Identification and functional analysis of LysM effectors from *Marssonina brunnea*

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Abstract Popular is not only an important tree species economically, but is also a model system for plant biology. Marssonina leaf spot is a serious leaf disease caused by *Marssonina brunnea*, which can result in significant economic losses. *M. brunnea* is considered to be a hemibiotrophic pathogen but its pathogenesis remains unclear. Full length cDNA of 24 LysM effector genes were obtained based on the sequencing of the *M. brunnea* genome. All of these genes belonged to a single gene family for which the characteristics of the amino acid sequences and protein domains were analyzed. Further detailed analysis was carried out on two representative gene family members. These two genes were upregulated during the infection process and, when transformed into *Arabidopsis thaliana*, reduced the plant's sensitivity to chitin thus might compromised its immune system. LysM effectors appear to show a specific affinity for chitin since the expressed proteins co-precipitated with insoluble chitin. We performed a series of studies into the molecular mechanism of LysM effectors from *M. brunnea* and carried out a preliminary analysis into the interaction between the pathogen and its host. This research lays a foundation for further investigation into forest tree resistance breeding.

Keywords Chitin · Effector · Gene family · Pathogenic fungi

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Introduction

Black spot disease, caused mainly by *Marssonina brunnea*, is a destructive fungal disease of poplar (Spiers 1983). When poplars are infected, punctiform black spots appear on the leaves and these then become enlarged resulting in premature defoliation. Consequently, the life cycle of the infected poplar is greatly shortened, together with a rapid reduction in the production of biomass (Wu et al. 1992). Since the Populus genus is the most important tree genus economically for this country, black spot disease has caused enormous losses over the last few decades.

Currently, few effective methods can prevent or control black spot disease. Chemical methods are restricted by the lack of suitable antifungal agents, whilst conventional disease-resistance breeding has limitations due to the low efficiency of selection and long breeding period. Hence, there is a need for molecular biology-based applications to be developed. In order to do this, a better understanding of the molecular mechanisms is required.

M. brunnea is a pathogenic hemibiotrophic fungus and as such is biotrophic, growing first on living tissue, and then later, necrotrophic, getting its nutrients from dead tissues by killing the host plant. *M. brunnea* displays a relatively long infection period before it finally kills the tissue. During this process, pathogen-associated molecular patterns (PAMPs) from the pathogenic fungus are recognized by *Populus*, eliciting the pathogen-triggered immunity (PTI) of host (Jones and Dangl 2006). Hence, *M. brunnea* also needs to take measures to suppress the host's defense reactions to enable further invasions. An effective strategy for doing so is for the pathogenic fungi to secrete small effector proteins that may aid the pathogen in evading recognition by the plant. Effectors play a crucial role in blocking signal transmission or can even directly suppress the expression of defense-related genes. Much of the research into plant immunity has focused

on effectors, however, it still remains in the early stages and only a few effectors have been identified so far (Giraldo and Valent 2013).

To discover more potential effectors, we previously isolated several secretory proteins from *M. brunnea* and performed full length cDNA cloning. The results showed that, of all the secretory proteins obtained, nine contained the same identical domain, the so-called LysM domain, and so were considered to belong to the same protein family (Cheng et al. 2010). Further investigation of the whole genome sequence of *M. brunnea* revealed the existence of several more family members and moreover, that this gene family is the largest one in *M. Brunnea* (Zhu et al. 2012).

Transcriptome analysis showed that the expression of these LysM genes tended to increase during the infection stage, indicating that they may be related to the pathogenicity of *M. Brunnea* (Zhu et al. 2012). Currently, LysM domains are known to exist in bacteria, fungi, animals and plants. These domains (Pfam PF 01476) range in length from 44 to 65 amino acids residues and recognize peptidoglycan and chitin. (Buist et al. 2008). Research on *Cladosporium fulvum* has shown that LysM domains may weaken host cell defense responses (de Jonge et al. 2010).

In order to further investigate the roles of the LysM domain in pathogen-host interaction, we identified 24 members of this gene family and then selected two representative genes for functional verification.

