Research

Plasmodiophora brassicae CBM18 Proteins Bind Chitin and Suppress Chitin-Triggered Immunity

Kevin Muirhead¹ | Edel Pérez-López^{2,3,†} |

¹ University of Calgary, Alberta, Canada

² Department of Plant Sciences, FSAA, Université Laval, Québec, Canada

³ Centre de recherche et d'innovation sur les végétaux (CRIV), Université Laval, Québec, Canada

† Corresponding author: E. Pérez-López; edel.perez-lopez.1@ulaval.ca

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Abstract

Plants have a sophisticated and multilayered immune system. However, plant pathogens, helped by effector proteins, have found several strategies to evade plant immunity. For instance, the clubroot pathogen *Plasmodiophora brassicae* is able to turn the roots of susceptible hosts into nutrient-sink galls suppressing pattern-triggered immunity (PTI) and effector-triggered immunity. Chitin, the main component of *P. brassicae* spore cell walls and a well-known pathogen-associated molecular pattern, can elicit PTI but is also the target of plant chitinases and chitin deacetylases. The fact that *P. brassicae* does not trigger PTI during infection of susceptible hosts motivated a genome-wide search of genes coding for secreted proteins with domains previously associated with chitin binding. We found that the *P. brassicae* genome encodes a repertoire of candidate-secreted effectors containing the chitin-binding domain carbohydrate-binding module family 18 (CBM18), chitinase, and chitin deacetylase domains. The role of these proteins in the pathogenicity of the clubroot pathogen is unknown. Here, we characterized two CBM18 proteins, *PbChiB2* and *PbChiB4*, which are transcriptionally activated during infection. Through coprecipitation, we found that recombinant *PbChiB2* and *PbChiB4* bind to spores and to chitin oligomers. We also showed that both proteins suppress chitin-triggered activation of the map kinase proteins MPK3 and MPK6 in the host *Brassica napus*. These findings suggest that *P. brassicae* CBM18 proteins act as effectors for protecting the clubroot pathogen and for suppressing chitin-triggered immunity during infection.

Keywords: chitin-binding protein, clubroot, canola, effectors, chitin-triggered immunity

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Pathogen recognition is essential to trigger immune responses that can limit infection (Zipfel 2014). Through pattern-recognition receptors located on the cell surface, plants can perceive pathogen-associated molecular patterns, and activation of pattern-triggered immunity (PTI) occurs (Dodds and Rathjen 2010). This first layer of immunity is characterized by the generation of reactive oxygen species, the activation of channels in the

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plant membrane, and the phosphorylation of mitogen-activated protein kinases leading to a wide array of antimicrobial defense mechanisms (Dodds and Rathjen 2010; Zipfel 2014). However, plant pathogens have developed very sophisticated strategies to escape PTI, infect the plant host, and complete their life cycle (Dodds and Rathjen 2010).

Chitin (polymer of β -1,4-linked N-acetylglucosamine [GlcNAc]_n) is an extensively studied pathogen-associated molecular pattern, a major component of fungal cell walls, and it has been shown that during infection, chitin oligosaccharides resulting from the activity of plant chitinases are recognized by receptor kinases, activating PTI (Sánchez-Vallet et al. 2015). To escape chitin-triggered immunity, fungal pathogens have developed several strategies, including the secretion of chitin-binding effectors (van den Burg et al. 2004). These effectors not only capture free chitin oligosaccharides but also act as a shield to protect pathogens from plant chitinases (Marshall et al. 2011; van Esse et al. 2007). The chitin-binding domains identified in fungal plant pathogen effectors can be divided into three major groups based on their carbohydrate-binding module (CBM) family: (i) CBM family 14 (CBM14), identified in chitin-binding effectors from fungal plant pathogens such as *Cladosporium fulvum* (van den Burg et al. 2004) and *Mycosphaerella fijiensis* (Stergiopoulos et al. 2010); (ii) CBM family 50 (CBM50), also known as LysM effectors, identified in *Cladosporium fulvum* (de Jonge et al. 2010), *Zymoseptoria tritici* (Marshall et al. 2011), *Magnaporthe oryzae* (Mentlak et al. 2012), *Colletotrichum higginsianum* (Takahara et al. 2016), and *Rhizoctonia solani* (Dörfors et al. 2019), among others; and (iii) CBM family 18 (CBM18), only identified in *Magnaporthe oryzae* (Mochizuki et al. 2011) and *Verticillium nonalfalfae* (Volk et al. 2019). In all these pathogens, the function of chitin-binding effectors can be grouped into (i) protection of the fungal cell wall from plant chitinases and (ii) suppression of chitin-triggered host immunity.

