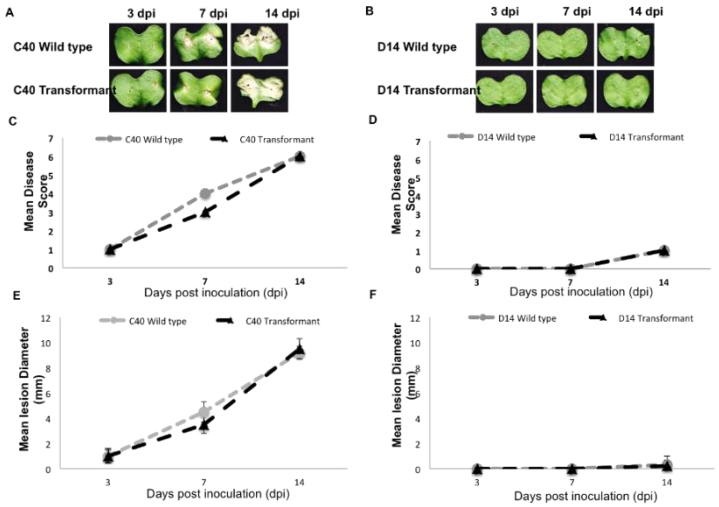

Fig. 6. Host pathogen interaction (time-course) and disease assessment of wild type and transformants on *B. napus* susceptible cv. westar cotyledons. A and B) symptoms of *L. maculans* and *L. biglobosa*. C and D) mean disease ratings on a 1-6 scale and E and F) mean lesion diameter (mm) of wild type and transformant. Mean of 26 plants (52 lobes) were taken.

4.2 Immunophilin (IMMs) in *Leptosphaeria maculans* and *L. biglobosa*

Conservation and characterization of IMM genes

A.

Species	Genes	accession no	a.a	pI/Mw	Localization	cNLS	TargetP	TM
L. maculans	*LmCYP1*	Lema_P042100.1	166	6.91/18.1	C	-	-	-
	LmCYP2	Lema_P051520.1	165	6.51/18.0	C	-	-	-
	LmCYP3	Lema_P023490.1	222	8.98/24.4	N	-	-	-
	LmCYP4	Lema_P059530.1	171	7.75/18.1	C	-	-	-
	LmCYP5	Lema_P083030.1	218	5.57/22.8	C	-	-	-
	LmCYP6	Lema_P062660.1	663	6.28/74.1	N	NLS	-	-
	LmCYP7	Lema_P000030.1	375	5.92/41.4	C	-	-	-
	LmCYP8	Lema_P080490.1	560	8.43/61.5	C	-	-	-
	LmCYP9	Lema_P020130.1	497	8.88/54.8	N	NLS	-	-
	LmCYP10	Lema_P067630.1	228	9.46/24.4	M	-	MTP	-
	LmCYP11	Lema_P091210.1	478	5.98/55.0	C	NLS	-	-
	LmCYP12	Lema_P077590.1	165	5.01/16.8	C	-	-	
L. biglobosa								
	LbCYP1	Lb_b35_P003046	166	6.97/18.1	C	-		-
	LbCYP2	Lb_b35_P006007	165	6.96/18.0	C	-		-
	LbCYP3	Lb_b35_P002298	181	8.44/19.8	C	-		-
	LbCYP4	Lb_b35_P006868	171	7.82/18.1	C	-		-
	LbCYP5	Lb_b35_P011036	211	7.88/22.8	C	-	SP	-
	LbCYP6	Lb_b35_P007186	375	5.85/41.1	C	-		-

	LbCYP7	Lb_b35_P007380	564	7.16/62.1	C	-		-
	LbCYP8	Lb_b35_P000291	498	9.12/54.8	N	NLS		-
	LbCYP9	Lb_b35_P011184	228	9.20/24.5	C	-	mTP	-
	LbCYP10	Lb_b35_P001062	474	5.84/54.3	C	-		-
	LbCYP11	Lb_b35_P009544	584	5.94/65.5	M	-	mTP	-

B.

Species	Genes	Accession no.	a.a	pI/Mw	Localization	cNLS	TargetP	TM
L. maculans	*LmFKBP1*	Lema_P030420.1	549	4.58/59.7	M	-	MTP	-
	LmFKBP2	Lema_P118050.1	134	4.87/14.5	EM	-	SP	-
	LmFKBP3	Lema_P011100.1	491	4.95/53.7	PM	-	SP	Y
	LmFKBP4	Lema_P077320.1	113	5.81/12.2	C	-	-	-
	LmFKBP5	Lema_P057060.1	124	5.13/13.2	C	-	-	-
L. biglobosa	*LbFKBP1*	Lb_b35_P007824	134	5.10/14.4	N	NLS	SP	-
	LbFKBP2	Lb_b35_P010150	367	4.88/39.2	M	-	mTP	-
	LbFKBP3	Lb_b35_P001362	498	4.62/54.0	PM	-	SP	Y
	LbFKBP4	Lb_b35_P008929	266	9.02/29.6	N	NLS	-	-
	LbFKBP5	Lb_b35_P006787	98	4.72/10.5	M	-	-	-