Materials and methods

Materials

M. brunnea f. sp. *multigermtubi* was isolated from *Populus canadensis* and streaked onto both potato dextrose agar (Sigma, USA) and simple liquid media (2 % w/v sucrose, 0.5 % w/v yeast extract) for subculturing (Cheng et al. 2010). The infection assay was performed as described (Cheng et al. 2010). the mycelia were collected with filter paper, frozen rapidly using liquid nitrogen and stored at −80 °C for later extraction of DNA and RNA.

Branches of *P. Canadensis* were taken from biennial cuttage seedlings and cultured in the greenhouse at 25 °C and 70 % relative humidity under a 16 h/8 h photoperiod.

Wild-type *A. thaliana* Col-0 were cultured in a constant temperature incubator at 23 °C under a 16 h/8 h light/dark cycle at 60 % relative humidity with supplemental lighting (5000 lux).

Full-length cDNA cloning

RNA was extracted from the mycelia of *M. brunnea* using a modified hexadecyltrimethylammonium bromide (CTAB) method (Jiang et al. 2011). First-strand cDNA was synthesized using 3'-Full RACE Core Set Ver. 2.0 (Takara, Japan) and 5'-Full RACE Kit according to the manufacturer's instructions (Takara, Japan), and then used as template for 3'RACE and 5'RACE. The PCR fragments were ligated into the pMD19-T (Takara, Japan) vector for sequencing. The full-length cDNA sequence was acquired by splicing the obtained fragments with the aid of Bioedit and these cDNA fragments were inserted into the pMD19 simple Vector for sequencing (Takara, Japan). Multiple sequence alignment was constructed using ClustalX2. Analysis of the molecular weight and isoelectric point (pI) of the proteins and their amino acid composition was performed using ProtParam (http://web.expasy.org/protparam/) The Pfam database was used to predict protein domains (http://pfam.sanger.ac.uk/). Predictions of signal peptides were made using SignalP (http://www.cbs.dtu.dk/services/SignalP) .

qRT-PCR Analysis

A modified CTAB method was used for the extraction of total RNA from poplar and *A. thaliana* (Xu et al. 2009). Total RNA was first treated with DNase I (Takara, Japan) to remove any genomic DNA contamination and then reverse-transcribed into cDNA using PrimeScript™ RT-PCR kit (Takara, Japan). The cDNA was diluted 1:3 with nuclease-free water to create the qRT-PCR template.

Reactions were carried out in a 20 μl reaction volume consisting of the following components: 10 μl 2×SYBR Premix Ex Taq^{TM} (Takara, Japan); 0.4 μl 10 μM PcCHI-f primers; 0.4 μl 10 μM PcCHI-r, 0.4 μl ROX reference dyeII; 2 μl cDNA template and 6.8 μl Milli-Q water. qRT-PCR was performed on an Applied Biosystems 7500 Real-time PCR system with the following cycling conditions: 95 °C for 30 s, 95 °C for 5 min and 60 °C for 34 s for 40 cycles. A dissociation curve was analyzed after the reaction to exclude the possibility of nonspecific amplification. Three replicates were performed for each reaction.

Primers for semi quantitative analysis were listed in Table S1, and *beta-actin* was chosen as an internal control gene (Morris et al. 2010) . Several defense-related genes were analyzed (Hamamouch et al. 2011; Czechowski et al. 2004; Galletti et al. 2011; Bi et al. 2010).

Protein expression

BL21 strain was used to perform the prokaryotic expression. Positive microbial suspensions were obtained after shaking at 23 °C for 24–30 h, then the suspensions were diluted in a ratio of 1:40 into 2 ml LB media (LB media: Tryptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}; Ampicillin 100 μg mL^{-1}, Chloromycetin 35 μg mL^{-1}). BL21 (DE3) plysS was used as a negative control. The microbial suspension was then diluted to

