Genome Wide Identification of the Immunophilin Gene Family in *Leptosphaeria maculans*: A Causal Agent of Blackleg Disease in Oilseed Rape (*Brassica napus*)

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Abstract

Phoma stem canker (blackleg) is a disease of world-wide importance on oilseed rape (*Brassica napus*) and can cause serious losses for crops globally. The disease is caused by dothideomycetous fungus, *Leptosphaeria maculans*, which is highly virulent/aggressive. Cyclophilins (CYPs) and FK506-binding proteins (FKBPs) are ubiquitous proteins belonging to the peptidyl-prolyl cis/trans isomerase (PPIase) family. They are collectively referred to as immunophilins (IMMs). In the present study, IMM genes, CYP and FKBP in haploid strain v23.1.3 of *L. maculans* genome, were identified and classified. Twelve CYPs and five FKBPs were determined in total. Domain architecture analysis revealed the presence of a conserved cyclophilin-like domain (CLD) in the case of CYPs and FKBP_C in the case of FKBPs. Interestingly, IMMs in *L. maculans* also subgrouped into single domain (SD) and multidomain (MD) proteins. They were primarily found to be localized in cytoplasm, nuclei, and mitochondria. Homologous and orthologous gene pairs were also determined by comparison with the model organism *Saccharomyces cerevisiae*. Remarkably, IMMs of *L. maculans* contain shorter introns in comparison to exons. Moreover, CYPs, in contrast with FKBPs, contain few exons. However, two CYPs were determined as being intronless. The expression profile of IMMs in both mycelium and infected primary leaves of *B. napus* demonstrated their potential role during infection. Secondary structure analysis revealed the presence of atypical eight β strands and two α helices fold architecture. Gene ontology analysis of IMMs predicted their significant role in protein folding and PPIase activity. Taken together, our findings for the first time present new prospects of this highly conserved gene family in phytopathogenic fungus.

Introduction

PLANTS BEING SESSILE IN NATURE, developed several adaptive responses towards various environmental abuses such as biotic and abiotic stresses (Agarwal et al., 2006). Taking into account the effect of environmental conditions on various agronomically important crops, it becomes relevant to study the molecular components involved in regulating plant responses to external stimuli (Mishra et al., 2013). *Brassica napus* is the third most important source of edible oil in the world after soybean and palm oil (El-Beltagi and Mohamed, 2010). In addition, the use of rapeseed oil in biodiesel production has also been investigated (Dmytryshyn et al., 2004) and is extensively produced in European countries (EASAC, 2012; Misra et al., 2013).

Phoma stem canker (blackleg) is a disease of world-wide importance on oilseed rape, (*Brassica napus*, canola, colza,

rapeseed, Raps) and can cause serious losses for crops globally (Howlett, 2004; West et al., 2001). The disease is caused by dothideomycetous fungi, *Leptosphaeria maculans* or *L. biglobosa* (Mendes-Periera et al., 2003). *Leptosphaeria maculans* was previously classified as highly virulent/aggressive or as A group type or Tox⁺ group, in contrast to *L. biglobosa*, which was classified as weakly virulent/nonaggressive or as B group type or Tox⁰ group (Rouxel et al., 2004; Shoemaker and Brun, 2001; Williams and Fitt, 1999). *Leptosphaeria maculans* and *L. biglobosa* were distinguished on the basis of morphological differences of pseudothecia (Shoemaker and Brun, 2001). Both fungi cause phoma leaf spots; however, it was suggested that *L. maculans* causes more serious phoma stem cankers, while *L. biglobosa* affects upper stem and causes superficial stem lesions (Fitt et al., 2006; Rouxel et al., 2004; Rouxel and Balesdent, 2005). The diversity between *L. maculans* and *L. biglobosa* species

complex was also investigated in order to decipher the phylogenetic relationship (Mendes-Pereira et al., 2003).

The family of immunosuppressant receptors called Immophilins (IMMs) includes cyclophilins (CYPs) and FK506-binding proteins (FKBPs) (Fischer et al., 1984; Harding et al., 1989). The family is characterized as having two important properties: peptidyl-prolyl cis/trans isomerase (PPIase) and drug binding. Cyclophilins bind to the immunosuppressive drug cyclosporin A (CsA) (Handschumacher et al., 1984), while FK506 binding proteins (FKBPs) bind to FK-506/rapamycin-binding proteins. Despite their lack of sequence and structural similarity, these two families share a common PPIase, catalyzing the cis/trans isomerization of proline imidic peptide bonds (Fisher et al., 1989).

All CYPs share a conserved domain, the cyclophilin-like domain (CLD), while FKBPs have a conserved FKBP_C domain. Depending upon the functional modules present, IMMs have been broadly classified into two groups: single domain (SD) and multidomain (MD) (Ahn et al., 2010). SD is characterized by the presence of a single catalytic CLD or FKBP domain exhibiting PPIase activity. In contrast, MD possesses other functional domains in conjunction with single or multiple catalytic CLD or FKBP domains. The additional functional domains include tetra-peptide repeat (TPR), WD40, coiled-coil domain (CCD), and internal repeats domain (RPT), Zinc Finger, and RRM, in addition to others (Ahn et al., 2010; Burkhard et al., 2001; Scheufler et al., 2000). The additional functional domains are involved in various processes, such as protein-protein interactions (Ke et al., 1993; Taylor et al., 2001) and RNA binding (Gasser et al., 1990; Luan et al., 1994).