Table 2. List of putative IMMs from *L. maculans* and *L. biglobosa* along with their nomenclature, molecular weight, isoelectric point, amino acid length (a.a), and predicted subcellular localization (C: cytoplasm; N: nuclear; M: mitochondria; EM: extracellular membrane; PM: plasma membrane). Nuclear localization signal (NLS), Mitochondria targeting peptide (MTP), Signal peptide (SP) and transmembrane. A. CYPs and B. FKBPs

Domain architecture of IMM in *Leptosphaeria*

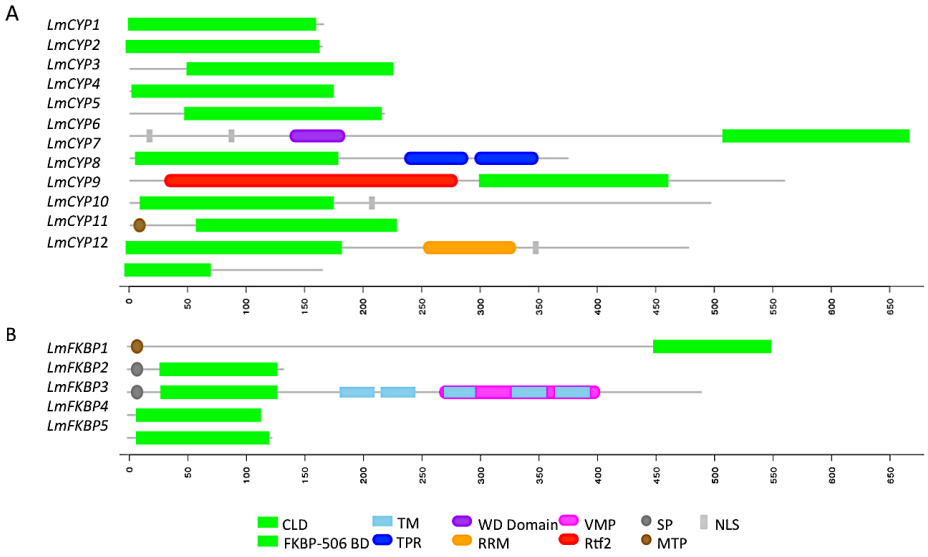

Fig 7. Domain architecture of the Leptosphaeria IMM gene family members:(A) CYPs and (B) FKBPs. CLD, cyclophilin-like domain; FKBP, FK506 binding protein; MTP, mitochondrial signal peptide; NLS, nuclear localized signal; Rtf2, replication termination factor domain; RRM, RNA recognition motif; SP, signal peptide; TM, transmembrane domains; TPR, tetratricopeptide repeats; VMP, vacular membrane protein.

Genomic distribution of IMMs

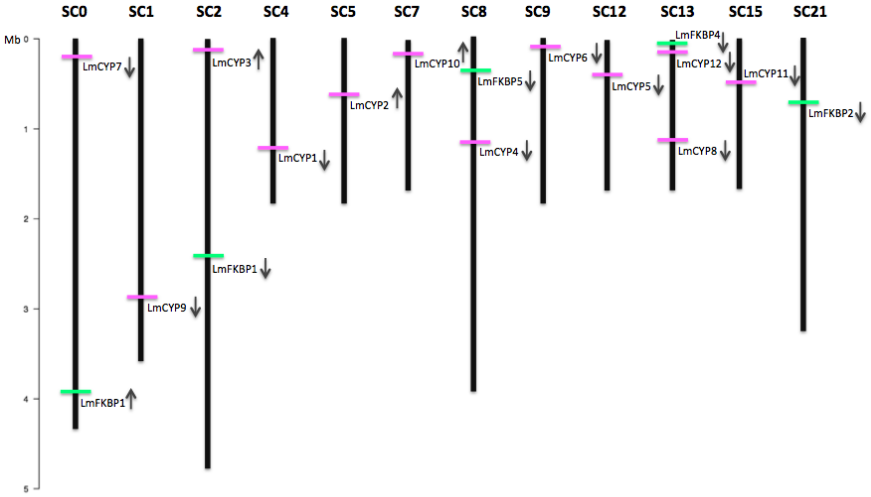

Fig 8. Genomic distribution of *L. maculans* IMMs on various chromosomes. CYPs and FKBPs are represented with horizontal green and pink bars, respectively. Arrows show the direction of the ORF specific to the gene encoding CYP and FKBPs proteins (i.e., lower for sense strand upper for antisense strand). Only the chromosomes having IMMs are shown; their number is indicated at the top. The chromosome length has been shown in Mb.