20 ml and incubated at 23 °C with shaking until the optical density at 600 nm (OD600) reached 1.5–1.6. IPTG was added (final concentration 0.4 mM) and the cells were incubated at 28 °C for 2 h. After this time, the microbial suspension was poured into a 50-ml centrifuge tube, centrifuged and the supernatant removed. Phosphate buffered saline (5 ml) (NaCl 8.0 g L^{-1}, KCl 0.2 g L^{-1}, Na$_2$HPO4 1.44 g L^{-1}, KH$_2$PO$_4$ 0.24 g L^{-1}, pH 7.4, 1%Triton X-100,1 mM PMSF) was added to resuspend the cells and this was then cleared by sonication. Total proteins were isolated by centrifuging the cell suspension at 8000×*g* for 20 min at 4 °C and then filtering off the supernatant containing the protein extract. Loading buffer (2.5 %*w/v* SDS, 25 % glycerol, 125 mM Tris-Cl (pH 6.8). 0.01 % w/v bromophenol blue) was added to the samples, which were then heated to 95 °C for 5 min. The samples were stained with R-250 for 5–10 min and destained with 10 % acetic acid. The results were observed, scanned and saved as photo files. Proteins from the prokaryotic expression were gently vortexed and incubated with insoluble chitin (Sigma USA) at room temperature for 3 h and then centrifuged at 13,000×*g* for 5 min. The supernatant was discarded and the sediment was rinsed three times with sterile water. Following this, 1 % sodium dodecyl sulfate (SDS) was added to the samples before being boiled for over 5 min. The supernatant was then used for Tricine SDS Polypropylene amide gel electrophoresis (PAGE). Separating gel with 8 % acylamide percentage was used. The total running time should be about 1 h for a 120 V voltage. Western blotting was performed as described using GST antibody (Burnette 1981).

Vector construction and plant transformation

A signal peptide of PR1a was added before LysM genes using overlapping PCR and constructed into binary vector PH35GS to express a fusion peptide which can secreted to intracellular space (Mitsuhara et al. 2000). The resulting vector was transformed into *Agrobacterium tumefaciens* strain EHA105 and then used to transform *A. thaliana* using a floral dip as previously described (Clough and Bent. 1998). After transformation, the wide-type plants and transgenic plants were both grown in the same environment.

Chitin treatment of *A. thaliana*

Chitooligosaccharides (tetrachitooligosaccharides, pentachitooligosaccharides and hexachitooligosaccharides) were purchased from the Megazyme Company (Germany). Solutions at a final concentration of 0.1 μM, 1 μM, 5 μM and 10 μM were prepared using sterile water. These were sprayed on comparable mature, intact *A. thaliana* leaves. Samples were taken at different timepoints (0 h, 0.5 h, 1 h, 1.5 h, 2 h) post application, rapidly frozen and stored in liquid nitrogen.

Results

Cloning of LysM gene family members from *M. brunnea*

The full length cDNA of the LysM gene family ranged from 690 bp to 1179 bp, with most of them being around 800 bp. The coding amino acid sequence was around 150 aa and the molecular weight ranged from 15.52 kDa to 16.67 kDa. All of the coding proteins contained an 18 bp or 19 bp signal peptide for protein secretion, and also known as a common feature of fungal effector proteins (Table 1). Eighty-nine scaffolds were acquired through the whole genome sequencing of *M. brunnea*, while LysM genes were distributed on 18 of them. We renamed all LysM genes according to their distribution.

The pI of the LysM effectors from *M. brunnea* vary greatly from those in prokaryotes. The theoretical pI of the *M. brunnea* effector proteins ranged from 4.34 to 8.54 with most of them being less than 5.5 (Table 1). Only MbLysM03, MbLysM04, MbLysM10, MbLysM20 and MbLysM24 had a pI over 7 and for MbLysM23 the pI was between 6 and 7. In contrast, in prokaryotes the pI of the LysM domain proteins ranged from 4 to 12, with most being between 5 and 10, notably within the interval from 8 to 10 (Buist et al. 2008). These results may reflect the innate differences between eukaryotes and prokaryotes.

Comparison of *M. brunnea* LysM effector family members

Multiple alignments showed that different members of this gene family had relative high homology (Fig. S1A). Two LysM domains were found in all family members based on the domain analysis of amino acid sequences. The first, LysM I, was located towards the middle of the sequence and its length was generally 41 amino acids. The second domain, LysM II, was found at the C-terminal of the proteins and was longer than LysM I with more variation in its length, ranging from 44 to 47 amino acids. The conserved region of the two LysM domains appeared within the first half of the domain sequence. Further analysis of the domain sequences revealed the presence of some highly conserved sites including the first amino acid, Tyr (Y); the 15th, Asp (D); the 17th, Cys (C) and the 28th Asn (N). Besides these, the second, seventh, 16th, 47th and 48th were also reasonably well conserved (Fig. S1B).