Biochemical and sequence analysis following genome sequencing projects have led to the identification of a large number of IMMs in various organisms. *Saccharomyces cerevisiae* contains 12 genes (8 CYPs and 4 FKBPs) (Arevalo-Rodriguez et al., 2004), *Caenorhabditis elegans*: 24 genes (16 CYPs and 8 FKBPs), and *Aspergillus nidulans*: 16 genes (11 CYPs and 5 FKBPs) (Pemberton, 2006). Human have a total of 24 CYPs and 18 FKBPs (Galat, 2003). Notably, photosynthetic organisms possess a large number of IMMs, with 52 genes (29 CYPs and 23 FKBPs) in *Arabidopsis* (Romano et al., 2004), 56 in rice (27 CYPs and 29 FKBPs) (Ahn et al., 2010), and 49 in *Chlamydomonas* (23 CYPs and 26 FKBPs) (Vallon, 2005). The higher number of IMMs in green photosynthetic organisms was predicted because they are more targeted towards the photosynthetic apparatus (He et al., 2004; Peltier et al., 2002). The numbers of IMMs predicted in phytopathogenic fungi are more or less similar to non-photosynthetic organisms except human (Singh, unpublished data).

IMMs are highly conserved ubiquitous proteins (Galat, 1999) localized in all cellular compartments and encode diverse functions (Wang and Heitman, 2005). They are involved in many processes, such as spliceosome assembly (Horowitz et al., 2002), protein trafficking and maturation (Ferreira et al., 1996), receptor complex stabilization (Leverison and Ness, 1998), apoptosis (Lin and Lechleiter, 2002), receptor signaling (Brazin et al., 2002), RNA processing (Krzywicka et al., 2001), development of asexual reproductive structures and cellular turgor generation in appressoria in fungi (Viaud et al., 2002), RISC assembly (Lki et al., 2012), and others. However, the diverse roles and still emerging novel functions suggest that the IMMs encompass far more

functions than the already defined function of protein folding in different organisms.

Several reports reveal the involvement of IMMs in response to different types of biotic and abiotic stresses (Ahn et al., 2010; Aviezer-Hagai et al., 2007; Marivet et al., 1992; Meiri et al., 2010; Pogorelko et al., 2014). The expression of IMM genes has also been previously shown to be induced by both biotic and abiotic stresses, including fungal infection (Godoy et al., 2000), viral infection, salicylic acid, ethephon (an ethylene releaser), and HgCl₂ (Marivet et al., 1995), cold shock, salt stress, drought, and heat (Ahn et al., 2010; Marivet et al., 1994; Scholze et al., 1999; Sharma and Singh, 2003). Very few studies of IMMs in fungi have been reported. In the phytopathogenic fungus *Mangnaportha grisea*, the CYP gene acts as a determinant of virulence during rice blast disease (Viaud et al., 2002). Cyclophilin A of *Cryptococcus neoformans* exhibited distinct functions that are important for growth and virulence (Wang et al., 2001). Thus, IMMs act as virulence factors for both plant and animal diseases caused by fungi.

Fungi, especially *S. cerevisiae*, have been used extensively as a reduced and easily manageable model system in biological research (Grutzmann et al., 2013). Combination of bioinformatics and omics-based tools has enabled the discovery of new members of several gene families in plants such as ABA repressor 1 (*ABR1*) (Mishra et al., 2013), homeobox gene family (Jain et al., 2008), glycerol-3-phosphate acyltransferase (GPAT) (Misra and Panda, 2013), protein phosphatase 2C family (Xue et al., 2008), nucleocytoplasmic transport receptors family (Huang et al., 2010), and others.

In spite of the fact that IMMs are highly conserved in all organisms, the genes are not well studied in phytopathogenic fungi due to unsequenced genomes. However, the availability of the high quality genome sequence of an important fungus, *L. maculans*, and the lack of information about this multigene family, motivated us to conduct this study. Therefore, in the present study, we performed genome-wide analysis of IMM genes using bioinformatics tools, and thus identified putative members of each family, respectively in the haploid strain v23.1.3 of *L. maculans* genome, and performed a comparative analysis with *S. cerevisiae*. Furthermore, comprehensive analysis of the putative identified genes, including domain architecture, chromosomal distribution, phylogenetic analysis, sequence alignment and secondary structure features, exon-intron structure, expression pattern in mycelium, as well as in primary leaf of *B. napus* during disease infection and Gene Ontology (GO) terms, has also been carried out.

Materials and Methods

Searching protein database, nomenclature, and domain analysis of IMMs

In order to identify the members of the IMMs in *L. maculans*, the Hidden Markov Model (HMM) profiles unique to cyclophilin (accession no. PF00160) and FKBP (accession no. PF00254) from Pfam database (version 26.0) (<http://pfam.sanger.ac.uk/>) were retrieved and then searched against the genome browser database of *L. maculans* (<http://urgi.versailles.inra.fr/>) using HMMER 3.0 software (<http://hmmer.janelia.org/>). All the significant hits with positive scores were selected for further classification. The genes identified were thus assigned names, such as “Lm” (*L. maculans*), followed by “CYP” (cyclophilin) and “FKBP” (FK506-binding

proteins), according to the catalytic domain present, and then followed by numbers in increasing order based on highest HMM scores. The identified IMM genes were then analyzed for the presence of the respective catalytic domain (i.e., cyclophilin-like-domain (CLD) in the case of CYPs and FKBP_C in FKBP s using Pfam databases). The scaled positions of domains were drawn using the gplots package of the open source package R using R scripts.