Although chitin is predominantly found in the cell walls of fungal plant pathogens, it is also the main carbohydrate present in resting spore cell walls in *Plasmodiophora brassicae* Woronin, the clubroot pathogen. Clubroot is a devastating disease affecting cruciferous crops worldwide. It is caused by the obligate parasite, biotrophic, soilborne protist *P. brassicae* (Dixon et al. 2009). The life cycle of *P. brassicae* takes place in the roots of the infected host. It is divided into two stages: (i) primary infection, characterized by the encysting of the spores, penetration of root hairs, and the subsequent formation of the zygote through other developmental stages, and (ii) secondary infection, characterized by formation of the secondary plasmodium and release back to the soil of the resting spores (Liu et al. 2020). Although *P. brassicae* secondary infection has drawn the most attention because it leads to the host death, primary infection remains the key step for infection (Chen et al. 2019). During analysis of the *P. brassicae* transcriptome in different hosts and developmental stages, several proteins with the chitin-binding (CBM18) domain were present in *P. brassicae* secretome (Schwelm et al. 2015), although the function of these proteins remains unknown. Similar results were obtained in another study examining effector proteins involved in *P. brassicae* primary infection (Chen et al. 2019).

Here, we performed a genome-wide search to identify all of the chitin-related proteins putatively secreted by *P. brassicae*, with special focus on those bearing CBM18 domains. Among the proteins identified, we characterized *PbChiB2* and *PbChiB4*, which have two CBM18 modules and a functional signal peptide. Both are secreted and transcribed during *P. brassicae* infection. We show that both proteins bind resting spores and chitin in vitro

and that these proteins are able to inhibit chitin-triggered immunity. We therefore hypothesize that these proteins act to “guard” *P. brassicae* throughout the infection.

MATERIALS AND METHODS

Plant material and growth conditions

Rapeseed (*B. napus*) Westar seeds, disinfected with 70% ethanol, 95% ethanol, 8% bleach, and sterile water, were sown on 1/2 Murashige and Skoog (MS) salts agar (1% w/v) plates (Sigma-Aldrich, Canada) with 1% sucrose. Plates were kept at 4°C for 4 days and then transferred to a growth chamber (Conviron ATC10, CMP4030 control system; 170 μ mol photons m² s⁻¹; 16/8 h light/dark cycle; 20°C) for germination and growth.

Clubroot infection

Ten-day-old rapeseed seedlings were transferred to Vèrada soil mix (Farad, Canada) with one plant in each square pot (3.5 × 3.5 inch). Seedlings were allowed to acclimatize in these pots for another 4 days before 500 μ l of 5 × 10⁷ resting spores per milliliter, extracted from canola galls (Pérez-López et al. 2020), was applied to each plant where the stem entered the soil. After 2 weeks, plants were transferred to 8-inch pots and grown until flowering to harvest the galls and the xylem sap as previously described (Kehr et al. 2005).

Bioinformatics analyses

To identify putative secreted chitin-related proteins, we performed a genome-wide search for proteins with a signal peptide prediction score ≥ 0.7 , and a length of <500 amino acids in the *P. brassicae* Pbe3.h15 genome (Schwelm et al. 2015). The resulting proteins were analyzed using Pfam (<https://pfam.xfam.org/>) to identify chitin-related carbohydrate-active enzymes (chitin-CAZymes). The proteins identified were characterized using several online tools as previously described (Pérez-López et al. 2020), including ApoplastP (Sperschneider et al. 2017) to predict apoplastic localization.