Domain analysis and expression of two LysM genes from *M. brunnea*

Two LysM genes from *M. brunnea* were selected for further detailed analysis. They were named *MbLysM5* and *MbLysM19* (Fig. 1A). These two genes encoded secretory proteins which had been detected by us before (Cheng et al.

Table 1 Summary of genes identified in this study

Name	Genbank accession number	cDNA length (bp)	ORF (bp)	Protein (aa)	MW (kDa)	PI	domain	Signal peptides
MbLysM1	JX294933	831	435	145	15.8	5.34	37–63;96–141	1–18
MbLysM2	JX294934	809	453	151	16.7	4.84	101–130	1–18
MbLysM3	JX294935	690	435	145	16.1	7.53	37–68;97–141	1–18
MbLysM4	JX294936	734	426	142	15.7	8.54	95–137	1–19
MbLysM5	JX294937	1179	429	143	15.9	4.84	37–77;96–139	1–18
MbLysM6	JX294938	792	426	142	16.0	5.40	102–140	1–19
MbLysM7	JX294939	770	426	142	16.0	5.13	41–63	1–19
MbLysM8	JX294940	837	435	145	16.1	5.27	38–66;97–141	1–19
MbLysM9	JX294941	805	435	145	15.8	4.97	37–77;96–141	1–18
MbLysM10	JX294942	842	429	143	15.7	8.23	95–139	1–18
MbLysM12	JX294944	760	432	144	15.8	4.66	96–140	1–19
MbLysM13	JX294945	779	432	144	16.1	5.42	97–140	1–19
MbLysM15	JX294947	800	432	144	15.8	4.48	96–140	1–19
MbLysM16	JX294948	730	432	144	15.5	4.49	37–65;96–140	1–18
MbLysM17	JX294949	779	429	143	15.7	4.34	95–123	1–19
MbLysM18	JX294950	761	432	144	15.7	5.34	37–64;96–140	1–18
MbLysM19	JX294951	962	435	145	16.1	5.00	38–65;97–141	1–19
MbLysM20	JX294952	691	432	144	15.7	7.54	41–69;96–140	1–19
MbLysM21	JX294953	960	435	145	15.9	5.39	97–141	1–19
MbLysM22	JX294954	754	438	146	16.1	5.21	99–142	1–21
MbLysM23	JX294955	824	441	147	16.2	6.15	98–143	1–19
MbLysM24	JX294956	815	444	148	16.0	8.26	99–144	1–19
MbLysM25	JX294957	798	432	144	15.9	4.62	38–78;97–140	1–19
MbLysM26	JX294958	803	435	145	15.7	5.29	98–141	1–19

2010). The full length cDNA of these two genes were 1179 bp and 962 bp, respectively, which encoded proteins of 143 and 145 amino acids, respectively. Further sequence analysis showed that the pI (5 and 4.84) of the two proteins and their amino acid compositions were similar, with Ala, Asp, Leu and Thr comprising a large proportion of all the amino acids present. The LysM II domains at the C-terminal of both proteins were conserved. However, the LysM I domains which located in the middle of these two proteins are different. The LysM I domain of *Mb*LysM5 is a integrate LysM domain, whereas the LysM I domain in *Mb*LysM19 only aligned with the first 28 amino acids of a typical LysM domain (Fig. 1B).

We inoculated poplars with *M. brunnea* to investigate the expression patterns of the *M. brunnea* LysM genes in the infection process. Four time point, 12 h, 24 h, 48 h and 96 h after inoculation were selected for qRT-PCR analysis (Fig. 1C). As the results shown, the expression of both genes was relatively stable without any remarkably change in the early stages, but had a significant up-regulated at 96 h after inoculation. It indicated that these two genes might come into play a role during this stage. Furthermore, the expression

patterns of *Mb*LysM5 and *Mb*LysM19 were similar to each other, which indicate the same function they might have..