Subcellular localization

Subcellular localization of IMM genes were predicted using PSORT (<http://wolfsort.org/>). Signal peptide cleavage sites 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/>).

Chromosomal distribution of IMM genes

Genomic distribution of putative IMM genes were determined using the *L. maculans* genome browser (<http://urgi.versailles.inra.fr/>) and mapped on their respective chromosomes. The scaled positions of IMM gene family CYPs and FKBP s on respective chromosomes were drawn using the gplots package of the open source package R using R scripts.

Exon–intron structure determination

In order to determine the gene structures for IMM genes, coding sequences corresponding to each gene (CYP/FKBP) were mapped onto genomic sequences including UTRs, exons, and introns. The coding sequences were obtained from the *L. maculans* genome database, while genomic sequences were identified from contigs and supercontigs (SCs). The contigs and SCs were aligned and further subjected to FGENESH software (<http://linux1.softberry.com/>) using the *L. maculans* genome as a query and with default parameters. The genomic sequences and coding sequences from each CYP/FKBP gene were thus mapped using Gene Structure Display Server (GSDS) (<http://gsds.cbi.pku.edu.cn/>) and cross checked by using Spidey (<http://www.ncbi.nlm.nih.gov/spidey/>). The final figures were drawn using GSDS.

Phylogenetic analysis and secondary structure features

Multiple sequence alignment of amino acid sequences belonging to CYP and FKBP from *L. maculans* and *S. cerevisiae* 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/>). Secondary structure features were predicted using Jpred3 server (<http://www.compbio.dundee.ac.uk/www-jpred/>). The phylogenetic trees were generated using ClustalX implementing neighbour-joining (NJ) method. Bootstrap values were calculated at 1, 000 iterations. The final phylogenetic tree was then edited using ITOL (<http://itol.embl.de/>).

Expression analysis of IMMs

The transcriptomes of *L. maculans* IMMs were analyzed in mycelium and infected leaves of *B. napus* at two time points:

7 and 14 disease post infection (dpi). The expression data were obtained from GSE27152 of Gene Expression Omnibus (GEO) (Soyer et al., 2014). The results were log transformed values of the average of three replicates. The hierarchical clustering analysis and the heatmaps were made using the gplots package of R software, as described earlier (Singh et al., 2011).

Conserved motif analysis and sequence logo

To determine the functional motifs within IMM genes, Multiple Em for Motif Elicitation (MEME) version 4.9.0 (<http://meme.nbcr.net/meme/>) from the Plant Intron Exon Comparison and Evolution database (PIECE) (<http://wheat.pw.usda.gov/piece/>) was employed with default parameters. Therefore, the conserved motifs in respective sequences were represented as block diagrams. The sequence logos of motifs were also generated using MEME suite.

Gene ontology (GO) term identification

The GO terms, including Biological Process (BP), Molecular Function (MF), and Cellular Process (CC) for IMMs were retrieved from the *L. maculans* genome database (<http://genome.jgi-psf.org/>). The datafile was obtained using BLAST with *L. maculans* predicted proteins (12, 469) against the nonredundant (nr) database to obtain the similarity with known sequences and thus mapped onto the Gene ontology, geneinfo, gene2accession, and PIR databases (Rouxel et al., 2011).

Results

Conservation and characterization of IMM genes in *L. maculans*

Members of the IMM genes in *L. maculans* genome were determined using HMM profiles unique to CYPs and FKBP s from Pfam (see Materials and Methods). All sequences returned by such searches were selected for further classification and then examined individually for accessory domains. Following this strategy, a total of 12 distinct CYPs (*LmCYP1-LmCYP12*) and 5 FKBP s (*LmFKBP1-LmFKBP5*) genes were identified (Table 1).

The characterization of 12 CYP and 5 FKBP genes, including nomenclature, accession number, length of amino acids (aa), isoelectric points (pI), molecular weights (Mw), subcellular localization, nuclear localized signal (NLS), signal peptide (SP), mitochondrial targeting peptide (MTP), and transmembrane (TM) profile are listed in Table 1. These reveal that the lengths of CYP proteins varied from 663 aa (*LmCYP6*) to 165 aa (*LmCYP12*; *LmCYP2*). However, in the case of FKBP s, the longest protein, *LmFKBP1*, has 549 aa, and the shortest, *LmFKBP4*, has only 113 aa (Table 1). The isoelectric point ranged from 5.01 (*LmCYP12*) to 8.98 (*LmCYP3*) in the case of CYPs, and from 4.58 (*LmFKBP1*) to 5.81 (*LmFKBP4*) (Table 1) for FKBP s. The molecular weight of CYP proteins were found to be lowest in *LmCYP12* (16.8 kDa) and highest in *LmCYP6* (74.1 kDa), in contrast to FKBP proteins, which ranged from 12.2 kDa in *LmFKBP4* to 59.7 kDa in the case of *LmFKBP3* (Table 1).