To investigate if the *P. brassicae* chitin-CAZymes are differentially transcribed based on pathogen host and/or developmental stage, the expression of the genes coding these proteins was analyzed using the RNAseq data previously reported (Schwelm et al. 2015) under the SRA accession numbers ERX1409397 to ERX1409403. RNAseq libraries were downloaded using the fastq-dump program of the NCBI SRA Toolkit v. 2.9.6 (Leinonen et al. 2011). The RNAseq libraries were assessed for quality using fastqc v. 0.11.8 (Andrews 2010), and TruSeq3 Illumina adapters were determined from the most represented sequences. Reads were quality-filtered and trimmed using Trimmomatic v. 0.39 (Bolger et al. 2014) with the following parameter settings: ILLUMINACLIP: TruSeq3-PE.fa:2:30:10:2:keepBothReads LEADING:3 TRAILING:3 MINLEN:36. Reads were mapped to the *P. brassicae* Pbe3.h15 genome using STAR v. 2.7.5c (Dobin et al. 2013). The STAR genome database was generated using the following parameter settings: –genomeSAindexNbases 11 –runMode genomeGenerate. STAR was executed using the following parameter settings: –runMode alignReads –outSAMtype SAM –outSAMattributes All –outSAMstrandField intronMotif. The SAM output files from STAR were processed using samtools v. 1.9 and analyzed using the Cuffdiff program of Cufflinks v. 2.1.1 (Trapnell et al. 2010). A heat map was generated using

cummeRbund 3.12 (Goff et al. 2020) package using the output generated by the Cuffdiff program and gene IDs shown in Table 1. The phylogenetic analysis of chitin-CAZymes was carried out using MUSCLE v. 3 (Edgar 2004) and MEGA v. 6 (Tamura et al. 2013). The phylogenetic tree was constructed using maximum likelihood, the James–Taylor–Thorthon model (Jones et al. 1992), and a bootstrap value of 1,000.

Signal peptide validation assay

To validate *PbChiB1*, *PbChiB2*, and *PbChiB4* signal peptides (22, 22, and 17 aa, respectively), the 66-bp (*PbChiB1sp* and *PbChiB2sp*) and 51-bp (*PbChiB4sp*) fragments were amplified by PCR from cDNA obtained from infected *Arabidopsis* plants using primer pairs introducing *EcoRI* at the N-terminal and *XhoI* (NEB, Canada) at the C-terminal end (Supplementary Table S1). The amplified signal peptide fragments were introduced into the plasmid pSUC2 (Jacobs et al. 1997) using T4 DNA ligase (Promega, U.S.A.). After sequencing, the pSUC2-*PbChiB1sp*, -*PbChiB2sp*, and -*PbChiB4sp* constructs transformed into *Saccharomyces cerevisiae* yeast strain YTK12. Plasmid pSUC2 contains the TRP1 marker that allows the yeast to metabolize tryptophan. If the signal peptide inserted in the plasmid is functional, the yeast can secrete an invertase to the supernatant, which can be detected through the colorimetric change of 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) to 1,3,5-triphenylformazan. After selection of positive clones by colony PCR and selection in CMD-W medium, we analyzed the invertase enzymatic activity as previously described (Yu et al. 2019). Two different clones expressing *PbChiB1*, *PbChiB2*, and *PbChiB4* signal peptides, respectively, were used in the invertase enzymatic activity assay twice with the same results.

Production of recombinant proteins

For protein expression, the cDNA for *PbChiB2* (Supplementary Fig. S1) and *PbChiB4* (Supplementary Fig. S1) without the signal peptide was synthesized and cloned into plasmid pET-14b (GenScript, U.S.A.). Plasmids were used to transform *E. coli* SHuffle T7 cells (NEB). *E. coli* transformants were grown in 50 ml of LB medium supplemented with ampicillin (100 µg ml⁻¹) at 37°C, 200 rpm, for 3 h. Protein expression was induced by the

addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, and growth was continued at 37°C, 200 rpm, for 2 h. After centrifugation at 3,200 × *g* for 15 min, cell pellets were lysed using a Branson sonicator equipped with a microtip (Branson Ultrasonics, U.S.A.). Pellets containing His-*PbChiB2* and His-*PbChiB4* were purified with Ni-NTA agarose (Thermo Fisher Scientific, Canada) under native conditions following the manufacturer's recommendations. Protein concentration was measured using a Qubit protein assay kit (Thermo Fisher Scientific).