Exon-intron architecture analysis

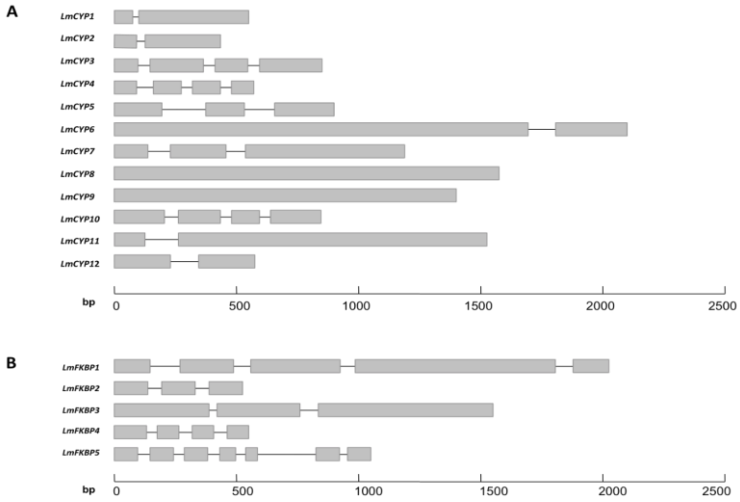

Fig 9. Exon–intron architecture of *L. maculans* IMMs. CYPs (A) and FKBPs (B) are shown. Exons are marked in *block*, while introns with *line*. Lengths of genes are shown in bp scale.

Transcriptomic expression analysis of IMMs

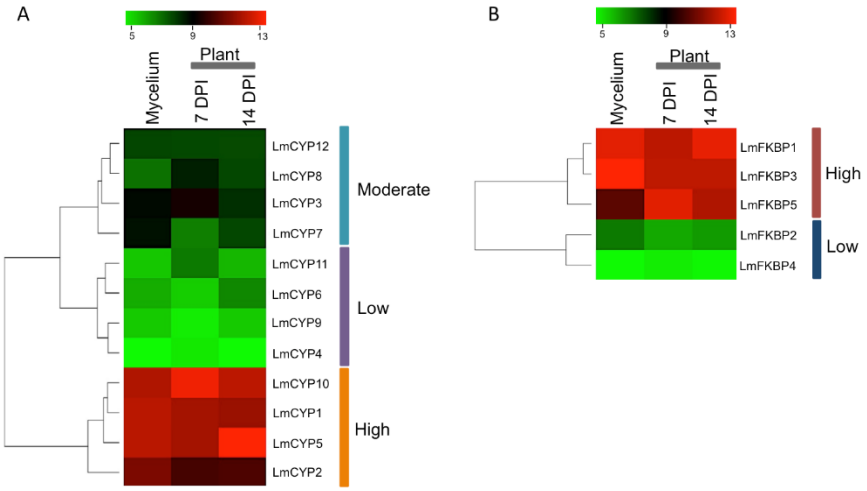

Fig 10. Expression analyses of *L. maculans* IMMs in mycelium and during *B. napus* leaf infection at 7 and 14 days post-infection (DPI). Heat maps showing expression of CYPs (A) and FKBPs (B). CYPs and FKBPs are classified into three and two main groups based on expression. Scale position is shown at the top of each heat map.

4.3 Cyclophilin 4 distinguishes *Leptosphaeria maculans* and *L. biglobosa*

Cyclophilin (*CYP4*) reveals a major difference between *L. maculans* and *L. biglobosa*

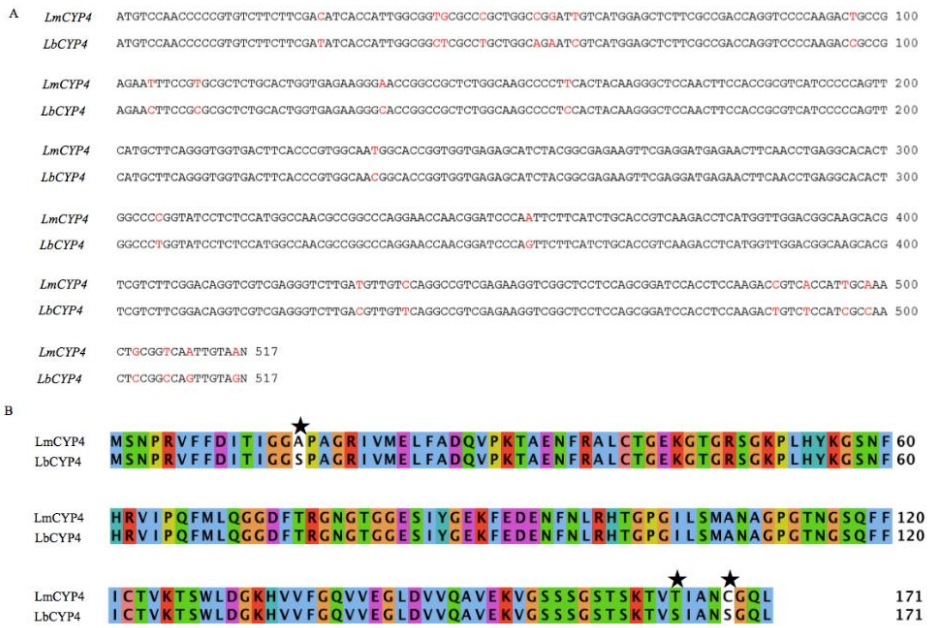

Fig 11. Multiple sequence alignment of cloned *LmCYP4* and *LbCYP4*, cDNA (A) and protein (B). In case of cDNA, 24 SNPs were observed as shown in red color. In protein three mutations are observed and highlighted with (*). The sequences shares 95 % homology at nucleotide level and 98% at protein level.