Domain architecture of IMM genes

Leptosphaeria maculans IMMs are categorized into Single Domain (SD) and Multi Domain (MD) depending upon the

TABLE 1. PUTATIVE IMM GENE FAMILIES IN PHYTOPATHOGENIC FUNGI *LEPTOSPHAERIA MACULANS*

Genes		INRA accession no	a.a	pI/Mw	Localization	cNLS	Nucpred	TargetP	TMHMM
A. CYPs									
<i>LmCYP1</i>	jgi Lepmul	9391 Lema_P042100.1	166	6.91/18.1	Cytoplasm	—	—	—	—
<i>LmCYP2</i>	jgi Lepmul	9823 Lema_P051520.1	165	6.51/18.0	Cytoplasm	—	—	—	—
<i>LmCYP3</i>	jgi Lepmul	7455 Lema_P023490.1	222	8.98/24.4	Nuclear	—	—	—	—
<i>LmCYP4</i>	jgi Lepmul	11701 Lema_P059530.1	171	7.75/18.1	Cytoplasm	—	—	—	—
<i>LmCYP5</i>	jgi Lepmul	2071 Lema_P083030.1	218	5.57/22.8	Cytoplasm	—	—	—	—
<i>LmCYP6</i>	jgi Lepmul	12014 Lema_P062660.1	663	6.28/74.1	Nuclear	NLS	NLS	—	—
<i>LmCYP7</i>	jgi Lepmul	3 Lema_P000030.1	375	5.92/41.4	Cytoplasm	—	—	—	—
<i>LmCYP8</i>	jgi Lepmul	2748 Lema_P080490.1	560	8.43/61.5	Cytoplasm	—	—	—	—
<i>LmCYP9</i>	jgi Lepmul	6039 Lema_P020130.1	497	8.88/54.8	Nuclear	NLS	NLS	—	—
<i>LmCYP10</i>	jgi Lepmul	10852 Lema_P067630.1	228	9.46/24.4	Mitochondria	—	—	MTP	—
<i>LmCYP11</i>	jgi Lepmul	3608 Lema_P091210.1	478	5.98/55.0	Cytoplasm	NLS	NLS	—	—
<i>LmCYP12</i>	jgi Lepmul	2458 Lema_P077590.1	165	5.01/16.8	Cytoplasm	—	—	—	—
B. FKBP									
<i>LmFKBP1</i>	jgi Lepmul	8148 Lema_P030420.1	549	4.58/59.7	Mitochondria	—	—	MTP	—
<i>LmFKBP2</i>	jgi Lepmul	6860 Lema_P118050.1	134	4.87/14.5	Extracellular	—	—	SP	—
<i>LmFKBP3</i>	jgi Lepmul	1110 Lema_P011100.1	491	4.95/53.7	Plasmamembrane	—	—	SP	Y
<i>LmFKBP4</i>	jgi Lepmul	2431 Lema_P077320.1	113	5.81/12.2	Cytoplasm	—	—	—	—
<i>LmFKBP5</i>	jgi Lepmul	11454 Lema_P057060.1	124	5.13/13.2	Cytoplasm	—	—	—	—

Included are nomenclature, molecular weight, isoelectric point, amino acid length (a.a), predicted subcellular localization, nuclear localization signal (NLS), mitochondria targeting peptide (MTP), signal peptide (SP), and transmembrane.

functional modules present (Ahn et al., 2010). In *L. maculans*, the number of SD IMMs is remarkably higher compared to MD. In the case of CYP, 8 out of 12 genes viz. *LmCYP1*, *LmCYP2*, *LmCYP3*, *LmCYP4*, *LmCYP5*, *LmCYP9*, *LmCYP10*, and *LmCYP12* were found to be SD (Fig. 1). However, 4 of 12 CYPs were found to be MD: *LmCYP6*, with a N-terminal WD40 domain; *LmCYP7*, that possesses a 2 C-terminal TPR; *LmCYP8* with a N-terminal Rtf2 domain; and *LmCYP11*, which has a C-terminal RRM domain (Fig. 1). Notably, there was no MD found with multiple catalytic domains in *L. maculans*. In the case of FKBP, 4 out of 5

genes have been classified as SD members (i.e., *LmFKBP1*, *LmFKBP2*, *LmFKBP4*, and *LmFKBP5*) and possess only single FKBP catalytic domain (Fig. 1). *LmFKBP3* is the only MD member, possessing vacuolar membrane protein (VMP) in addition to FKBP (Fig. 1).

Subcellular localization of IMM genes in *L. maculans*

IMMs are present in all major subcellular compartments. In *L. maculans*, the majority of the proteins were found to be cytosolic, nuclear, and mitochondria localized. Out of 12 CYP,

FIG. 1. Domain architecture of the *L. maculans* IMM gene family members: (A) CYPs and (B) FKBP. 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, vacuolar membrane protein.

7 viz. *LmCYP1*, *LmCYP2*, *LmCYP4*, *LmCYP5*, *LmCYP7*, *LmCYP8*, and *LmCYP11* were predicted to be localized in cytoplasm (Table 1). 4 CYPs, viz. *LmCYP3*, *LmCYP6*, *LmCYP9*, and *LmCYP11* are localized in nucleus (Table 1). *LmCYP6* possesses an N-terminal NLS, while both *LmCYP9* and *LmCYP11* have NLS at the C-terminal (Fig. 1). *LmCYP10* is the only CYP localized in mitochondria (Table 1) and also possesses an N-terminal MTP (Fig. 1). There were no CYP found to be present in endoplasmic reticulum (ER) and chloroplast.