Western blot analysis

Proteins were separated by 15% SDS-PAGE and transferred to 0.2-µm polyvinylidene fluoride membranes (Bio-Rad, Canada) for 1 h at 0.4 V cm⁻¹ in a Mini-PROTEAN Tetra Cell (Bio-Rad). His-tagged proteins were detected using monoclonal anti-polyHis-HRP conjugate (Sigma-Aldrich). Phosphorylated mitogen-activated protein kinases (MAPKs) were detected using phospho-p44/p42 MAPK primary antibody (Cell Signaling Technology, Leiden, Netherlands), followed by a goat anti-rabbit secondary antibody HRP conjugate (Sigma-Aldrich) following the manufacturer's recommendations. Membranes were blocked with 5% skim milk, and HRP activity was detected using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) with an Azure imager C300 (Azure Biosystems, Canada) to detect the chemiluminescent signal.

Carbohydrate sedimentation assay

This assay was performed as described by Volk et al. (2019). Briefly, 20 µg of recombinant *PbChiB2* or *PbChiB4* resuspended in 20-mM Tris (pH 8.0) was independently mixed with 2 mg of chitin magnetic beads (NEB), chitin from shrimp shells (Sigma-Aldrich), chitosan from shrimp shells (Sigma-Aldrich), cellulose (Sigma-Aldrich), or xylan (Thermo Fisher Scientific) and incubated at room temperature for 2 h with shaking at 300 rpm. After centrifuging for 5 min at 13,000 × *g*, the supernatant was precipitated with cold acetone and resuspended in 40 µl of 2× SDS-PAGE buffer. The pellets were washed with 800 µl of 20-mM Tris pH 8.0 and resuspended in 40 µl of Laemmli sample buffer with 13% β-mercaptoethanol. The presence of *PbChiB2* and *PbChiB4* was detected using western blot as described above.

TABLE 1

Description and identity of the putative secreted chitin-related carbohydrate-active enzymes reported in this study^y

Name	Gene ID	Accession number	No. amino acids	MW (kDa)	IP	Signal peptide cleavage	TM domain (Y/N)	Apoplastic ^z (Y/N [score])
<i>PbChi1</i>	PBRA_005464	CEO96860	477	50.3	4.9	21–22	N	Y (0.8)
<i>PbChi2</i>	PBRA_005765	CEO97161	357	37.9	6.3	18–19	N	Y (0.63)
<i>PbChi3</i>	PBRA_003743	CEO94930	400	44.3	5.8	23–24	N	N (0.66)
<i>PbChiB1</i>	PBRA_003759	CEO94946	222	22.9	7.6	22–23	N	Y (0.72)
<i>PbChiB2</i>	PBRA_001907	CEP01301	137	14.4	5.3	22–23	N	Y (0.77)
<i>PbChiB3</i>	PBRA_002543	CEP02278	336	35.5	7.7	20–21	N	N (0.54)
<i>PbChiB4</i>	PBRA_002958	CEP03198	172	17.9	4.2	17–18	N	Y (0.81)
<i>PbChiD1</i>	PBRA_007091	CEO98977	314	34.1	6.7	21–22	N	N (0.54)
<i>PbChiD2</i>	PBRA_002230	CEP01965	263	29.6	5.6	18–19	N	N (0.71)
<i>PbChiD3</i>	PBRA_008962	CEP02378	328	35.3	4.5	21–22	N	N (0.59)
<i>PbChiBD1</i>	PBRA_003161	CEP03401	387	40.8	4.8	29–30	N	Y (0.84)
<i>PbChiBD2</i>	PBRA_005081	CEO96410	372	40.3	4.7	18–19	N	Y (0.59)
<i>PbChiBD3</i>	PBRA_002551	CEP02584	355	37.7	4.2	21–22	N	Y (0.8)
<i>PbChiBD4</i>	PBRA_007776	CEP00042	402	43.5	6.8	18–19	N	N (0.61)
<i>PbChiBD5</i>	PBRA_001295	CEP09389	302	32.3	4.9	32–33	N	Y (0.91)
<i>PbChiBD6</i>	PBRA_008942	CEP02358	367	39	4.9	20–21	N	Y (0.77)
<i>PbChiBT1</i>	PBRA_004239	CEO95513	440	48.9	8.6	21–22	N	N (0.73)

^y MW = molecular weight; IP = isoelectric point; and TM = transmembrane.