Disease severity in *B. napus* shows differences between *L. maculans* and *L. biglobosa*

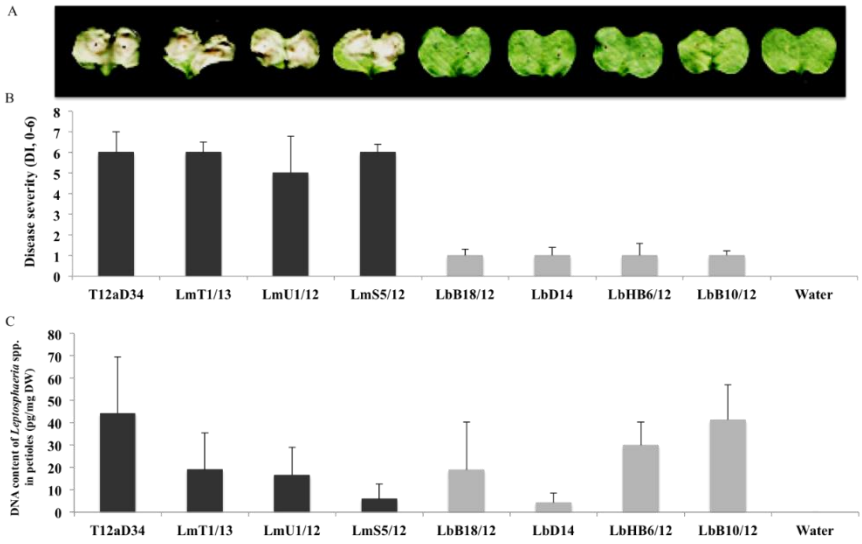

Fig. 12. Disease symptoms (A) and severity (B) in cotyledons after artificial inoculation of the susceptible *B. napus* cv. westar with isolates of *L. maculans* (highly aggressive) and L. biglobosa (weakly aggressive), respectively. For inoculation, each lobe of cotyledons was wounded and supplemented with 10 µL of fungal spore suspension (10^6 spores/ml). Water inoculated plants served as control. Plants were incubated for 14 days. Disease severity was assessed 14 days after inoculation (dai) using IMASCORE (0-6 scale). Error bars show ± standard deviation, n = 9. *L. maculans* isolates (named by prefixing 'Lm') and *L. biglobosa* isolates (named by prefixing 'Lb'). C) DNA content (pg/mg DW) of *Leptosphaeria* spp in petioles of *B. napus* cv. Westar inoculated with *L. maculans* and L. biglobosa. The amount of *Leptosphaeria* spp. DNA in plant tissue was measured with species-specific primers, based on the *CYP4* gene. *Leptosphaeria* actin primers have been used as an internal control. Fungal DNA of the isolates C40 and Na21, respectively, served as DNA for standard curves for *L. maculans* and L. biglobosa quantification. Error bars show ± standard deviation, n = 4.

***Leptosphaeria CYP4* is highly expressed in highly aggressive *L. maculans* as compared to weakly aggressive *L. biglobosa* isolates**

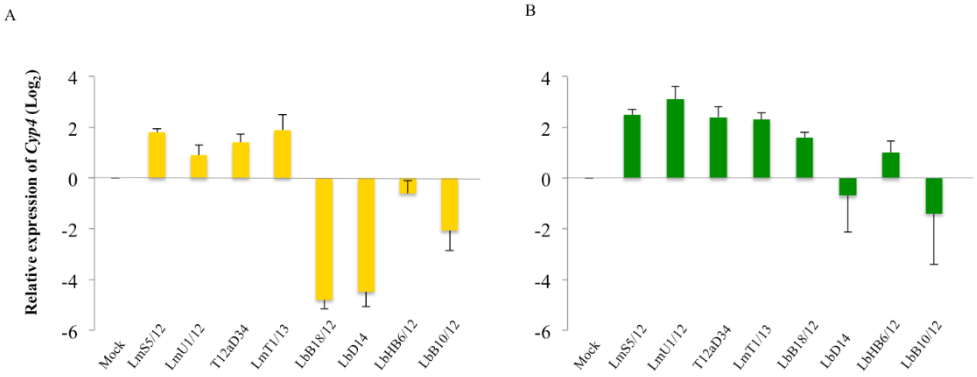

Fig 13. Relative gene expression of *LmCYP4* and *LbCYP4* in vitro and in planta. Mycelium (7 dpi) and artificial inoculated *B. napus* (petiole) cv. Westar (14 dpi) were used for gene expression analyses. Bar graphs depict mean fold change (log2 scale) in expression of *LmCYP4* and *LbCYP4*, as obtained using qRT-PCR. Error bars show ± standard deviation, n = 3. *L. maculans* isolates (named by prefixing 'Lm') and *L. biglobosa* isolates (named by prefixing 'Lb'). *Leptosphaeria* actin has been used as an internal control.

