

MSc thesis on

Functional analysis of LysM-domain containing effector proteins in *Botrytis cinerea*



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Wageningen, April 2011

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Dedicated to my family

Abstract

The LysM Motif (LysM) is a protein domain present in different prokaryotes and eukaryotes. LysM-domain containing effector protein, Ecp6 from *Cladosporium fulvum* has chitin binding ability. By binding with the appoplastic chitin fragments by the Ecp6 *C. fulvum* can avoid chitin fragment recognition as a PAMP by the host plant. Recently four genes *Bclysm1*, *Bclysm2*, *Bclysm3* and *Bclysm4* have been identified in the *Botrytis cinerea* genome, they have a number of LysM-domains. The *Bclysm2* is a homologous of Ecp6. It has been predicted that *B. cinerea* LysM-domain containing four of the genes or at least *Bclysm2* has chitin binding ability and likely can avoid plant recognition during pathogenicity. In QPCR it was found that all of the four *Bclysm* genes have different level of transcription during growth in nutrient medium and in planta infection. Individual knockout mutants of three genes, *Bclysm1*, *Bclysm2*, and *Bclysm4* did not express any difference in virulence assay in tobacco and tomato plant than the wild type of *B. cinerea* means individual *Bclysm* genes are not essential for virulence. Study shows that without any of single gene of the four *Bclysm* genes, *B. cinerea* is not sensitive to different stress conditions. *Bclysm* mutants are also not performed any difference in their growth than the wild type on different nutrients medium. *Bclysm* mutants have grown better on tobacco leaf extract than tomato. However, on tobacco leaf extract mutants have grown slowly than the wild type. Studied three of the *Bclysm* mutants (*Bclysm1*, *Bclysm2*, and *Bclysm4*) and wild type of *B. cinerea* are not sensitive to the chitinase *in vitro*. Better germination has found for *Bclysm* mutants and wild type in chitinase at the presence of fructose. In this study it has confirmed that BcLysM2 is able to bind with chitin, however binding with other carbohydrates are not confirmed.

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1. Introduction

The Lysine Motif (LysM) is a protein domain first reported by Garvey *et al.*, (1986) in lysozyme, which is a polysaccharide hydrolyzing enzyme that can degrade bacterial cell wall (Garvey 1986; de Jonge and Thomma 2009). LysM domains are found in various enzymes such as peptidases, chitinases, esterases, reductases or in nucleotidases (Buist *et al.*, 2008). Some plant proteins such as extracellular receptor-like kinases also have LysM domains. It can help to sense chitin fragments that are released from fungal cell walls during fungal attachment to a plant surface (Buist *et al.*, 2008; de Jonge and Thomma 2009; Zhang *et al.*, 2007). This chitin recognition can generate defense responses in plant against fungi. In legume plants LysM containing receptor-like kinases act as a receptor for nodulation factors that helps to form nodules during symbiosis with nitrogen fixing *Rhizobium* bacteria. This receptor can sense the presence of lipo-chitin oligosaccharide signaling molecule released by *Rhizobium* as a nodulation factor (Limpens *et al.*, 2003). LysM domain are also present in fungal proteins. Three LysM domains were recently reported in Ecp6, one of the extracellular effector proteins of *Cladosporium fulvum* (de Jonge and Thomma 2009; Buist *et al.*, 2008; Bolton *et al.*, 2008). Apoplastic secretion of the LysM domain-containing Ecp6 protein can increase *C. fulvum* virulence in susceptible tomato plants (Bolton *et al.*, 2008). Subsequently it has been demonstrated that purified Ecp6 protein from *C. fulvum* can bind with chitin but not with xylan, cellulose or chitosan (de Jonge *et al.*, 2010).

Plants can sense Pathogen Associated Molecular Patterns (PAMPs)(Janeway 2002) and this PAMP recognition can induce resistance to the pathogen by PAMP Triggered Immunity (PTI) (Boller and He, 2009). A pathogen can secrete effector proteins to counteract the effects of PAMPs. Effectors can prevent PAMP recognition by the plant and promote pathogen establishment in host plant, which is called effector trigger susceptibility (ETS) (Jones and Dangl 2006). Furthermore a specific resistance gene of a plant can mediate effector triggered immunity (ETI) (Jones and Dangl 2006) by recognizing some of the pathogen effectors. In many plant-pathogen interactions, ETS is a step for pathogen where the pathogen can avoid recognition of chitin as a PAMP by the plant, and it is important for pathogen viability and causing disease.

Fungal cell walls have a high amount of chitin, a polymer of N-acetylglucosamine. Chitin is a molecule that is typical for fungi and insects, but it does not appear in plants. Plants are able to sense chitin fragments as a PAMP and this sensing can induce PTI as a defense response. For example, if a tomato plant can recognize chitin that is released from the *C. fulvum* cell wall, it will result in a defense response that includes the production of enzymes that degrade fungal cell walls. In such a case, the chitin binding ability of Ecp6 would scavenge chitin fragments from the apoplast, thereby reducing defense responses and promoting disease (de Jonge and Thomma 2009; Bolton *et al.*, 2008; de Jonge *et al.*, 2010). It might be suggested that Ecp6 is one of the effector proteins that contributes to ETS. Another chitin-binding effector protein, AVR4 of *C. fulvum* has no LysM domain. Recognition of Avr4 by the tomato resistance gene Cf4 triggers resistance to *C. fulvum* (Bolton *et al.*, 2008). In vitro experiments showed that AVR4 could protect *C. fulvum* hyphae from degradation and lysis by chitinase, whereas Ecp6 was unable to protect fungal hyphae from chitinase (de Jonge *et al.*, 2010). It was suggested that the LysM domain can bind with chitin fragments and mediate ETS by avoiding recognition of PAMPs. It can be predicted that the avoidance of PAMP recognition results in reduced or delayed chitinase induction by the plant.

During defense responses, plants can produce various fungal cell wall degrading enzymes, toxins and can induce an oxidative burst by producing active oxygen species (AOS), which can cause death of their own cells around the invaded fungal hyphae (Baker and Orlandi 1995). This defense mechanism is called programmed cell death (PCD) and can be visible as a necrosis called hypersensitive response (HR) (Greenberg *et al.*, 1994; Gilchrist 1998). HR can effectively stop fungal growth especially for biotrophic and hemibiotrophic fungi, as these fungi need living host tissues for their nutrients and eventually for their survival. In this situation PCD would be a threat for these fungi. They are unable to spread through the plant and the plant will demonstrate ETI and be resistant.

Botrytis cinerea is a necrotrophic plant pathogen. It has a wide host range of over 200 plant species (Williamson *et al.*, 2007). Recently LysM domains have been identified in *B. cinerea*. The *B. cinerea* genome contains four putative genes, *Bclysm1*, *Bclysm2*, *Bclysm3* and *Bclysm4*, all of which encode proteins containing different numbers of LysM domains. It has been described before that LysM has mostly chitin binding ability (Bolton *et al.*, 2008; Petutschnig *et al.*, 2010). One of those *Bclysm* genes, *Bclysm2* is orthologous to a *C. fulvum* gene encoding the chitin-scavenging protein Ecp6. *Bclysm* proteins of *B. cinerea* might also have chitin scavenging properties to avoid recognition of chitin as a PAMP by the plant. This might prevent plant to show any defense against chitin which would be called

ETS. Unlike biotrophic and hemibiotrophic fungi, *B. cinerea* doesn't need living tissues for growth. The fungus has a necrotrophic lifestyle and can utilize dead tissue like a saprophyte. In this context PCD might not be the effective way for a plant to prevent *B. cinerea*. In contrast, PCD may provide a favorable environment for the necrotrophic *B. cinerea* to grow. Moreover, according to Govrin et al., (2000) *B. cinerea* can induce oxidative burst and HR in *Arabidopsis*. A HR deficient *Arabidopsis* mutant *dnd1*, was resistant to *B. cinerea*. It can be assumed that ETI would not be beneficial for a plant in case of *B. cinerea* interaction. Therefore the study was focused on to investigate four *Bclysm* genes in *B. cinerea* in chitin binding ability. Accordingly it would be thought-provoking to explore the function of *Bclysm* genes of *B. cinerea* in plant infection, carbohydrate binding preference and cell wall integrity.