^z The score indicates the probability that a tested instance will belong to either the apoplastic or nonapoplastic class.

A similar protocol was followed to study the ability of *PbChiB2* and *PbChiB4* to bind resting spores. Each recombinant protein (20 µg) was incubated with 1×10^{15} resting spores per milliliter extracted from canola galls. In parallel, xylem sap from canola clubs was used to study the effect of chitinases on the interaction of *PbChiBs* with resting spores. It is well known that xylem sap is rich in plant chitinases released during pathogen infection (Volk et al. 2019). To study this, resting spores preincubated with xylem sap were incubated with *PbChiB2* or *PbChiB4*. Another treatment, consisting of adding the xylem sap to spores preincubated with the recombinant proteins, was included. After each treatment, the spores were pelleted and washed with 20-mM Tris pH 8.0, and the presence of *PbChiB2* and *PbChiB4* was analyzed as described above. All the sedimentation assays were repeated twice with identical results.

MAPK activation assay

B. napus cultivar Westar was grown for 2 weeks in 1/2-MS liquid medium after germination, and seedlings were incubated either with (i) water (mock), (ii) 1 µM chitin ([GlcNAc]6; Santa Cruz Biotechnology, U.S.A.), (iii) *PbChiB2* or *PbChiB4* (10 µM), or (iv) chitin preincubated with recombinant *PbChiB2* or *PbChiB4* (10 µM) for 1 h at room temperature (Supplementary Fig. S2). Three seedlings per treatment were pooled, and protein extraction and phosphorylated MAPK3 and MAPK6 detection were performed as previously described (Tsuda et al. 2009) after 4 min of treatment. The intensity of the bands was analyzed using ImageJ (<https://imagej.nih.gov/>), and the significance of the difference was analyzed through a *t* test. This experiment was repeated twice with the same results.

RESULTS

Chitin-related *P. brassicae* CAZymes secreted repertoire

During the first description of the *P. brassicae* genome, a set of more than 20 chitin-CAZymes was reported (Schwelm et al. 2015). However, in that study, the authors reported chitin-CAZymes that could and could not be secreted by the club-root pathogen, whereas here, using more restricting parameters, we identified only 16 putative secreted chitin-CAZymes in the *P. brassicae* genome (Table 1, Fig. 1A). These proteins were divided into four main categories: chitinases (*PbChi*), chitin-binding (*PbChiB*), chitin deacetylases (*PbChiD*), and chitin-binding domains (*PbChiBD*). An additional candidate with a chitin-binding domain tyrosinase (*PbChiBT*) was also found (Fig. 1A, Table 1).

The domain present in the *PbChi* proteins was identified as a glycosyl hydrolase, family 18 (GH18) belonging to the GH18 cluster A as previously reported (Schwelm et al. 2015). These proteins are less than 500 aa in length, encode a predicted signal peptide, and contain no transmembrane domains (Table 1). The nine *PbChiD* and *PbChiBD* were identified as members of the carbohydrate esterase family 4 (CE4). This family of esterases catalyzes the deacylation of polysaccharides. A NodB-like domain, characteristic of this family, was identified in all nine proteins (Fig. 1A). Of the nine proteins identified as chitin deacetylases, four were identified as putative apoplastic proteins (Table 1). In six of the nine proteins, in addition to the NodB-like domain, we found a chitin-binding domain (*PbChiBDs*; Table 1), the carbohydrate-binding module family 18 (CBM18), with five proteins containing two copies of the domain (*PbChiBD1* to *PbChiBD4*, *PbChiBD6*) and *PbChiBD5* with only one (Fig. 1A).

The last four chitin-related *P. brassicae* proteins detected in this study were proteins with only the CBM18 domain (*PbChiBs*) (Asenio et al. 2000; Fig. 1, Table 1). *PbChiB1* have only one module of the CBM18 domain, whereas *PbChiB2* and *PbChiB4* have two, and *PbChiB3* have three modules (Fig. 1A). *PbChiBs* with one and two modules of the CBM18 domain are predicted to be apoplastic, a common feature of chitin-binding effectors (Volk et al. 2019; Table 1), whereas *PbChiB3* do not seem to be apoplastic (Table 1).