In the case of FKBP, 3 out of 5 are localized in cytosol. Similarly, one is localized in the plasma membrane and one in mitochondria. Cytosolic FKBP include *LmFKBP2*, *LmFKBP4*, and *LmFKBP5* (Table 1). *LmFKBP2* is a cytosolic CYP possessing an N-terminal signal peptide (SP), compared to *LmFKBP4* and *LmFKBP5* (Fig. 1). Although *LmFKBP3* is located in the plasma membrane, it also contains SP and transmembrane (TM) profiles, which make this protein unique among other FKBP family members. Mitochondrial *LmCYP1* has MTP at the N-terminal (Fig. 1).

Genomic distribution of IMMs

The *L. maculans* genome has been assembled into 17–18 chromosomes. There are 10 chromosomes that are completely annotated while others are incomplete. IMMs were found to be unevenly distributed on different chromosomes. In the case of complete chromosomes, Chromosome 0 (SC0) is the longest among all (4.25 Mb) and possesses *LmCYP7* and *LmFKBP3*. Chromosome 1 (SC1; 3.37 Mb) has *LmCYP9*, and chromosome 4 (SC4; 1.91 Mb) contains *LmCYP1*. *LmCYP2* was located on chromosome 5 (SC5; 1.86 Mb), and chromosome 7 (SC7; 1.76 Mb) possesses *LmCYP10*. *LmCYP6* was located on chromosome 9 (SC9; 1.77 Mb). Chromosome 21 (SC21; 3.30 Mb), which comprises two other supercontigs (i.e., SC20 and SC23), is 3.30 Mb long and possesses only single FKBP gene, *LmFKBP2* (Fig. 2) (Rouxel et al., 2011).

Partial chromosomes, including SC2, SC8, SC12, SC13, and SC15, are also included among IMM genes. Chromosome 2 (SC2) and chromosome 19 (SC19) may either form a single chromosome of size 4.87 Mb or two chromosomes sized greater than 3.43 Mb. The exact size is difficult to establish due to lack of one telomere and presence of rDNA repeats. Therefore, we combined both chromosomes (SC2 and SC19 together) and formed one single chromosome named chromosome 2. The chromosome becomes the longest amongst all. Chromosome 2 (SC2) has *LmCYP3* and *LmFKBP1*. The other two chromosomes, chromosome 8 (SC8) and chromosome 10 (SC10), were also assembled together to form a single chromosome of size 3.75 Mb, and named chromosome 8. Notably, one CYP (*LmCYP4*) and one FKBP (*LmFKBP5*) were found to be localized on chromosome 8. Chromosomes 12 (SC12), chromosome 15 (SC15), and chromosome 13 (SC13) are dubiously assembled, and thus uncertain. Therefore, we kept the aforementioned chromosomes separate. Chromosome 12 (SC12) has size 1.63 Mb and possesses *LmCYP5*, and chromosome 15 (SC15) comprises the *LmCYP11* gene. Chromosome 13 is still not assembled and had no genetic map evidence (Rouxel et al., 2011). Interestingly, it was found to have the maximum number of IMM genes [i.e., 3, irrespective of its size (1.63 Mb)]. It consists of two CYPs viz. *LmCYP8* and *LmCYP12*, and one FKBP gene, *LmFKBP4*, as shown in Figure 2 (Rouxel et al., 2011).

Phylogenetic analysis and secondary features of IMMs

Comparative evolutionary relationships between IMM gene family CYPs and FKBP, from *L. maculans* and *S. cerevisiae*, were analyzed by constructing phylogenetic trees supported by bootstrap values with 1000 replications using the clustalX software program. Homologous gene pairs with more than 90% bootstrap values were grouped together

FIG. 2. Genomic distribution of *L. maculans* IMMs on various chromosomes. CYPs and FKBP represented with horizontal green and pink bars. Arrows show the direction of the ORF specific to the gene encoding CYP and FKBP proteins (i.e., lower for sense strand upper for antisense strand). Only the chromosome having IMMs are shown; their number is indicated at the top. The chromosome length has been shown in Mb.

(Fig. 3A and 3B). In the case of CYPs, Group1 includes *CPR2*, *CPR5*, Group 2 includes *LmCYP2* and *LmCYP8*, Group3 includes *CPR4* and *CPR8*, and Group4 includes *LmCYP9*, *LmCYP11*, and *LmCYP12* (Fig. 3A). FKBP s formed three major groups: Group5 with *FPR3* and *FPR4*, Group 6 with *LmFKBP4* and *LmFKBP5*, and finally Group7 with *LmFKBP2* and *LmFKBP3* as shown in Figure 3B. Different orthologous gene pairs between two genomes are shown in Table 2. The secondary structure features were also compared from the deduced aa. sequences of CYPs and FKBP s from both genomes. Remarkably, typical 8 anti-parallel β strands and 2 α helices were observed in the case of CYPs (Fig. 4). In contrast, there were 8 beta sheets and only 1 helix observed in FKBP s (Fig. 5).