2. Materials and Method

2.1 QPCR analysis of *Botrytis cinerea* genes during plant infection

To measure the expression of *Bclysm* genes in *Botrytis cinerea*, cDNAs were used as templates that were obtained from different sources. Two cDNA samples were made on RNA isolated from liquid cultures of *B. cinerea* grown in medium containing glucose or pectin as carbon sources. Two other cDNA samples were made on RNA isolated from *B. cinerea*-infected tomato and tobacco plants. QPCR was done using primer pairs Q1F+Q1R, Q2F+Q2R, Q3F+Q3R and Q4F+Q4R (supplementary Table 1) for four *Bclysm* genes *Bclysm1*, *Bclysm2*, *Bclysm3* and *Bclysm4* of *B. cinerea* respectively. Relative quantification (RQ) of each of the gene transcripts were performed by comparing with an internal standard.

2.2 Making knockout mutants in *Botrytis cinerea*

Two flanking regions at 5' and 3' for each of the genes were selected for amplification. At 5' flanking region of each gene, two forward-F (5.1 + 5.2), one reverse-R (5.3) and at 3' flanking region of each gene two reverse-R (3.1 + 3.2), one forward-F (3.3) primers were designed respectively (supplementary Table 2). 5' and 3' flanking regions of each of the genes were amplified by using genomic DNA (10-50 µg) of *B. cinerea* as a template for all of the genes. PCR mixture was made with 5 µl of 5x Go tag buffer; 0.25 µl of Go tag polymerase; 0.5 µl of dNTP, and 0.5 µl of each primers forward and reverse for each of the genes. Primer pair 5.1F and 5.3R for 5' and primer pair 3.1R and 3.3F for 3' were used for each of the genes respectively. Volume was adjusted by adding milli-Q water up to 25 µl. About 550-670bp in length, two flanking regions 5' and 3' from both side of each of the gene were amplified and the fragments were denoted by *Bclysm1*-5'flank, *Bclysm1*-3'flank, *Bclysm2*-5'flank, *Bclysm2*-3'flank, *Bclysm3*-5'flank, *Bclysm3*-3'flank, , *Bclysm4*-5'flank, and *Bclysm4*-3'flank,.

Purified fragment contain 5' and 3' flanking region were separated for each of the genes separately. Two purified flanking regions 5' and 3' were overlapped with hygromycin cassette (2.4 kb), primers 5.2 F + 3.2 R for each of the genes. Reaction mixture was contain 20 ng of each flanking region, 20 ng of hygromycin cassette, 5 µl of 10x high fidelity buffer, 0.5 µl of

high fidelity polymerase, 5 µl of dNTP, 1µl of each primer of 5.2F and 3.2R for each of the genes and the volume was adjusted up to 50µl with milli-Q water. The PCR program overlap PCR (Appendix 1a) was used. PCR products with 1kb ladder were run through the gel to see the expected bands about 3.5 kb. For transformation 10-15 µg DNA was needed. The amount of fragments were increased by using overlap PCR. Protoplast transformation of *B. cinerea* and PCR-based screening of those transformants were done according to the description of Kars *et al.*(2005).

2.3 Southern blot

Genomic DNA of all the mutants and wild type was digested with HindIII. 3' flanking regions of each gene were used as the gene probe. The probe for detecting the HPH cassettes was amplified using pLOB7 as a template with primers LZ92+LZ93. Southern hybridization was performed with the manufacturer's instructions for DIG nucleic acid detection kit (Roche, Germany).

2.4 Virulence assay

Tobacco and tomato plants were used for infection assay. Droplets of a suspension of conidia of wild type and mutants (2 µl, 10⁶ conidia/ml in potato dextrose broth, 1.2g/l) were inoculated on opposite sides of the central vein of tobacco and tomato leaves. For tomato, 3-4 droplets per leaf half and for tobacco, 1-2 droplets per leaf half were used. For tomato, 4 leaves with 4 leaflet were treated. For tobacco, 4-5 individual leaves per plant were treated. Lesion size were measured with a digital caliper at 3dpi (day post inoculation). Each mutant was tested in two independent experiments.

2.5 Growth assay

Radial growth assays were done on B5 medium containing 50mM glucose, 10mM KH₂PO₄ and 1.5% agar. For stress treatments, 0.5M sorbitol, 0.5M NaCl, 50 µg/ml calcofluor white, 0.05 µg/ml iprodione, 0.02 µg/ml carbendazim and 10 mM, 15 mM, 30 mM and 50 mM of H₂O₂

were used. Another plate growth assay was performed to test the growth preference of mutants and wild type on B5 medium containing different carbon sources: 50 mM galactose, 50 mM fructose, 0.1% and 1% cellulose, 0.1% and 1% pectin and 0.1% and 1% xylan. Tomato and tobacco leaf extract were also used as nutrient medium. All mutants and wild type were grown in each of the treatment with three replications of each. B5 medium without any treatment were used as a control for all mutants and wild type. For iprodione and H₂O₂ mycelium containing agar plugs were used and for other treatments 5 µl of 10⁵ spores/ml were used.

2.6 Chitinase treatment

Fresh spores were collected from ten days old culture of *B. cinerea* *Bclys*m mutants and wild type. Spores of *Trichoderma viride* were collected from the stock of *Verticillium* Lab. Spore concentrations (6x10⁴ spore/ml) were adjusted by using hemocytometer and dilution procedure. Spores of *B. cinerea* mutants and wild type were incubated for 3 hours in B5 containing 10mM fructose for pre-germination. Spores of *T. viride* were incubated in PDB (Potato Dextrose Broth) for 24 hours for pre-germination. After germination, 30% (15 µl) chitinase was added into 35 µl of spore suspension in a microtiter plate. Next incubation was performed for 4 hours. For germination test 30% chitinase with B5 containing fructose solution, only 30% chitinase with B5 medium without fructose and only B5 containing fructose solution were used in same setting

2.7 BcLysM1 and BcLysM2 protein production and characterization

2.7.1 Expression of BcLysM1 and BcLysM2 in *Pichia pastoris*

2.7.1.1 Primer design and amplification of *Bclysm1* and *Bclysm2* genes

To amplify *Bclysm1* two forward and one reverse primer were designed. Of two forward primers, one primer that did not contain any restriction site and HIS-FLAG tags (Bcl1F) and the other one containing a restriction site (EcoRI) and HIS-FLAG tags (Bcl1LF) were designed (supplementary Table 3). Both forward primers were started just after the signal peptide of the gene and cover the whole 5' exon before the intron and part of the second exon. The reverse primer (Bcl1R) was designed with restriction site NotI. Bcl1F and Bcl1R primers were used first to amplify the gene without 5' restriction site and tags by using PCR. Later the first PCR product was used as a template and primers Bcl1LF and Bcl1R were used to add restriction site EcoRI and the HIS-FLAG tags. For *Bclysm2* one pair of primer Bcl2F (forward) and Bcl2R (reverse) were designed and used to amplify the gene by using gDNA of B05.10. PCR mix was prepared with 5 µl of 5x buffer, 0.5 µl of dNTP, 0.25 µl of GoTaq (PROMEGA), 0.5 µl of each forward and reverse primer for each gene, 0.5µl of template DNA or PCR product and the volume was adjusted up to 25 µl with milli-Q water. PCR products were run on an agarose gel. Expected DNA bands were purified from gel by using GE Healthcare illustraTM GFXTM PCR DNA and Gel band purification kit. Purified fragments were ligated into pGEMT vector.