Chitin-related encoding genes are differentially expressed in planta

To study the expression of the chitin-CAZymes and the chitin-binding proteins, we used RNAseq data obtained from *P. brassicae*-infected *B. rapa*, *B. napus*, and *B. oleracea* var. *capitata* during secondary infection (35 days post-inoculation), as well as life stage-specific RNAseq data generated from germinating resting spores, maturing resting spores, and plasmodia (Schwelm et al. 2015; Fig. 1B). In general, we observed a differential expression for each gene based on the host. Transcripts of seven of the proteins were in the same range in *B. rapa* and *B. napus* (Fig. 1B), whereas only two presented the same transcription profile in the three hosts (*PbChiB1* and *PbChiB2*; Fig. 1B). The remaining 10 proteins showed a differential transcription for each host analyzed and for each life stage. It was interesting to find that the transcript of proteins such as *PbChiB4* was preferentially detected in germinating spores and plasmodia but not in maturing spores (Fig. 1B), whereas the transcripts of other proteins such as *PbChiB2* were mostly detected in maturing spores and plasmodia but not in germinating spores (Fig. 1B).

PbChiB proteins have a functional signal peptide

To investigate the functionality and role of the effectors in chitin-triggered immunity and activity in *P. brassicae* pathogenicity, we focused on *PbChiB* proteins with one and two modules of the CBM18 domain: *PbChiB1*, *PbChiB2*, and *PbChiB4*, mainly because they were the smallest. The first step was to confirm that these proteins have a functional signal peptide. For that, the secretory activity directed by *PbChiB1*, *PbChiB2*, and *PbChiB4* signal peptides was tested individually. The three signal peptides showed secretory activity in yeast, as shown on CMD-W plates and by the reduction of 2,3,5-triphenyltetrazolium chloride to insoluble red-colored 1,3,5-triphenylformazan (Fig. 2). The results confirmed that *PbChiB1*, *PbChiB2*, and *PbChiB4* signal peptides are functional and might enable the secretion of these proteins to susceptible hosts during *P. brassicae* infection.

PbChiB2 and *PbChiB4* bind chitin in vitro

After analyzing the chitin-binding domain present in the three *PbChiB* proteins, we noticed that one of the glycines of the consensus sequence was substituted by alanine in both *PbChiB4* CBM18 β-hairpin loops (Supplementary Fig. S4). In *PbChiB1* and *PbChiB2* CBM18 domains, we observed the insertion of an alanine and leucine, respectively, in the consensus sequence (Supplementary Fig. S4). In none of the proteins was the fourth disulfide bond of the expected four-disulfide core present (Supplementary Fig. S4). Based on this observation and the fact that *PbChiB1* has only one CBM18 module and weak transcripts in the three hosts analyzed and the different life stages (Fig. 1B), only *PbChiB2* and *PbChiB4* were retained for further analyses (Fig. 3A).

FIGURE 1

Phylogeny, structure, and expression of *Plasmodiophora brassicae* chitin-related carbohydrate-active enzymes (chitin-CAZymes) reported in this study. **A**, Phylogeny of the amino acid sequence of the chitin-CAZymes based on a maximum-likelihood tree. The protein domain organization is shown on the right side of the tree. **B**, Expression profile of chitin-CAZymes measured using RNAseq data from Schwelm et al. (2015). Overexpressed (dark orange) or underexpressed transcripts (light yellow) are presented as log fragments per kilobase of transcript per million mapped reads+1 fold changes relative to the mean expression measured for three *P. brassicae* hosts: *B. rapa* (Br), *B. napus* (Bn), and *B. oleracea* var. *capitata* (Bo) during secondary infection (35 days post-inoculation), and life stage-specific RNAseq data generated from germinating resting spores (Gs), maturing resting spores (Ms), and plasmodia (Pl).

Recombinant and purified *PbChiB2* and *PbChiB4* (Fig. 3B) were used in a sedimentation assay with an array of carbohydrates. Both proteins were able to bind to chitin immobilized in magnetic beads and shrimp shell chitin, but not to shrimp shell chitosan or to the plant cell wall cellulose or xylan (Fig. 3C). Curiously, although we did not study the affinity of the interactions, we were able to detect in the pellet and the supernatant *PbChiB4*, whereas *PbChiB2* was only detected in the pellet, pointing to a difference in affinity between the proteins (Fig. 3C).