LysM effectors can bind with chitin in vitro

To find the possible relationship between chitin and LysM effectors. We constructed recombinant vectors to express *Mb*LysM19-GST and *Mb*LysM5-GST fusion proteins using prokaryotic expression system. Total proteins were extracted and then detected by SDS PAGE. With expectation, a band between 33 kDa and 45 kDa was detected. BL21 (DE3) plysS was used as a negative control and no bands were detected (Fig. 2A). We then mixed the fusion proteins with insoluble chitin. After 3 hours incubation, the supernatant was discarded, and the sediment was washed for three times. Then the sediment was boiled and analyzed by SDS PAGE. As the results shown, the band with correct size was still visible indicating that the fusion proteins were capable of binding to chitin (Fig. 2B). Western blotting assays was performed to confirm that the proteins binding to chitin were the LysM-GST fusion proteins (Fig. 2C).
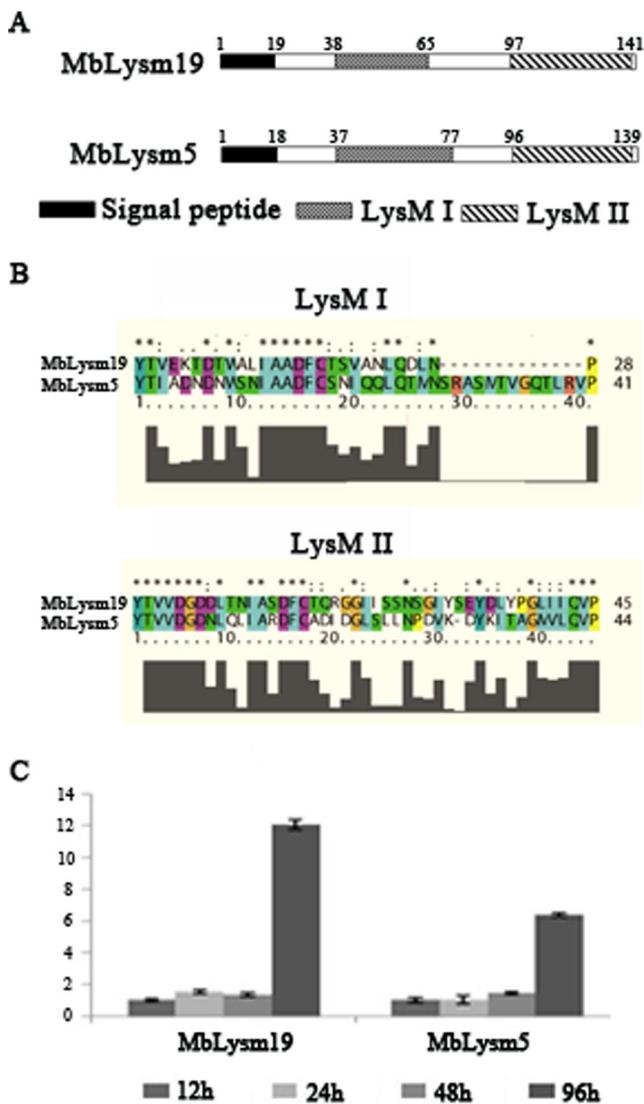
Fig. 1 Domain analysis, sequence alignment and expression patterns of two LysM genes in *Marssonina.brunnea*. **a**. Signal peptide and LysM domains in LysM genes; **b**. Sequence alignment of LsyM domains bwtween two LysM genes; **c**. Expression analysis of *Marssonina.brunnea* LysM genes during infection. Four time points (12hpi, 24hpi, 48hpi, 96hpi) were selected