2.7.1.2 Cloning of genes

Two cloning steps were performed before expressing both genes into *Pichia pastoris*. At first PCR product were cloned in to pGEMT vector. 10 µl of ligation solution was prepared with the mixture of 5 µl of 2x buffer, 1 µl of pGEMT vector, 2 µl of purified PCR product, 1 µl of T4 ligase and 1 µl of milli-Q water. The mixture was incubated at room temperature for 1 hour. 90 µl of chemically competent cellsdH5α were mixed with the ligation solution and kept on ice for 20 minutes. Heat shock was given to the mix at 42°C for 90 seconds and immediately plated on LB plate with ampicillin selection. Plates were incubated overnight at 37°C.

After overnight incubation 16 colonies for each gene were selected for colony PCR. Bcl1F, Bcl1R and Bcl2F, Bcl2R primer pairs were used to do the colony PCR. Colony PCR was performed with 40 cycles (Appendix 3). According to the PCR result three positive colonies

were selected that contained the cloned fragment. Colonies were cultured overnight in ampicillin containing liquid LB medium. Plasmids from each of the colonies were isolated by using QIA prep spin. Miniprep kit (250). 15 µl of plasmid with the concentration about 50-100ng/µl were prepared and sent for sequencing.

pGEMT plasmids with correctly sequenced genes were cut with restriction enzymes NotI and EcoRI to get the cloned gene-fragment. For restriction digestion 5 µl of plasmid, 1 µl of NotI, 2 µl of 10xbuffer and 1 µl of BSA were used to make the reaction mixture and the volume was adjusted up to 20 µl. Reaction mixtures were incubated in 37° C for 2-3 hours. After incubation the mixtures were put at 65°C for 15 minutes to stop the restriction enzyme activity. Then 1 µl of BSA, 1 µl of restriction enzyme EcoRI and 4 µl of 10x buffer were added in the previous reaction mixtures. Finally the volume was adjusted up to 40 µl by adding milli-Q water. The mixtures were incubated at 37° C for another 3 hours. The expected bands were purified from gel for each of the genes by using DNA purification kit.

In the second cloning step, purified fragments of each of the genes were ligated into pPic9. For ligation 5 µl of 2x buffer, 1 µl of pPic9 vector, 2 µl of gene fragment, 1 µl of milli-Q water and 1 µl of T₄ ligase were mixed and incubate in 14°C overnight. 90µl of chemically competent cells were mixed with each of the ligation reactions. Mixtures were kept on ice for 20 minutes then heat shock was performed at 42°C in water bath for 90 sec and kept on ice after the heat shock for another 90 seconds. 700µl of LB medium was added to the mixture. Mixtures were incubated in 37°C while shaking for 1 hour and then centrifuged. 100 µl of resuspended pellet was plated on ampicillin containing LB agar medium plates. Plates were incubated overnight in 37°C. Next day, 16 colonies from each of the constructs were selected for colony PCR by using primers pairs Bcl1F + Bcl1R and Bcl2F + Bcl2R for *Bclysm1* and *Bclysm2* respectively. PCR products were run on gel and for *Bclysm1* and *Bclysm2* the expected band sizes are visible.

One of the positive colonies from each of the constructs was cultured overnight in ampicillin containing liquid LB medium. Plasmids were isolated by using QIA prep spin. Miniprep kit (250). The concentration of plasmid DNA was measured by using Nanodrop. Isolated plasmids were cut with the restriction enzyme SacI to make linear plasmid DNA. Restriction digestion mixtures were made by using 5 µl of plasmid DNA of each of the constructs, 1 µl of SacI, 2 µl of buffer and the volume was adjusted up to 20 µl by adding MQ. Mixtures were incubated at

37°C for 3 hours. After incubation the reactions were placed at 65°C for 15 minutes to stop the enzymatic reaction of Sac1. DNA purification kit was used to purify linear plasmid DNA.

2.7.1.3 Transformation into *Pichia pastoris*

To culture *Pichia pastoris* strain GS115, YPD liquid medium was made by 1% of yeast extract, 2% of peptone and 2% of dextrose. *P. pastoris* was pre-cultured in 5ml of YPD medium and incubated at 28°C while shaking at 225-259 rpm for 8 hours. Pre-cultured medium was transferred to 75ml of YPD medium in a 300 ml erlenmeyer flask to produce more *Pichia* cells. Flasks were kept overnight at the same conditions.

The culture was centrifuged for 5 minutes at 1500x g at 4°C. Supernatant was discarded and the pellet was resuspended and washed two times, the first time with 50ml and the second time with 25ml ice cold water. Each time the resuspended pellet was centrifuged at the same condition. Finally the pellet was resuspended in to 10ml of ice cold 1M sorbitol and was centrifuged. After discarding the supernatant the pellet was again resuspended in 1ml of ice cold 1M sorbitol. 80µl of the resuspended *Pichia* cells were mixed with the linearized plasmid DNA of each of the genes separately. Mixtures were transferred in to pre-cooled electroporation cuvettes. After 5 minutes incubation the cuvettes were pulsed at charging voltage 1500V and resistance of 200Ω. Immediately after the pulse 1ml of 1M sorbitol was added to each of the cuvettes and transferred to an eppendorf tube. 200µl of electroporation mixture was used for plating on MD (minimal dextrose) plate. Plates were incubated at 28°C for 4 days.

After 4 days incubation, single colonies were taken by toothpick and transferred to 30µl of 25mM NaOH. Mixtures were boiled in the PCR machine for 10 minutes at 94°C. 0.5µl of boiled colony for each of the constructs were used for the PCR reaction. PCR was done by using primers Bcl1F, Bcl1R and Bcl2F, Bcl2R for *Bclysm1* and *Bclysm2* respectively. Positive colonies showed a band for the gene fragment with expected size on gel.

2.7.1.4 Protein production of BcLysM1 and BcLysM2 in recombinant *Pichia pastoris*

Recombinant *P. pastoris* strains were cultured for protein production by following the protocol of Invitrogen with slight modifications. One colony for each of the constructs was taken to grow into 10 ml of BMGY(Buffered Glycerol-complex Medium) medium (Appendix 2) at pH6.0 and with addition of 1% glycerol. Cultures were incubated in the incubator at 28-30°C while shaking at 250-300 rpm for 22 hours. After incubation optical density (OD₆₀₀) of the cultures were measured. Value of OD₆₀₀ was adjusted to 0.1 by making a dilution of the culture with a final volume of 10ml. 1% of methanol was added to medium which was incubated in the incubator, at 28°C-30°C shaking at 250-300 rpm for next 72 hours. Methanol induces the expression of the heterologous gene construct. Therefore, every 24 hours 1% of methanol was added to the medium. After 3 days incubation *P. pastoris* transformant cultures were centrifuged and the supernatant was stored in -20°C.

2.7.2 Characterization of expressed proteins

2.7.2.1 Western blotting

To do the western blot SDS (sodium dodecyl sulfate)- polyacrylamide gel was prepared. 20µl of protein sample was mixed with 5µl of 5x loading buffer and incubated at 95°C while shaking, for 10 minutes. Samples were run through the gel. Subsequently, gels were used for western blotting to detect the presence of the protein.

2.7.2.2 Carbohydrate binding assays

1ml of *Pichia* medium containing the BcLysM2 protein was added to 3 mg of five different carbohydrates (crab chitin, chitin beads, chitosan, xylan and cellulose). Mixtures were incubated on a rotary shaker at -4°C overnight. After incubation, mixtures were centrifuged for 3 minutes at maximum speed. Supernatants were separated and kept as a 'supernatant' sample. Pellets were washed three times with milli-Q water. After the final wash 200µl of 1% SDS was added to the pellets which were boiled 10 minutes in a heat block at 95°C while shaking. Finally, the pellet samples were centrifuged and the supernatants were collected in a separate tube. These samples

were kept as 'pellet' samples. Both from supernatant and pellet samples 20µl was taken and 5 µl of loading buffer was added. These samples were boiled for 10 minutes at 95°C in a heat block while shaking. Two SDS polyacrylamide gels were run, one with supernatant and another with pellet samples. Western blots were developed for both of the gels to detect the presence of protein in supernatant and/or pellet samples.