FIGURE 2 Analysis of *PbChiB1*, *PbChiB2*, and *PbChiB4* signal peptide functionality using a yeast invertase assay. Schematic representation of the constructs containing the signal peptide sequence of *PbChiB* proteins and empty vector (EV) pSUC2 in yeast YTK12 were plated on CMD-W (Trp depleted), and the enzymatic activity of secreted invertase was determined using the reduction of 1,3,5-triphenyl tetrazolium chloride to the red-colored formazan (1,3,5-triphenyl formazan) assay. Images are representative of three replicates using independent biological samples. The full sequences of the predicted signal peptides of *PbChiB1*, *PbChiB2*, and *PbChiB4* are presented in Supplementary Figure S3. Created with <https://biorender.com/>.

PbChiB2 and *PbChiB4* bind resting spores in vitro

To study the interaction with resting spores, recombinant *PbChiB2* and *PbChiB4* were used in a sedimentation assay (Fig. 4A). When *PbChiB2* and *PbChiB4* were incubated with resting spores isolated from canola clubroot galls, we were able to detect the His-tagged proteins in the pellet (Fig. 4B). Similar results were obtained when the xylem sap was added after the spores were preincubated with the recombinant proteins (Fig. 4B). The interaction was abolished when the resting spores were first preincubated with the xylem sap before addition of the recombinant proteins (Fig. 4B).

PbChiB2 and *PbChiB4* suppress chitin-binding immunity

To determine if *PbChiB2* and *PbChiB4* were able to compete with plant chitin receptors and interfere with early chitin-triggered immunity, we treated *B. napus* seedlings with 1 μ M of chitin ([GlcNAc]₆) and registered the activation of MAPK3 and MAPK6 4 min after application (Fig. 5A). When *PbChiB2* and *PbChiB4* were preincubated with chitin, MAPK activation was clearly reduced with *PbChiB2* and to a lesser extent with *PbChiB4* (Fig. 5B). The analysis through ImageJ of the intensity of the bands showed that MAPK3 expression was significantly reduced after preincubation with *PbChiB2* ($P < 0.0001$) and *PbChiB4* ($P = 0.0005$; Supplementary Table S2). Something similar occurred with the expression of MAPK6, which was extremely significantly reduced when preincubated with *PbChiB2* ($P < 0.0001$) and significantly reduced when preincubated with *PbChiB4* ($P = 0.05$; Supplementary Table S2). The results obtained with *PbChiB4* agree with what we observed

FIGURE 3 *Plasmodiophora brassicae* *PbChiB2* and *PbChiB4* bind specifically to chitin and chitin oligomers. **A**, Scheme of the recombinant *PbChiB2* and *PbChiB4*. **B**, SDS-PAGE 15% electrophoresis of the recombinant and purified *PbChiB2* and *PbChiB4* (red star). **C**, Affinity precipitation of *PbChiB2* and *PbChiB4* in the presence of chitin beads, shrimp shell chitin, shrimp shell chitosan, cellulose, and xylan. The supernatant and the pellet samples were analyzed after centrifugation. Created with <https://biorender.com/>.

to the glycosyl hydrolase GH18 family, lost its chitinase activity during the pathogen evolution but kept its chitin-binding ability, preventing chitin-triggered immunity during infection (Fiorin et al. 2018).

Here, we provide evidence suggesting a dual function of *P. brassicae* chitin-binding effectors: The first function is to protect the spores from plant chitinase releases by the host during immune response depletion. The second function is to suppress chitin-triggered immunity, capturing chitin oligomers released during *P. brassicae* penetration to the host cell, but the role of *PbChiB2* and *PbChiB4* during *P. brassicae* infection needs to be further investigated. A role for the apoplast in *P. brassicae* infection has not yet been identified. Given the predicted apoplastic localization of several putative *P. brassicae* effectors, including the chitin-binding *PbChiB2* and *PbChiB4*, this will be a fertile area for future investigation.

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