4.4 Genome-wide analysis of Immunophilin (IMMs) *Plasmodiophora brassicae*

P. brassicae harbor multiple copies of immunophilin (IMM) proteins

IMM sub-family	Gene	ENA accession no.	AA Length	pI/Mw	Domain Architecture[a]	pSORT[b]	Nucpred[c]	TargetP[d]	TMHMM
CYPs									
	PbCYP1	PBRA_003988	645	5.40/70.2	MD	C	–	–	–
	PbCYP2	PBRA_008091	169	5.79/18.7	SD	C	–	–	–
	PbCYP3	PBRA_003184	164	8.62/17.5	"	C	–	–	–
	PbCYP4	PBRA_001833	188	6.41/20.6	"	C	–	–	–
	PbCYP5	PBRA_005408	163	5.91/17.2	"	C	–	–	–
	PbCYP6	PBRA_004353	409	5.92/47.0	MD	N	–	–	–
	PbCYP7	PBRA_006873	208	9.37/22.8	SD	C	–	–	–
	PbCYP8	PBRA_004546	665	9.33/73.8	"	N	NLS	–	–
	PbCYP9	PBRA_006640	260	6.13/28.4	"	M	–	mTP	TM
	PbCYP10	PBRA_004862	325	5.30/36.4	MD	C	–	–	–
	PbCYP11	PBRA_001554	350	8.79/38.4	SD	M	–	mTP	–
FKBPs									
	PbFKBP1	PBRA_006340	428	5.53/45.9	MD	C	–	–	–
	PbFKBP2	PBRA_007950	137	9.56/14.8	SD	M	–	mTP	–
	PbFKBP3	PBRA_000431	382	4.83/41.9	"	C	–	–	–
	PbFKBP4	PBRA_006099	192	9.48/20.3	"	EC	–	SP	TM
	PbFKBP5	PBRA_004213	120	8.93/12.7	"	C	–	–	–
	PbFKBP6	PBRA_006954	352	6.22/38.1	MD	N	–	–	–
	PbFKBP7	PBRA_004571	314	7.00/34.3	MD	C	–	–	–
PARVULINs									
	PbPAR1	PBRA_004934	292	9.20/32.2	SD	N	NLS	–	–
	PbPAR2	PBRA_000749	118	9.30/12.8	MD	M	–	mTP	–

Table 3. List of putative IMMs identified from *P. brassicae* along with their nomenclature, European Nucleotide Archive accession ID (ENA), amino acid length, isoelectric point, molecular weight (kDa), and predicted intracellular localization.

[a] SD: Single-domain proteins and MD: Multi-domain proteins

[b] C: cytosol, M: mitochondria, N: Nuclear, EC: Extra cellular

[c] NLS – Nuclear localized signals

[d] mTP: Mitochondrial target peptide and SP: signal peptide

***P. brassicae* IMM clustered into single and multi-domain proteins based on domain architecture**

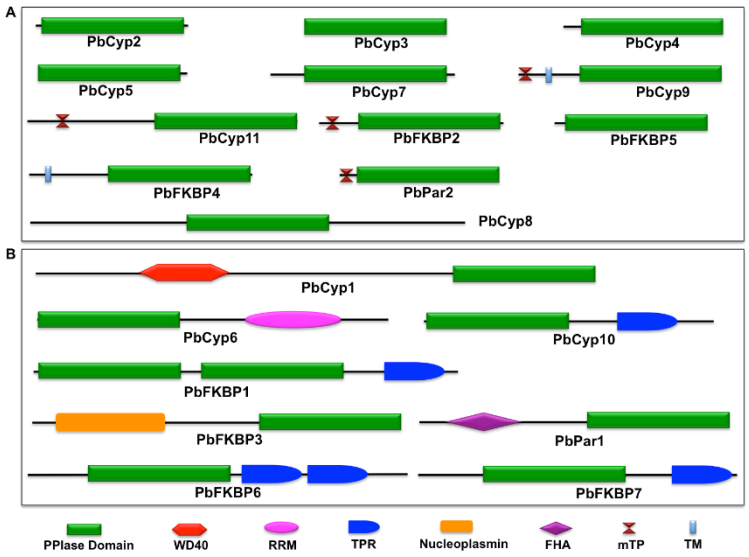

Fig. 14. Functional domain architecture of putative IMMs from *P. brassicae*. A) Single-domain proteins showing only single catalytic PPIase domains (green) and B) Multi-domain proteins showing additional domains coupled with the PPIase domain. CYPs contain a cyclophilin-like domain (CLD), FKBPs possess FKBP_C and parvulins contain a rotamase domain. Additional domains/motifs, such as WD40, RRM- RNA recognition motif, TPR- tetracopeptide repeat, FHA- forkhead-associated domain, mTP- mitochondrial target peptide, and TM- transmembrane domain, have been highlighted in multicolor scheme.