3. Results

3.1 *Bclysm* genes in *Botrytis cinerea*

Seven genes encoding LysM domain-containing proteins were identified in the genome of *Botrytis cinerea*. Some of the LysM domain-containing proteins of *B. cinerea* have additional protein domains. *Bclysm1*, *Bclysm2*, *Bclysm3* and *Bclysm4* are the four genes of *B. cinerea* that have only a number of LysM domains and they lack other domains. The proteins BcLysM1 and BcLysM2 both are predicted to have a signal peptide (signalP 3.0 server) and they contain one and two copies of LysM domains respectively. The BcLysM3 and BcLysM4 proteins have no signal peptide and each of them contains one LysM-domain (Fig 1). BcLysM2 has homology with Ecp6, the effector protein of *Cladosporium fulvum* which contains three LysM domains (Bolton *et al.*, 2008).

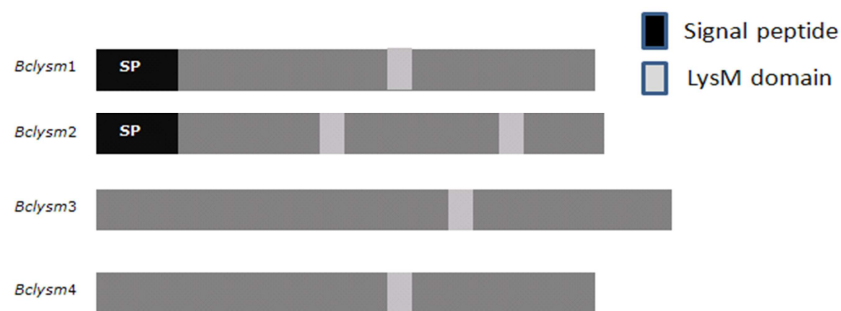


Figure (Fig) 1. Structure of LysM-domain containing proteins of *Botrytis cinerea*; sp: signal peptide, the grey boxes represent LysM domains.

3.2 Expression analysis of *Botrytis cinerea* *Bclysm* genes

To determine the expression profile of *Bclysm* genes in *B. cinerea*, their mRNA level was monitored by real-time PCR in cultures containing glucose and pectin as the sole carbon source and during infection on tomato and tobacco. *Bclysm1*, *Bclysm2* and *Bclysm3* show close to equal transcript level during growth in pectin containing medium, as well as in glucose containing medium. *Bclysm4*, however, shows a very low transcript level both in pectin and glucose culture. (Fig 2.).

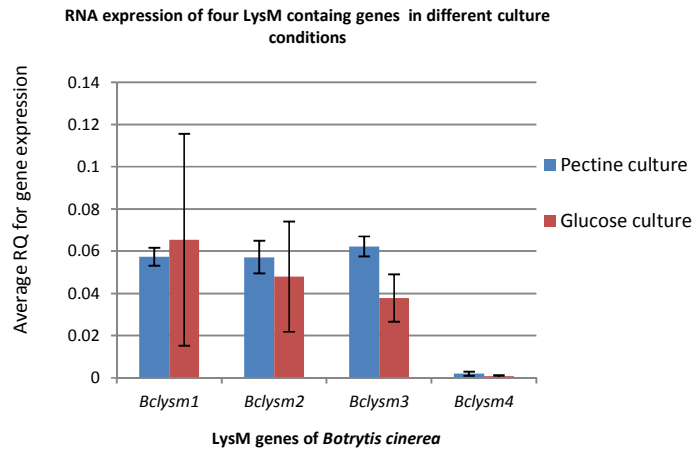


Fig 2. Transcript levels of *Bclysm1*, *Bclysm2*, *Bclysm3*, and *Bclysm4* in different carbon sources, assessed by real-time PCR. Cultures were sampled for RNA extraction at 3 h after transfer from a pre-culture with glucose as carbon source. mRNA levels of the genes were normalized to the constitutive reference gene *Bcrp15*. Data are represented as mean \pm standard deviation. Three technical replicates were analyzed.

In the first plant infection experiment, *Bclysm3* showed higher transcript levels compared to other genes both in tomato and tobacco. For *Bclysm3* the level was very similar at 2dpi and 3dpi. *Bclysm1* and *Bclysm2* showed very low transcript levels, both in tobacco and tomato. For *Bclysm4* the transcript level was more than two fold higher in tobacco and 0.5 fold higher in tomato plant at 3dpi than 2dpi (Fig 3).

The results obtained by using template RNA from the first experiment were not reproducible in the second experiment. Only *Bclysm4* showed comparable transcript level with the first experiment. In tobacco 3dpi, the transcript levels for *Bclysm1*, *Bclysm2* and *Bclysm3* were more than 3 fold higher compared to 2dpi. In tomato the transcript levels of *Bclysm1*, *Bclysm2* and *Bclysm3* at 3dpi were more than 2 fold higher compared to 2dpi.

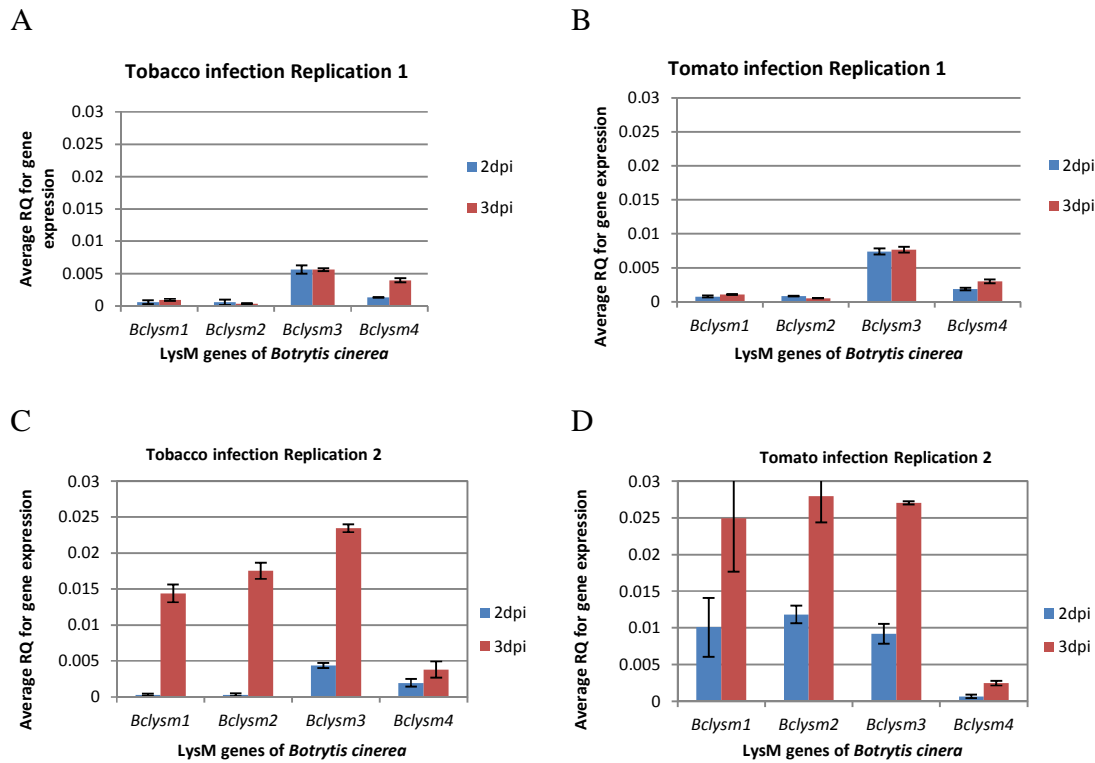


Fig 3. QPCR result average RQ (relative quantification) for each of the genes in two different plant infection. A and C-QPCR done by using RNA as a template from infected tobacco plant from 2 dpi and 3 dpi. B and D-QPCR done by using RNA as a template from infected tomato plant from 2dpi and 3dpi. Both experiment have done with two biological repeats. The figure shown 1st repeat (A, B) and 2nd repeats (C,D) and three technical replicates of each repeat were analyzed.