Fig. 2 Prokaryotic expression of LysM-GST fusion protein and their binding with insoluble chitin. **a**. Prokaryotic expression of *Mb*LysM19-GST and MbLysM5-GST fusion proteins; **b**. The fusion protein co-precipitate with insoluble chitin; **c**. Western blotting assays confirmed the protein co-precipitate with insoluble chitin were *Mb*LysM19-GST and MbLysM5-GST fusion proteins

chitooligosaccharides for inducement (Fig. 3B). The degree of chitooligosaccharides polymerization also affected the induction. We treated *A. thaliana* with chitooligosaccharides with three types of polymerization degree. Although all resulted in the increased expression of *PDF1.2*, the upregulation was much more significant when treated with hexachitoologosaccharides (Fig. 3C). In *A. thaliana*, 0.5 h after treatment with chitoologosaccharides, increases in the expression levels of *PDF1.2* could be observed. As the incubation time increased, the expression level of *PDF1.2* also increased gradually, reaching its peak of expression at around 1.5 h, and decreasing slightly after this time point (Fig. 3D)..

LysM effectors weaken chitin-induced defense responses in *A. thaliana*

As described above, a possible mechanism for LysM effectors may come from their binding to chitin to avoid recognition by the host. Hence, they would be expected to be localized in the extracellular matrix. To investigate this, we added a tobacco PR1a signal peptide at the N-terminal of the LysM genes to aid the secretion of the LysM genes from the plant cells (Mitsuhara et al. 2000). We then re-construct vectors based on PH35GS, the recombinant plasmid PH35GS-*Mb*LysM19 and PH35GS-MbLysM5 were introduced into *A. thaliana* Col-0 using the floral dip technique (Clough 2005). The T1 generation seeds were screened with hygromycin in MS media and resistant seedlings were obtained. All these

Resistance-related genes in *A. thaliana* are induced under chitin treatment

Using the housekeeping gene *beta-actin* as a reference genes, we performed semi-quantitative analysis on known *A. thaliana* early defense genes (*PR1, PR2, PDF1.2, WRKY22, WRKY29* and *FRK1*). All of these defense genes, except *PR2*, showed increased expression levels. Among them, *PDF1.2* showing the most significant change (Fig. 3A).

The expression level of *PDF1.2* increased concurrently within the chitooligosaccharides concentration until it arrived the peak when 5 μM chitooligosaccharides was used. No further changes in expression were observed even using 10 μM
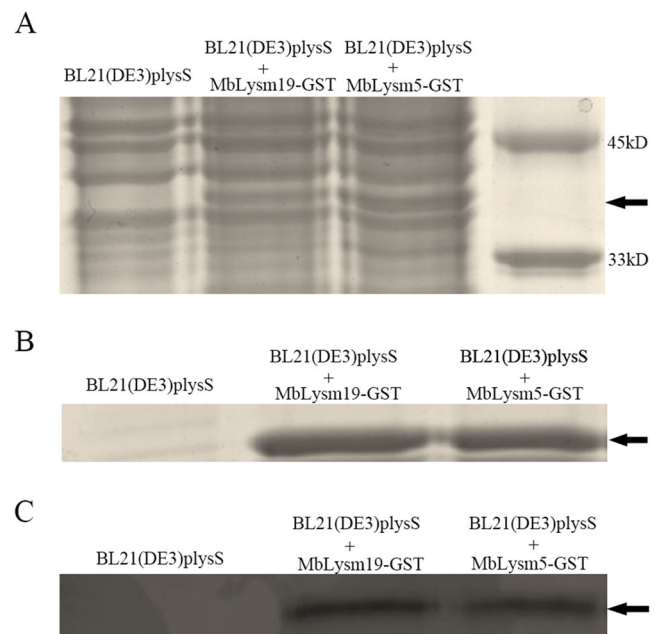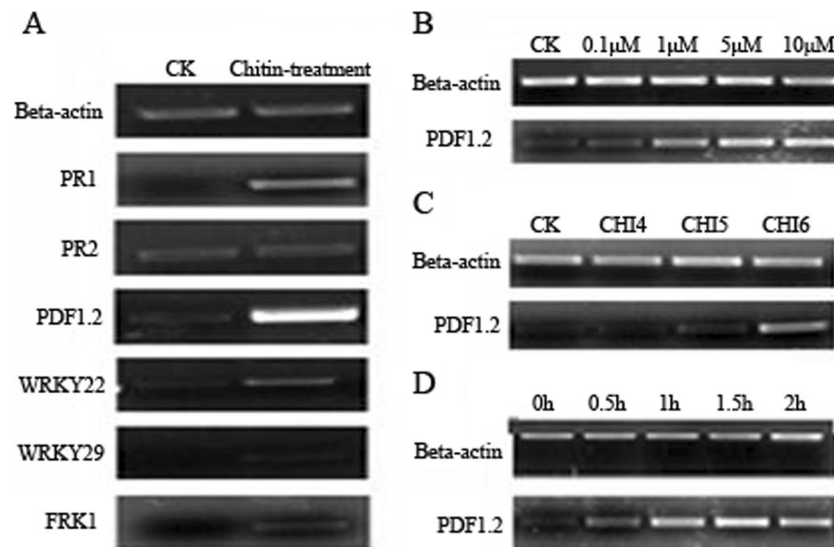
Fig. 3 The defense-related genes of *A.thaliana* were induced by chitooligosaccharides.**a**. Expression of seveal defense-related genes (PR1, PR2, PDF1.2, WRKY22, WRKY29, FRK1) induced by chitooligosaccharides in *A.thaliana*. **b**. Expression of PDF1.2 gene induced by chitooligosaccharides with different concentration (0 μM, 0.1 μM, 1 μM, 5 μM and 10 μM); **c**. Expression of PDF1.2 gene induced by chitooligosaccharides with different polymerization degree. CHI4 (tetra chitooligosaccharides), CHI5 (pentachitooligosaccharides) and CHI6 (hexachitooligosaccharides); **d**. Expression of PDF1.2 gene treated with chitooligosaccharides for different time (0 h, 0.5 h, 1 h, 1.5 h and 2 h)