Immunophilins were differentially regulated in various *P. brassicae* life-stages and infected *Brassica* plants

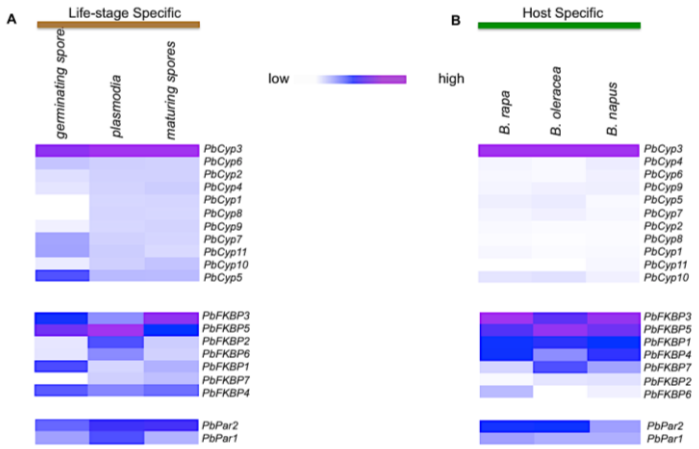

Fig. 15. Transcriptome of the putative IMMs from *P. brassicae*. The heat maps show the RNA-seq based expression profile of IMM during (A) *Plasmodiophora brassicae* life stages, including germinating spores, plasmodia and maturing spores and (B) expression in clubroot-

infected *Brassica* hosts *B. rapa*, *B. napus* and *B. oleracea*. The heat maps were drawn using the gplots package of R statistical software.

Comparative analysis of IMMs in five other pathogens of *Brassica*

A

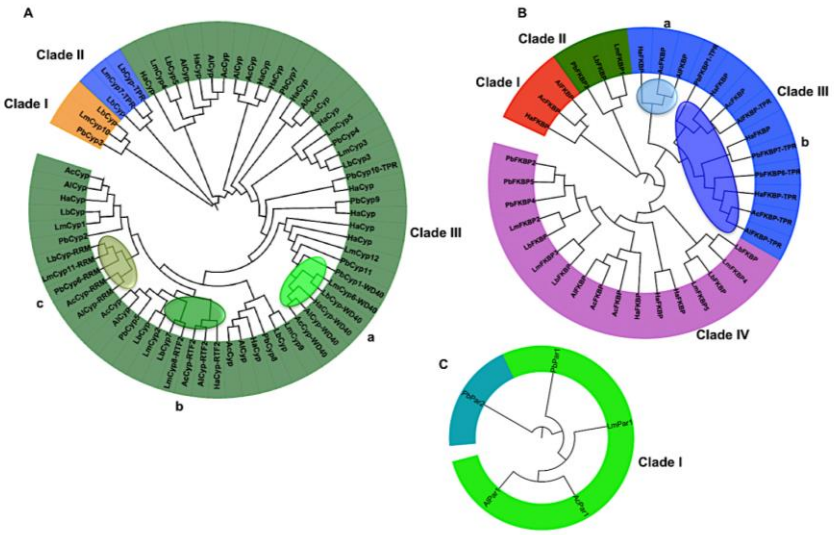

Fig. 16. Phylogenetic analysis of putative IMMs from various *Brassica* pathogens. A phylogenetic tree was constructed to determine the evolutionary relationship among the members of each of the subfamilies: (A) Cyclophilins (B) FKBPs and (C) Parvulins from *Plasmodiophora brassicae* (‘Pb’), *L. maculans* (‘Lm’), *L. biglobosa* (‘Lb’) and *Albugo candida* (‘Ac’), *Albugo laibachii* (‘Al’), and *Hyaloperonospora arabidopsidis* (‘*Ha*’). Clades and sub-clades are also shown.

4.5 Heterologous Expression of a *Plasmodiophora brassicae PbCYP3*

***Plasmodiophora brassicae* cyclophilin *PbCYP3* shares high similarity with phytopathogenic fungi and other organisms**

The deduced amino acid sequence of *PbCYP3* showed maximum identity homology with cyclophilin A from various organisms: *Magnaporthe oryzae* (62%), *L. maculans* (73%), *L. biglobosa* (73%), *P. nodorum* (70%), *P. tritici-repentis* (71%), *T. aestivum* (69%), *O. sativa* (68%), *Z. mays* (70%), *P. trichocarpa* (68%), *B. napus* (69%), *A. thaliana* (73%), *Phytophthora infestans* (71%), *P. nicotiana* (73%), *P. sojae* (73%), *P. parasitica* (73%), *Saprolegnia parasitica* (75%), and *H. sapiens* (71%). Dendrogram drawn from the amino acid sequences showed a closer relationship of *PbCYP3* with other members of plants, human, oomycetes and phytopathogenic fungi (Fig. 36A).