Exon–intron architecture analysis

To understand the structural components of *L. maculans* IMM s, their exon–intron organization was analyzed. Interestingly, we found that IMM genes in *L. maculans* contain short introns compared to exons (Fig. 6), as is the case in other fungi (Grutzmann et al., 2014). Based on their intron length, in the case of CYPs, *LmCYP1* has the smallest intron of 48 bp, while the longest one is in *LmCYP5*, with 188 bp. However, the length of exons in CYPs varies drastically, from 1854 bp of the longest one in *LmCYP6* to 33 bp of the smallest one in *LmCYP1*. In the case of the FKBP family, *LmFKBP3* contains the shortest intron (49 bp), and *LmFKBP5* has the longest one (303 bp). Similarly, *LmFKBP1* has the longest exon with 715 bp, compared to the shortest one in *LmFKBP5* with 21 bp. Interestingly, we found that 2 genes are intronless, namely *LmCYP8* and *LmCYP9*.

In terms of the number of exons and introns, *LmCYP4*, *LmCYP3* and *LmCYP10* possess 4 exons and 3 introns each

(Fig. 6). *LmCYP5* has 3 exons and 2 introns. Likewise, *LmCYP1*, *LmCYP2*, *LmCYP6*, *LmCYP7*, *LmCYP11*, and *LmCYP12* all equally possess 2 exons and 1 intron. Despite IMM gene family CYPs and FKBP s sharing a common peptidyl-prolyl isomerase (PPIase), catalyzing the cis/trans isomerization of proline imidic peptide bonds (Fisher et al., 1989), the exon–intron architecture varies drastically. It was observed that FKBP s in *L. maculans* contain remarkably higher number of exons and introns compared to CYPs, irrespective of size (Table 2). *LmFKBP5* consists of 7 exons and 6 introns, *LmFKBP1* possesses 5 exons and 4 introns, both *LmFKBP2* and *LmFKBP3* have 3 exons and 2 introns, and *LmFKBP4* has 4 exons and 3 introns.

Transcriptomic expression analysis of IMM s

We have used publicly available transcriptome data for carrying out expression analysis of IMM genes identified in this study (“Materials and Methods”). Heat maps showing information about mycelium and primary leaf infection specificity of the expression of IMM genes of *L. maculans*, have been generated using gplots from the R-statistical software package (Fig. 7). Based on the expression patterns of CYPs, they have been subgrouped into three classes, viz. Highly expressed, Moderately expressed, and Low expressed (Fig. 7A). IMM genes, such as *LmCYP1*, *LmCYP2*, *LmCYP5*, and *LmCYP10*, were constitutively highly expressed in mycelium and infected primary *B. napus* leaves, thereby indicating their enormous significance during infection. In contrast, IMM genes, such as *LmCYP3*, *LmCYP7*, *LmCYP8*, and *LmCYP12*, were found to be moderately expressed during *in vitro* growth (mycelium), as well as during primary *B. napus* leaf infection. Interestingly, *LmCYP3* and *LmCYP7* were found to be more expressed during mycelium growth

FIG. 3. Phylogenetic tree depicting the evolutionary relationship between *L. maculans* and *S. cerevisiae* IMM s. CYPs (A) and FKBP s (B). The tree was constructed using neighbor-joining method using 1000 bootstrap replicates. Homologous gene pairs with more than 90% bootstrapped value have grouped and shown in solid box.

FIG. 4. Multiple sequence alignment of CYPs from *L. maculans* and *S. cerevisiae*. The secondary structures are also represented (i.e., α helix (red bar) and β sheets (green bar)).

FIG. 5. Multiple sequence alignment of FKBP from *L. maculans* and *S. cerevisiae* FKBP genes. The secondary structures are also represented (i.e., α helix (red bar) and β sheets (green bar)).

FIG. 6. Exon-intron architecture of *L. maculans* IMM genes. CYPs (A) and FKBP (B). Exons are marked in block, while intron with line. Lengths of genes are shown in bp scale.

TABLE 2. *S. CEREVISIAE* IMM GENE FAMILY AND COMPARISON WITH *L. MACULANS*

<i>S. Cerevisiae</i> CYPs	<i>L. maculans</i> orthologue	Localization	a.a	Mw	pI
A. CYPs					
CPR1	LmCYP4	Cytosol	162	17.39	690
CPR2	LmCYP1	Extracellular	205	22.76	574
CPR3	LmCYP10	Mitochondria	182	19.91	881
CPR4	LmCYP2	Extracellular	318	35.77	642
CPR5	LmCYP3	ER	225	25.32	535
CPR6	LmCYP7	Cytosol	371	42.07	584
CPR7	LmCYP5	Cytosol	393	45.13	526
CPR8	LmCYP6	Mitochondria	308	34.94	652
B. FKBP					
FPR1	LmFKBP4	Cytosol	135	14.0	534
FPR2	LmFKBP2	Extracellular	114	12	572
FPR3	LmFKBP1	Nuclear	411	46.5	436
FPR4	LmFKBP5	Nuclear	392	43.9	458

Included are subcellular localization, amino acid length (a.a), molecular weight (Mw), and isoelectric point (pI).

compared to *LmCYP12* and *LmCYP8*, showing their limited effect during plant infection (Fig. 7A). The expression of *LmCYP4*, *LmCYP6*, *LmCYP9* and *LmCYP11* were found to be low in mycelium as well as in infected primary *B. napus* leaves, suggesting them less significant during infection (Fig. 7A).