transformants was verified by PCR. These seedlings were transferred onto soil once four leaves had appeared. The seedlings were kept in normal growth conditions until they started flowering and were then treated with 5 μM hexa-chitooligosaccharides for 1.5 h.

Semi-quantitative PCR analysis showed that the expression levels of *PDF1.2* only significantly increased after treatment with chitooligosaccharides in *A. thaliana* transformed with PH35GS. In comparison, *A. thaliana* transformed with *Mb*LysM19-PH35GS and MbLysM5-PH35GS were insensitive to *PDF1.2* induction by chitooligosaccharides, especially LysM5, which showed almost no change in *PDF1.2* expression (Fig. 4). This indicates that the LysM genes suppressed the defense response of plants normally induced by chitoologosaccharides.
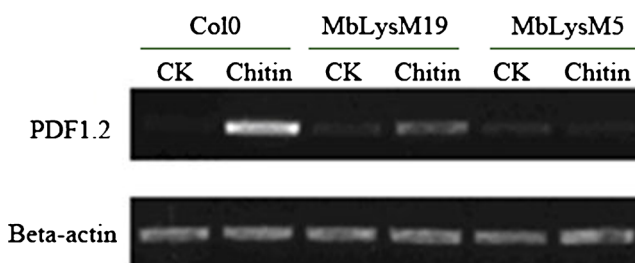

Fig. 4 The expression of PDF1.2 gene with and without chitin treatment *A.thaliana col0* was used as control. The PDF1.2 genes was obviously up-regulated when treated with chitooligosaccharides. But in the *A.thaliana* transformed with *Mb*LysM19-PH35GS, the expression of PDF1.2 was only slightly increased and in *A.thaliana* transformed with *Mb*LysM5-PH35GS, the expression of PDF1.2 was even not changed

Discussion

Pathogenic fungi can be divided into three categories: biotrophic, necrotrophic and hemibiotrophic according to their nutrition acquisition methods. Biotrophs live in a parasitic style, acquiring their nutrition from the host's living tissues. Necrotrophs kill the host first, and then decompose their tissues for survival. Hemibiotrophic fungi could also be regarded as necrotrophs but with weaker virulence, although they do also kill the hosts (Lee and Rose 2010). During a long-term infection process, they may additionally acquire nutrition from the host's living tissues. *M. brunnea* belongs to this latter group. There is often a long period of time from the initial infection to the eventual death of the host, and during this period, pathogenic fungi face recognition by the host and the resulting immune response. As a result, pathogenic fungi have evolved effector proteins, which are released into the host and suppress the host's defense reactions (Chisholm et al. 2006). Fungi have more complex genomes and a higher degree of evolution and thus possess a greater variety of means for infection. Hence, it is more difficult to perform functional analysis on fungal effectors than on those from bacteria. Until now, only a few fungal effectors have been identified, including the LysM effectors, which have a close relationship with the virulence of the pathogenic fungi. They are so-named because they only possess LysM domains. The LysM effectors exist widely in all types of fungi, although their function during infection is unclear. De Jonge and co-workers discovered that the LysM effector, ECP6, could bind to chitin (De Jonge et al. 2010). Since then, research on LysM effectors