A

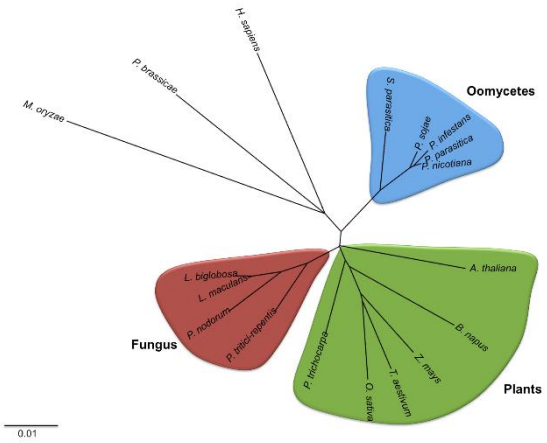

Fig 17. A) Unrooted phylogenetic tree of cyclophilin A showing the evolutionary relationship between *P. brassicae*, *M. oryzae*, *L. maculans*, *L. biglobosa*, *P. nodorum*, *P. tritici-repentis*, *T. aestivum*, *O. sativa*, *Z. mays*, *P. trichocarpa*, *B. napus*, *A. thaliana*, *Phytophthora infestans*, *P. nicotiana*, *P. sojae*, *P. parasitica*, *Saprolegnia parasitica*, and *H. sapiens*.

PbCYP3 is highly expressed in _Magnaporthe_ deletion mutant

PbCYP3 was cloned into *Bam*HI and *Kpn*I sites of expression vector pCB15322 to generate pCB15322:*PbCYP3* construct (Fig 18A). Transformation of *PbCYP3* using protoplast produced 20 transformants. qRT-PCR screening for the overexpression of *PbCYP3* was performed with five positive strains. These strains were putative overexpressed strains (Fig 18B). These overexpressed strains were named Pb6-Pb10. The *Magnaporthe* deletion strain *Δcyp1* mutant was used as a control (Fig 18B). All the overexpressed strains were differentially expressed in the deletion strains. This indicates that the *PbCYP3* is expressed ectopically. Amongst all Pb6 showed high expression while Pb7 showed the lowest expression (Fig 18C).

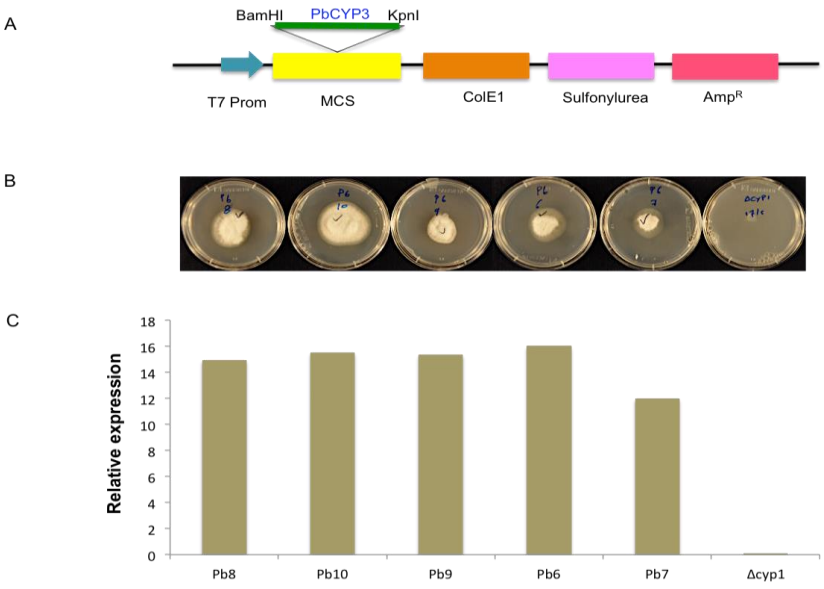

Fig 18. A. Schematic diagram of pCB15322:*PbCYP3* construct used for the transformation in *Magnaporthe oryzae*. The vector pCB15322 possesses T7 promoter, MCS site, Colicin E1 (the *cea* gene) and selection markers such as sulfonylurea (herbicide) and ampicillin. B. Protoplast transformation plates showing different *PbCYP3* overexpressed lines Pb6-Pb10. The transformants were selected on BDCM medium (without sucrose) containing 150µg/ml chlorimuron ethyl (sulfonylurea). C. Bar diagram showing relative expression of *PbCYP3* in various overexpressed lines and in deletion mutant (*Δcyp1*) of *M. oryzae* analyzed using qRT-PCR. Total RNA was extracted from mycelia after two-week incubation at 25 °C in dark.