3.3 Identification and molecular characterization of homokaryotic mutants

3.3.1 Pure mutants screening

Four genes have been targeted for knockout mutant construction. The mutants $\Delta Bclysm1$, $\Delta Bclysm2$ and $\Delta Bclysm4$ were found to be pure, homokaryotic knockout mutants. However, it appeared that for $\Delta Bclysm3$, all transformants in which homologous recombination had occurred, were heterokaryotic and had retained wild type nuclei besides the transformed (mutant) nuclei. PCR was done for checking the 5' and 3' flanking region and for checking the presence of the wild type gene. Results shows that homokaryotic knockout mutants showed a band for either one or both flanking fragments, but they showed no amplification product for the

wild type gene (Fig 4). Transformants obtained with the *Bclysm3* construct showed both of the flanking fragments, but also the wild type *Bclysm3* gene (Fig 4). It indicates that the *Bclysm3* was replaced by the hygromycin cassette but the transformants still contained the wild type gene. Mutant screening was continued for *Bclysm3* by subsequent rounds of single spore plating on selective medium. Even after six cycles of single spore plating, all homologous recombinants tested were heterokaryotic, and a homokaryotic *Bclysm3* knockout mutant was not obtained, Further experiments were done with the homokaryotic mutants in the *Bclysm1*, *Bclysm2* and *Bclysm4* genes.

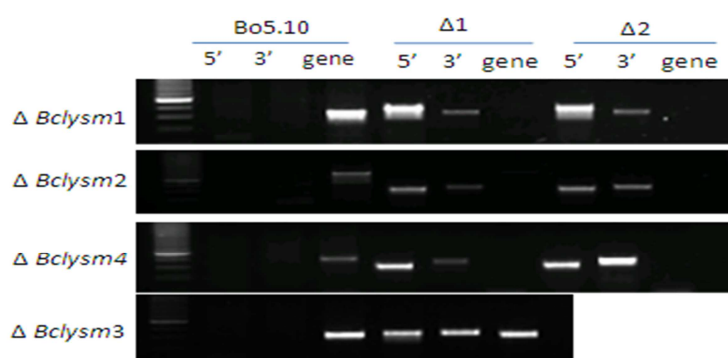


Fig 4. Two individual mutants of each gene are compared with wild type strain B05.10. Δ1 and Δ2 are the individual mutant for each of the genes. For *Bclysm1*; Δ*Bclysm1*-10a and Δ*Bclysm1*-14c, *Bclysm2*; Δ*Bclysm2*-4b and Δ*Bclysm2*-6c, *Bclysm4*; Δ*Bclysm4*-4b and Δ*Bclysm4*-6a, are the individual mutants. Δ *Bclysm3* has 3', 5'flank and *Bclysm3* gene (one of more than 20 construct).

3.3.2 Southern blot

Southern blot of wild-type strain B05.10 and knockout mutants was performed. Genomic DNA of each strain was digested with HindIII and hybridized to a probe mixture containing probes of *Bclysm1*, *Bclysm2*, and *Bclysm4*. HindIII digestion led to hybridization signal on the blot as a band in kilobase (kb) (Table 1, Fig 5) Some nonspecific bands were found on the blot. It indicates that additional ectopic integrations may have occurred besides the homologous integration. Nonspecific bands could also be due to the use of too much probe, insufficient washing time and blocking time during blotting. The hygromycin probe detected bands only for mutants (Table 1, Fig 5). No band was found for B05.10 as expected. During loading of digested DNA the sample for *Bclysm4.6a* was lost, therefore in the blot hybridized with the hygromycin probe no band was found for *Bclysm4.6a*.

Table 1: Gene probes and hygromycin probes hybridization signal (kb) for all of the mutants and wild type.

Gene probes		HPH probe	
Gene names	Mutants (kb)	Wild type (kb)	Only for mutants (kb)
<i>Bclysm1</i>	2.3	3.5	3.0
<i>Bclysm2</i>	1.9	6.7	8.5
<i>Bclysm4</i>	2.1	9	9

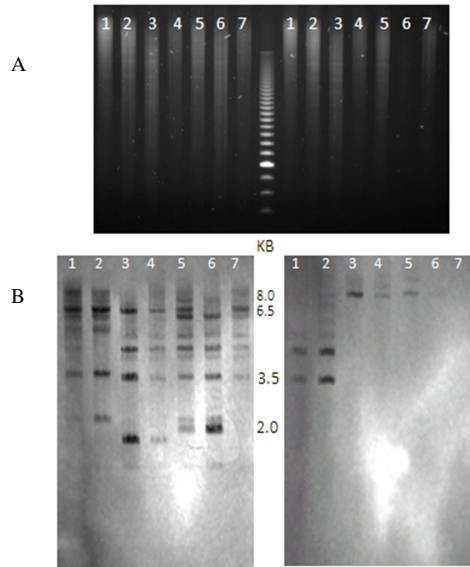


Fig 5. Southern blot. Two individual mutants of each of three *Bclysm* mutants and wild type. 1-7 are *Bclysm1.10a*, *Bclysm1.14c*, *Bclysm2.4b*, *Bclysm2.6c*, *Bclysm4.4b*, *Bclysm4.6a*, and B05.10 respectively. A- the gel picture of HindIII digestion. B- southern blot for gene probe (left) and hygromycin probe (right).

3.4 *Bclysm* genes are not essential for virulence on tomato and tobacco

To determine the function of *B. cinerea* *Bclysm* genes in virulence, two individual mutants of three knockout mutants $\Delta Bclysm1.10a$, $\Delta Bclysm1.14c$, $\Delta Bclysm2.4b$, $\Delta Bclysm2.6c$, $\Delta Bclysm4.4b$, $\Delta Bclysm4.6a$ and wild type strain B05.10 were used for tobacco and tomato plant infection assays.

Lesion sizes were measured after 3dpi. When the experiment was performed the first time, all of the mutants made significantly smaller lesions than the wild type strain B05.10, both in tobacco and tomato infection assay (supplementary Fig 1). However, the spore concentration used in the first experiment was lower for all mutants than for the wild type. This could be a possible reason for the reduction of lesion size in all mutants. In the second experiment, special care was taken to ensure that equal spore numbers were used for the wild type and the mutants. When the experiment was repeated, none of the mutants showed any difference in lesion size both in tomato and tobacco, compared to wild type (Fig 6).