and chitin has been performed in other pathogenic fungi. However, previous studies have only managed to identify one or two genes from any one species, and there have been no reports on the identification and characterization of a whole LysM gene family.

We cloned a LysM effector gene family containing 24 genes, based on the analysis of the whole genome sequence of *M. brunnea*. The conserved regions of the *M. brunnea* LysM genes were mainly in the LysM domains. Some highly conserved sites were found within the LysM domains, which are likely to be essential for the stability of the domain structure and may contribute to chitin binding. Analysis on the expression patterns of the LysM genes showed that the highest expression was during the later stages of infection. Since it is known that chitinases decompose the cell walls of fungi generating a large amount of chitin fragments, we suggest that the chitinases of poplars are likely to perform their function during the late stage of infection. To prevent these chitin fragments from triggering the poplar immune system, *M. brunnea* releases LysM effectors that bind to the chitin fragments thus weakening the immune response of poplar.

Functional verification in *A. thaliana* proved that LysM effectors of *M. brunnea* could indeed suppress the expression of resistance-related genes which might be the downstream targets of chitin-induced signaling pathway. This confirmed that as fungal effectors, LysM proteins from *M. brunnea* can inhibit the PAMPs-triggered immunity. It also indicated that this suppression of immune responses might be universal for this group of effectors since it was not only restricted to poplars, but also occurred in *A. thaliana*.

The number of LysM domains within a protein may also affect its function. Among the LysM effectors reported previously, three LysM domains were found in ECP6 and Mg3LysM, two LysM domains in slp1, and only one in Mg1LysM (de Jonge et al. 2010; Marshall et al. 2011; Mentlak et al. 2012). These effectors can bind to chitin directly and the number of LysM domains has no effect on binding. However, Mg1LysM from *Mycosphaerella graminicola*, which possesses only one LysM domain (Marshall et al. 2011), could not suppress the chitin-induced defense responses and so it appeared that LysM genes containing two or more LysM domains were necessary for this function. Most of *M. brunnea* LysM genes have two LysM domains. However, of the two genes analyzed, *Mb*LysM5 with two integrated LysM domains suppressed the host immunity more significantly than *Mb*LysM19 with only one integrated LysM domain, indicating that the integration of the LysM domain is essential for LysM effectors to play a role.

LysM effectors in pathogenic fungi can weaken the host's defense responses because they can compete with the chitin elicitor receptors in the host. CEBIP in rice is a typical LysM receptor (Shimizu et al. 2010; Hayafunea et al. 2014). Recent studies have revealed that the effector, slp1, from the rice blast pathogen *Magnaporthe oryzae*, which contains LysM domains, can compete with CEBIP to bind to chitin (Mentlak et al. 2012). The homolog of CEBIP exists in Populus and we believe that it may have a competitive relationship with one of the LysM effectors that we have identified. Furthermore, CERK and CEBIP of rice can form a dimer when elicited by chitin, however, it has not yet been shown whether dimerization also occurs in poplar, nor whether the LysM effectors of *M. brunnea* can form a heterodimer with CERK in poplars. The latter deserves further attention, since, if they can form a heterodimer, the CERK and CEBiP dimer will presumably be damaged, and thus the immunity response will be weakened.

The long-term interaction between *M. brunnea* and poplar means that they have co-evolved with new genes appearing to substitute for ones that had been recognized by each other. It should be a possible reason for the existence of so many LysM domain-containing genes with a similar sequence and structure in both species. Studies on such a gene family will lay a foundation for the understanding of co-evolution between pathogen and host.

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