FKBPs were classified into two major classes (expressed at high and low levels) (Fig. 7B). IMM, such as *LmFKBP1*, *LmFKBP3*, and *LmFKBP5*, were found to be highly expressed during both mycelium growth, as well as during primary *B. napus* leaf infection. This clearly demonstrates their functional significance during infection. Strikingly, *LmFKBP5* expressed strongly in leaves compared to myce-

lium. In contrast, the expression of *LmFKBP2* and *LmFKBP4* were found to be constitutively lower during both mycelium growth as well as during primary *B. napus* leaf infection (Fig. 7B).

Conserved motifs in IMMs

In order to determine the conserved motifs present in *L. maculans* IMMs, aa sequences were subjected to MEME suit ("Materials and Methods"). It was observed that IMM genes possess highly conserved motifs with significant *p* value (<0.0001). The conserved motifs and sequence logo for CYPs and FKBP are thus represented in Supplementary Figures S1 and S2, respectively.

Gene Ontology (GO) term identification

GO terms, including Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) were retrieved for all IMMs: BP, including protein folding (GO:0006457) (Supplementary Table S1), MF, peptidyl-prolyl cis trans isomerase activity (PPIase) (GO:0003755), cyclophilin-type peptidyl-prolyl cis trans isomerase activity (GO:0042027), FK506-sensitive peptidyl-prolyl cis trans isomerase activity (GO:0030051), ubiquitin-protein ligase activity (GO:0004842), and nucleic acid binding (GO:0003676) (Supplementary Table S1). Cellular components (CC), including ubiquitin-ligase complex (GO: 0000151) were thus represented in Supplementary Table S1.

Discussion

IMM gene families code for ubiquitous proteins with conserved peptidyl-prolyl cis/trans isomerase (PPIase) activity, and thus are conserved in all organisms. We have systematically retrieved and analyzed the putative IMMs from phytopathogenic ascomycetous fungus *L. maculans*, the causal agent of phoma stem canker (blackleg) which is a

FIG. 7. Expression analysis 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 FKBP (B). CYPs and FKBP are classified into three and two main groups based on expression. Scale position is shown at the top of each heat map.

disease of world-wide importance on agronomically important crop (i.e., oilseed rape) (Fitt et al., 2006; Howlett, 2004; West et al., 2001). IMM genes have been implicated in a wide variety of processes. The conservation of IMM genes in phytopathogenic fungi shows their crucial role in a vast majority of processes. IMM genes were demonstrated to play a potent role as virulence determinant in both plant and animal diseases.

In *C. neoformans*, the CPA1 and CPA2 cyclophilins proteins play a shared role in cell growth, mating, and virulence (Wang et al., 2001). *CYP1*, a cyclophilin-encoding gene in the phytopathogenic ascomycetes *M. grisea*, seems to regulate virulence-related functions including aporesium turgor generation and lipid generation (Viaud et al., 2002). Similarly, IMM gene family cyclophilin (*CYPA*) in the phytopathogenic fungus *Botrytis cinerea* were shown to be involved in different aspects of morphogenesis and virulence (Viaud et al., 2003). Similar to other non-photosynthetic organisms, such as *S. cerevisiae*, *C. elegans*, and *A. nidulans* (Arevalo-Rodriguez et al., 2004; Pemberton, 2006), in which putative IMM genes have been identified, *L. maculans* has also been shown to possess low number of IMM genes [i.e., 17 altogether (12 CYPs and 5 FKBP)]. The number of IMM genes in photosynthetic organisms, such as *Arabidopsis*, rice, and *Chlamydomonas*, are remarkably higher, which shows that they are more targeted toward the photosynthetic apparatus (Ahn et al., 2010; He et al., 2004; Peltier et al., 2002; Romano et al., 2004; Vallon, 2005). *L. maculans* IMM proteins exhibited wide variation on the basis of their amino acid (aa) length, molecular weight (Mw), isoelectric point (pI), and presence of signal peptides at N and C-terminal region; however, their catalytic domains were found to be highly conserved, supporting the previously made observations (Wang and Heitman, 2005). IMM genes in *L. maculans* are also categorized into single domain and multi domain (MD), such as in the other organisms that have been studied to date (Ahn et al., 2010). In the case of MD, the presence of other functional domains, such as tetratricopeptide repeat (TPR), WD40 repeats, RNA-binding domain (RNP) or RNA recognition motif (RRM) and vacuolar membrane protein (VMP), in addition to CLD/FKBP, may also reveal their role in diverse functions (Bannikova et al., 2013; Li et al., 2007; Scheufler et al., 2000; Taylor et al., 2001).

IMM gene family CYPs and FKBP are localized throughout the cell and thus perform various functions (Wang and Heitman, 2005). In the present study, in *L. maculans*, they were predominantly localized in three main compartments (i.e., cytoplasm, nucleus, and mitochondria). However, only one gene was found to be localized in plasma membrane. Cytoplasmic cyclophilin A is a well-known target for the binding of immunosuppressive drug CsA in human (Handschumacher et al., 1984). Cyclophilin gene *CYP1*, from *M. grisea*, encodes a protein localized in mitochondria and cytosol, thus playing a crucial role during plant infection causing rice blast disease (Viaud et al., 2002). A similar, bifunctional *CYPA* from *B. cinerea* is localized in the mitochondria (Viaud et al., 2003). It is possible that the IMM genes from *L. maculans* can also act as virulence determinants against blackleg disease in *B. napus*. However, further experimental validation is needed to check the exact role of IMM genes in *L. maculans*.