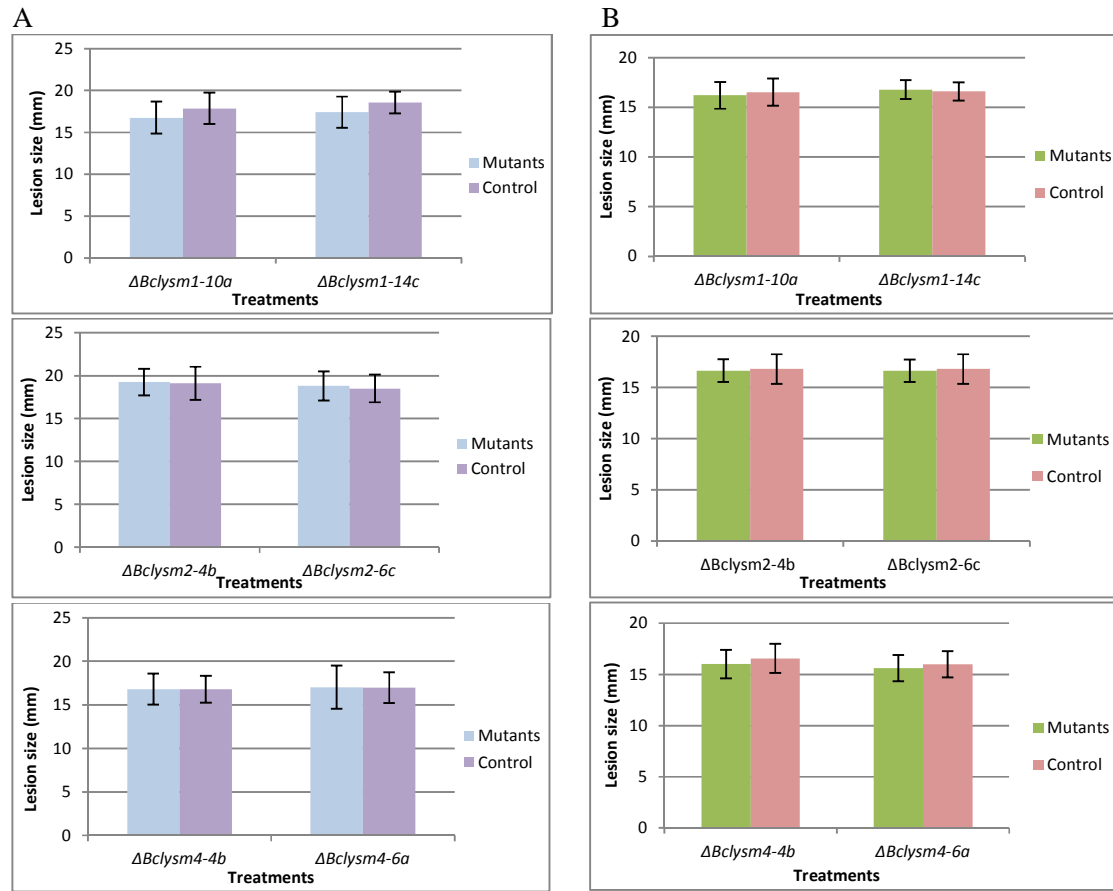


Fig 6. Two individual mutants of each of three knockout mutants induced lesion size average in millimeter. Two individual clusters in each of the graph shows individual mutants and corresponding wild type lesion size as a control. Results shows the average lesion size caused by 186 droplet of inoculum on tobacco (A) and 409 droplet of inoculum on tomato (B) leaves.

3.5 Growth assay of *BclysM* mutants on different carbohydrates and stress conditions

Three different kinds of five different stress mediums were used in this experiment: sorbitol and sodium chloride (NaCl) as an osmotic stress, calcofluor white for inhibitor of chitin and $\beta(1,3)$ -glucan synthases (Roncero and Duran 1985), iprodione and carbendazim, as a fungicides. Spores of mutants and wild type were inoculated on the medium with the different stress-inducing chemicals. The colony diameter of the mycelium was measured every day after spore germination. Results show that for each of the treatment all mutants grew as well as the wild type (supplementary Fig 2). On non-treatment plate the mycelium of all mutants and wild type were grown nearly in equal diameter to the treatment plates, however the mycelium density was too much on the non-treatment plates than the treatment plates. Result shows that $\Delta BclysM1$, $\Delta BclysM2$, $\Delta BclysM4$ and wild type (B05.10) are equally sensitive to H_2O_2 concentrations of 10mM and 15mM. In 30mM and 50mM H_2O_2 , however, mutants were less sensitive than the wild type (Fig 7). The wild type was unable to grow in 50mM of H_2O_2 , whereas the mutants formed a colony of 1 cm, approximately 20% of the colony size on medium lacking H_2O_2 .

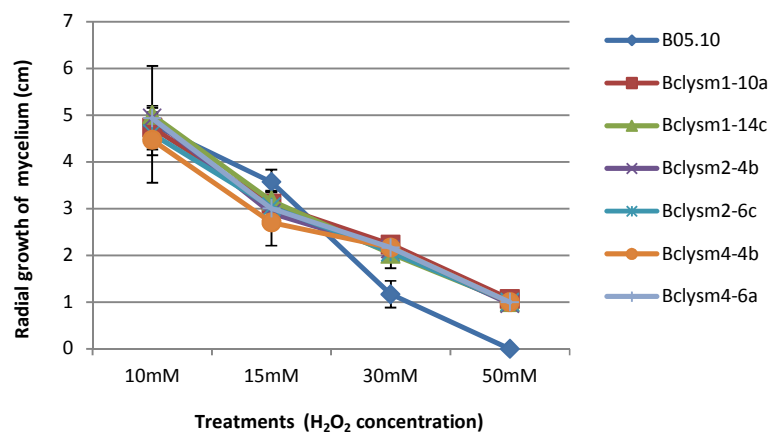


Fig 7. Radial growth (3rd day) of LysM mutants (two individual mutants of each *BclysM* mutants) and wild type (B05.10) of *Botrytis cinerea* on B5 agar medium containing 10 mM, 15 mM, 30 mM and 50 mM of H_2O_2 . Each of the value is the average of three replicates. Data were collected up to 8th day. From 4th day to 8th day have shown result as like as 3rd day (supplementary Fig 3).

Radial growth on galactose (15 mM), fructose (15 mM), cellulose (0.1% and 1%) and 0.1% pectin was similar for all mutants compared to wild type (supplementary Fig 4F). On 1% pectin all mutants grew slightly more than the wild type.

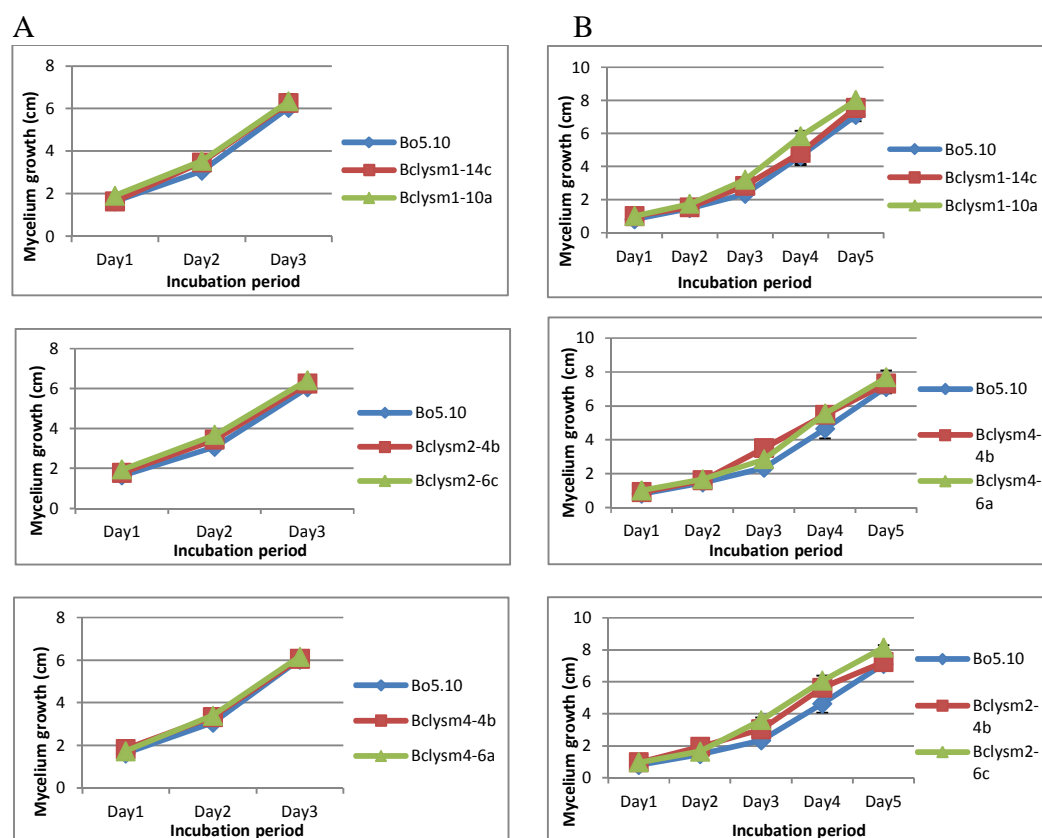


Fig 8. Mycelium growth of two individual mutants of each of three *Bclysm* mutants of *Botrytis cinerea* and wild type (B05.10) on 1% pectin (A), 1% xylan (B) containing B5 agar medium. In each of the graph wild type is same. Each value shows the average of three replications.