***In planta* quantification of overexpressed line Pb6 showed similar amount like wild type**

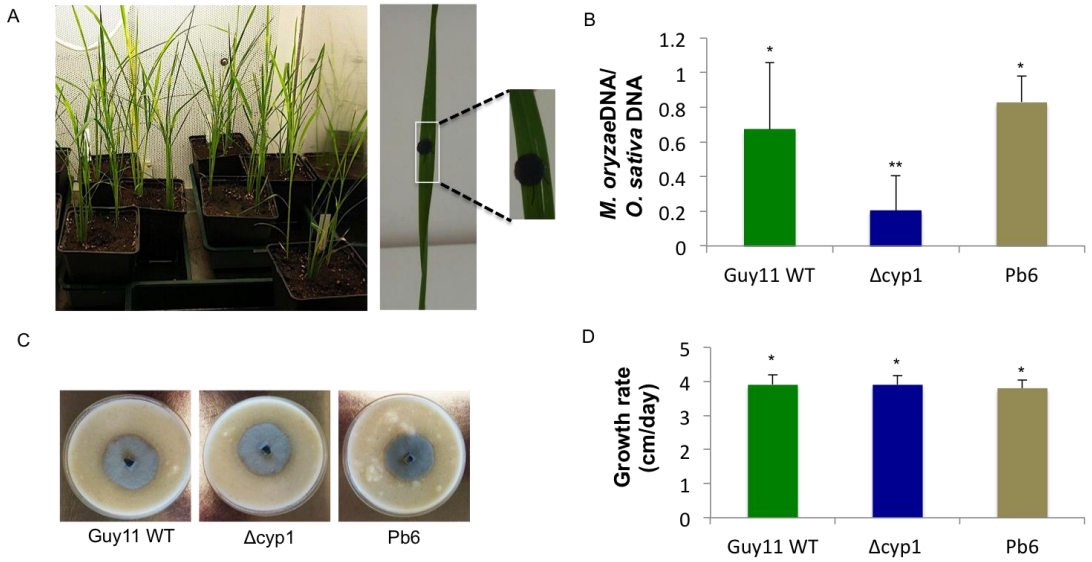

Fig 19. A) Four-week-old rice plants were artificially inoculated with wild type Guy11, *Δcyp1* mutant and overexpressed line Pb6. Agar plugs (4 cm diameter) derived from 2 weeks old cultures of wild type Guy11, deletion strain *Δcyp1* mutant and overexpressed line Pb6 were used. B) DNA quantification (qPCR) of *M. oryzae* was performed using actin primer from *M. oryzae* (MgActF/R) and elongation factor primer from *Oryza sativa* (OsElfF/R) (listed in Table 4). Different symbols (*, **) indicate statistically significant differences (*p value* < 0.05) within experiments based on the Tukey's HSD test. C, D) Colony morphology showed no significant variations between wild type Guy11, deletion strain *Δcyp1* mutant and overexpressed line Pb6. Cultures were maintained for 2 weeks at 25 °C in dark.

5. Conclusions

IMMs are a highly conserved protein family in phytopathogens. Reported here are the identification of the putative IMM repertoire in *Brassica* infecting phytopathogens including *L. maculans, L. biglobosa* and *P. brassicae.* The numbers of putative IMMs in these phytopathogens are highly correlated with other fungi and lower eukaryotes. The occurrence of IMMs in multiple copies highlights their pivotal role in various cellular processes in these phytopathogens. Subcellular localization prediction showed that IMMs in phytopathogens are localized in all major cellular compartments with majority targeted in cytosol. Similarity in domain architecture of the putative IMMs in *L. maculans, L. biglobosa* and *P. brassicae* revealed that they are co-evolved during evolution. Furthermore, it would be intriguing to know the role of additional domain/motifs present in the putative IMMs, which can also lead to discover novel roles related to protein folding and binding to RNA in the phytopathogens.

Higher expression of *CYP4* in *L. maculans* mycelium as well as in artificially inoculated hosts as compared to *L. biglobosa* demonstrated their role as determinants of virulence. The mutation at ^{168}Cysteine to ^{168}Serine in *L. biglobosa* presumably is the possible reason of low transcript abundance of *CYP4* as compared to *L. maculans*. Structure model of *LmCYP4*, *LbCYP4* and *PbCYP3* showed the presence of conserved patterns, i.e. eight anti-parallel β-pleated sheets, two α-helices and one β-loop region like other known cyclophilins A reported so far. Strikingly, *LmCYP4* and *LbCYP4* have a conserved divergent loop ^{48}RSGKPLH^{54}, which is a characteristic feature for plants cyclophilins in contrary to *P. brassicae.* Thus it shows that the *LmCYP4* and *LbCYP4* co-evolved with their hosts more closely compared to *P. brassicae.*

Complementation analysis of *PbCYP3* in *M. oryzae* served as appropriate platform to functionally characterize the gene due to its obligate nature. So, far IMMs in particular cyclophilin A in phytopathogens have been associated abundantly with the infection processes and/or virulence of plant infection. Unlike plant and human counterparts, many of their roles in phytopathogens are not thoroughly investigated yet. Therefore, characterizing other IMMs in phytopathogens may provide more insight into their roles and functions for pathogenicity and the infection process of yet not completely understood host-pathogen interactions. Future studies may aim to *implement RNAi mechanism to establish the role of LmCYP4 in pathogenicity of L. maculans.* Deciphering the role and mechanisms of pathogenicity based on cyclophilin activity might be the basis for new control strategies of *L. maculans, L. biglobosa* and *P. brassicae,* providing new targets for pesticides and/or genetically engineered plant defense.

6. References

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