Nuclear IMM genes are required during embryogenesis (Carol et al., 2001). Mitochondrial and nuclear encoding IMM genes in

L. maculans also possess mitochondrial targeting peptide (MTP) and nuclear localized signal (NLS). Additionally, mitochondrially localized IMM genes are significantly involved in mitochondrial permeability, which leads to apoptosis (Zoratti and Szabo, 1995). In fungi, mitochondrial cyclophilin also accelerates folding of newly imported proteins within the matrix as part of a complex that includes the chaperones Hsp60 and Hsp70 (Matouschek et al., 1995; Rassow et al., 1995). There is no evidence available regarding endoplasmic reticulum (ER) and chloroplast localized genes. Perhaps, *L. maculans* lacks IMM genes that are localized in chloroplast, a plant-specific organelle, thereby explaining the fact that the plant IMM gene has a conserved function in photosynthesis (He et al., 2004). *L. maculans* genome was found to encode only one IMM, *LmFKBP3*, with predicted targeting to the signal peptides (SP) possibly suggesting their roles in secretory pathways as well (He et al., 2004).

Comparative phylogenetic analysis between *L. maculans* and *S. cerevisiae* IMM genes was performed to understand the relationship between the two genomes, using clustalX and a bootstrap value of 1000 iterations. Notably, the bootstrap value of the parent node, as well as the node of individual clades, was found to be low, indicating high sequence variations in proteins in both genomes. The variation in IMM gene family CYPs and FKBP from *L. maculans* and *S. cerevisiae* possibly indicates their divergent evolution. However, only a few homologous and orthologous gene pairs could be identified. Similarly, homologous genes were clustered in different groups based on their high bootstrap value and thus marked on the phylogenetic tree. The orthologous gene pairs between the two genomes are shown in Table 2. Secondary structure prediction revealed that the CYPs and FKBP in *L. maculans* have conserved secondary structure features, such as other organisms studied to date (Ahn et al., 2010; Kay, 1996). In the case of CYPs, typical eight β strands and two α helices were observed, while FKBP has eight β strands and one α helix. GO analysis of IMM genes in the phytopathogenic fungus *L. maculans* also suggests their role in diverse processes, such as protein folding, PPIase activity, ubiquitin-protein ligase activity, and nucleic acid binding (Ahn et al., 2010; He et al., 2004).

Despite the observation that IMM gene family CYPs and FKBP share a common PPIase, catalyzing the cis/trans isomerization of proline imidic peptide bonds, there are variations in exon-intron architecture. Gene structure analysis of IMM genes in *L. maculans* showed the presence of smaller introns compared to exons. The small size of introns in genes is due to a compact genome (45.12 Mb), suggesting a correlation between intron and genome size. Intron and exon lengths within a genome can reflect the constraints imposed by splicing. In *S. cerevisiae*, due to a compact genome, genes usually have small introns (Grutzmann et al., 2014). They also show extended consensus sequences for the 5' canonical splice site (SS) and the branchpoint region (Kupfer et al., 2004). These features facilitate a structural interpretation of intron sequences, and they suggest low-complex alternate splicing patterns, both of which make fungi attractive models for mechanistic studies of alternative splicing (Grutzmann et al., 2014). Notably, in *L. maculans*, two CYPs were found to be intronless. However, more strikingly, the FKBP genes in *L. maculans* contain remarkably higher number of exons and introns compared to CYPs, irrespective of size.

The expression of IMM genes has previously been shown to be induced by both biotic and abiotic stresses (Chou and Gasser, 1997; Godoy et al., 2000; Marivet et al., 1992). Although there are several reports about the potential role of IMMs in various biotic stresses, only few studies are available about phytopathogenic fungi IMMs and their expression during plant infection (Viaud et al., 2002). Therefore, present studies also demonstrated the expression profiles of IMM genes of *L. maculans*, depicting their potential role in infection *in vitro* (mycelium) as well as in infected plant leaves at two time points viz. 7 and 14 dpi. *L. maculans* IMMs were shown to be differentially expressed in mycelium and *B. napus* leaves. In total, 7 IMMs out of 17 IMMs were shown to be highly expressed during mycelium and leaves infection. This demonstrates their significant role in disease development and regulation during infection. However, 10 IMMs were found to be either moderately or weakly expressed, thereby suggesting downregulation of these genes in mycelium as well as in infected in *B. napus* leaves.

Conclusions

Immunophilins have not been well studied in phytopathogenic fungi and their precise number, architecture, role in biotic stress, and transactional regulation is poorly understood. However, their role in biotic stress derived from previous studies have provided us knowledge to identify this family in phytopathogenic fungi *L. maculans* causing economical important disease 'Blackleg' in oilseed rape. Thus the fungus is pivotal in agrigenomics-related research. Our study, for the first time, has identified and classified this important gene family in *L. maculans* and compared them with model organism *Saccharomyces cerevisiae*. The expression profile in both mycelium and infected primary of *B. napus* leaves demonstrated their potential role during disease infection. Taken together, our findings present new prospects of this important gene family in phytopathogenic fungi. Further studies may aim to functionally characterize the differentially regulated immunophilins, furthering our understanding of the underlying regulatory network of gene expression and delineating the precise role of *L. maculans* immunophilins during disease infection in *B. napus*.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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