In 0.1% xylan mutants grew similar to the wild type (supplementary Fig 4E). At higher percentage (1%) of xylan, the wild type strain grew slower than all of the mutants (Fig 8). In this experiment two individual mutants have also shown difference in their growth, which is not much, as day 1 and day 5 individual mutants and wild type show nearly equal colony diameter. All of the mutants grew better than wild type on tomato leaf extract, but not on tobacco leaf extract (Table 2).

Table 2: Mycelium growth (cm) of *Bclysm* mutants and wild type per day on tomato leaf extract \pm indicates standard error. Growth was observed for 5 consecutive days from the 1st day after spore inoculation

Medium	Fungal strains	<i>Bo5.10</i>	<i>Bclysm1-10a</i>	<i>Bclysm1-14c</i>	<i>Bclysm2-4b</i>	<i>Bclysm2-6c</i>	<i>Bclysm4-4b</i>	<i>Bclysm4-6a</i>
Tomato leaf extract	Growth /day (cm) with standard deviation	1.35 \pm 0.11	1.76 \pm 0.14	1.45 \pm 0.02	1.86 \pm 0.00	1.48 \pm 0.40	1.86 \pm 0.00	1.68 \pm 0.02
Tobacco leaf extract	Growth /day (cm) with standard deviation	2.26 \pm 0.14	2.05 \pm 0.02	2.06 \pm 0	2.05 \pm 0.02	1.08 \pm 0.02	2.11 \pm 0.07	2.08 \pm 0.07

3.6 *Bclysm* mutants are unaltered in sensitivity to chitinase

It has been reported that the LysM domain-containing effector Ecp6 from *C. fulvum* can bind with chitin and it can constrain chitin-induced alkalinization in tomato cell suspensions. Nevertheless Ecp6 is unable to protect fungal hyphae from degradation by chitinase (de Jonge *et al.*, 2010). On the other hand it has been found that an endochitinase overexpressing transgenic tobacco line shows reduction and in some cases total resistance to *B. cinerea* (Lorito *et al.*, 1997). This information made it interesting to study *B. cinerea* wild type and *Bclysm* mutants for their sensitivity to chitinase.

Three of the *Bclysm* mutants and wild type of *B. cinerea* (B05.10) did not show any differences in mycelium growth upon chitinase treatment. *Trichoderma viride* was used as a positive control in this experiment. In the presence of chitinase, *T. viride* mycelium was largely degraded but $\Delta Bclysm1$, $\Delta Bclysm2$, $\Delta Bclysm4$ and the wild type strain B05.10 grew as well in the presence of chitinase as in the control without chitinase (Fig 9A). Additionally, it has found that *B. cinerea* germination is better in combination of chitinase and fructose. Where only in fructose solution their germination is comparatively less (Fig 9B).

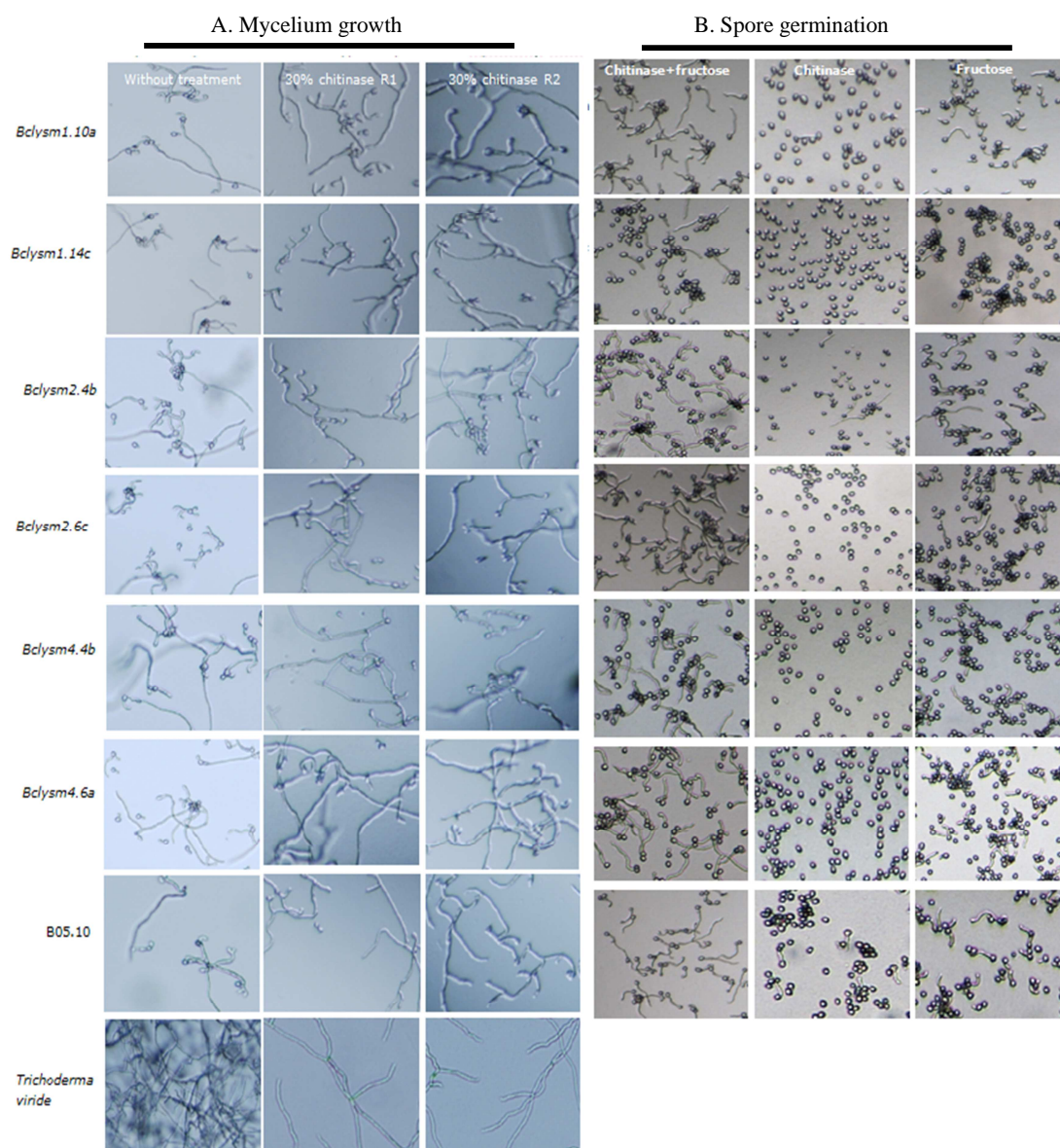


Fig. 9. A- Mycelium growth of three *Bclysm* mutants and wild type in presence of chitinase and without chitinase. *Trichoderma viride* has used as a positive control. B- Germination of three *Bclysm* mutants and wild type in presence of 3chitinase with fructose, only chitinase and only fructose. Pictures are showing one from three of the replications.

3.7 Production of BcLysM1 and BcLysM2 protein in *Pichia pastoris*

Recombinant *P. pastoris* strains containing the *Bclysm1* or *Bclysm2* gene construct were cultured for protein production. In both gene constructs, the region encoding the *B. cinerea* signal peptide was replaced by a yeast signal peptide sequence, to ensure that the protein is secreted in the culture medium. Western blot showed protein signal only for BcLysM2 (68-69 KD) but not for BcLysM1 (24-25 KD) (Fig. 10).

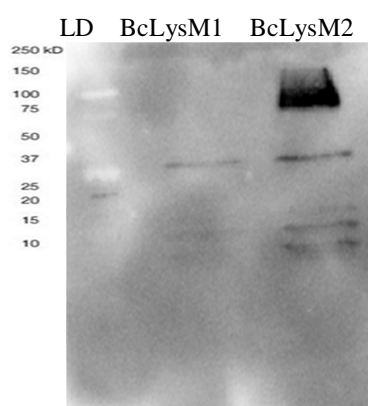


Fig.10 Western blot of proteins BcLysM1 and BcLysM2, produced in recombinant *Pichia pastoris*.

3.8 BcLysM2 binds with chitin but not with other carbohydrates

Five insoluble carbohydrates (crab chitin, chitin beads, chitosan, xylan, and cellulose) were used to check carbohydrate binding affinity of BcLysM2 protein. Protein precipitating with an insoluble carbohydrate in the pellet sample indicates carbohydrate binding. Protein remaining in the supernatant but not in the pellet shows the inability of protein to bind with the carbohydrate.

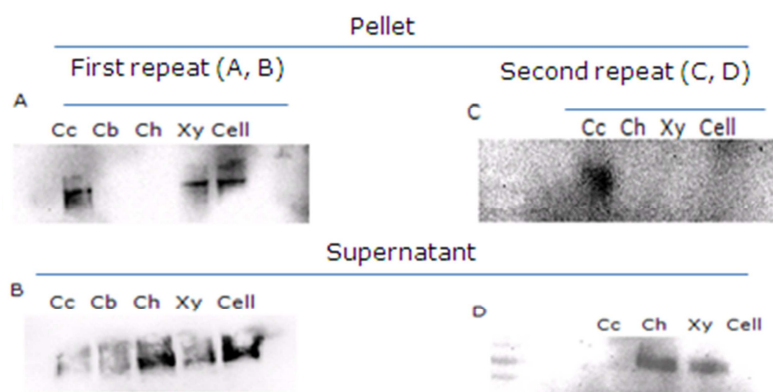


Fig. 11 Western blot. Carbohydrate binding affinity of BcLysM2 protein shows protein signal in the pellet sample (A, C). Protein cannot bind with carbohydrate shows protein signal in supernatant (B, D). Figure A, B and C,D are the western blot result for two repeats of the one experiment. In second repeat chitin beads are not included. In this figure Cc-Crab chitin, Cb-Chitin beads, Ch-Chitosan, Xy- Xylan, and Cell- Cellulose.

Western blot shows that during the first experiment, protein was detected in the crab chitin pellet sample and no signal was found in the supernatant fraction (Fig. 11 A, B). This indicates that BcLysM2 protein binds with crab chitin. The same result was obtained in the second experiment (Fig. 11C, D).

Protein signal was found both in pellet and supernatant samples of xylan and cellulose in the first experiment (Fig.11 A, B). In the cellulose supernatant fraction, there was a stronger protein signal than in the xylan supernatant fraction. However, the second experiment has not been reproducible for xylan and cellulose. There was no protein signal for either xylan or cellulose in the pellet samples (Fig.11 C, D). Moreover in this experiment, there was no signal at all in the pellet or in the supernatant samples of the cellulose binding assay.

4. Discussion

Botrytis cinerea is a necrotrophic fungus. Recently four LysM containing genes, *Bclysm1*, *Bclysm2*, *Bclysm3* and *Bclysm4* have been identified in the *B. cinerea* genome. Two of them, *Bclysm1* and *Bclysm2* have a signal peptide and the other two, *Bclysm3* and *Bclysm4* don't. It is therefore predicted that the BcLysM1 and BcLysM2 proteins will be secreted from the hyphae, whereas the BcLysM3 and BcLysM4 proteins remain in the cytoplasm. Expression analysis for the four *Bclysm* genes was performed *in vitro* with pectin and glucose as carbon sources and *in planta*. *Bclysm1*, *Bclysm2* and *Bclysm3* were transcript more *in vitro* experiment than *in planta* (Fig.2 & 3). The transcript levels for *Bclysm1*, *Bclysm2* and *Bclysm3* during tobacco and tomato infection were not reproducible between experiments. Only *Bclysm4* was expressed equally in two experiments, both in tobacco and tomato. The reason for the difference between experiments remains unclear, it will be essential to perform additional biological repeats for this experiment. Knockout mutants were made by using homologous gene replacement for four of the *Bclysm* genes in *B. cinerea*. Pure, homokaryotic knockout mutants were obtained for *Bclysm1*, *Bclysm2* and *Bclysm4*. Transformants obtained with the *Bclysm3* knockout construct showed successful homologous recombination, but these transformants always carried nuclei that contained the wild type gene. Even multiple rounds of single spore plating on selective medium failed to result in the isolation of a homokaryotic *Bclysm3* mutant. This observation suggests that the *Bclysm3* gene may be essential for viability, but the underlying mechanism is difficult to explain. The BcLysM3 protein is cytoplasmic, while the supposed substrate (chitin fragments) occur outside the fungal cell. Furthermore, BcLysM3 has no known protein domain other than the LysM domain. Therefore the biological function of BcLysM3 remains unknown.

Further experiments were performed with the pure mutants in the remaining three genes. Southern blot (Fig.5A) and gel picture (Fig.5B) of the three pure mutants demonstrated that all of the mutants have the hygromycin cassette inserted into the wild type gene. But multiple copies of 3' flanking region was also detected in each of the mutants. This might be due to insertion of the construct in another region of the genome by chance.

As one of the *Bclysm* genes has homology with Ecp6 of *C. fulvum*, it was assumed that at least BcLysM2 (and possibly other BcLysM proteins) of *B. cinerea* may also be able to sequester fungal chitin and thereby avoid chitin-induced defense response by the host plant. The avoidance of chitin-induced defense might enable wild type *B. cinerea* to make larger lesions. The *Bclysm*

mutants might show reduced lesion sizes as compared to the wild type if they are unable to sequester chitin fragments and may be exposed to plant chitinase or toxin. In tobacco and tomato infection assays, the lesion size caused by *Bclysm* mutants were the same as for the wild type. It demonstrates that none of the three *Bclysm* genes tested is essential for virulence.

It is possible that in single knockout mutants, only one of the four *Bclysm* genes are mutated whereas the other three *Bclysm* genes are still present. These genes might take over the function of the mutated gene, and no phenotype would be observed. In this study it was also found from chitinase treatment that none of the mutants and wild type were sensitive to chitinase. This result can be explained the obtained result from *in plant* infection assay. For an example, if *Bclysm* genes are able to sequester chitin fragments then *Bclysm* mutants are unable to sequester their chitin fragments and faced chitinase as one of the plant defense responses during plant infection assay, as *Bclysm* mutants and wild type are not sensitive to chitinase thereby they grew well through the plant. That might cause the equal lesion size as well as the wild type.

Two of the *Bclysm* genes *Bclysm3* and *Bclysm4* have no signal peptide. It indicates that the protein encoded by these two genes will not secrete and remain inside the cytoplasm. As they have chitin binding LysM domain and chitin is in outside of the cell, then they might be involve in chitin or other carbohydrates synthesis to the cell wall. If the *Bclysm* genes are important for cell wall composition, then the deletion of those genes may affect the cell wall thickness or stability. This might result in mutants being more sensitive to stress than wild type. The mycelium growth of mutants under stress conditions was compared to that of the wild type *B. cinerea*. In this experiment all mutants grew as well as the wild type in different concentrations of sorbitol, and sodium chloride (NaCl) as an osmotic stress, calcofluor white for inhibitor of cell wall and iprodione and carbendazim, two fungicides. It indicates that all of the genes *Bclysm1*, *Bclysm2* and *Bclysm4* of *B. cinerea* is equally sensitive to osmotic stress, chitin synthases inhibitor and fungicide of applied concentration. All of the mutants and wild type grew on control plates to equal diameter as in the stress treatment plates, but the mycelium was more dense in control plates. The medium was not sufficiently stressful for causing reduction of radial growth.

Active oxygen species (AOS) are produced by the plant during interaction with pathogen (Baker and Orlandi, 1995). AOS can induce programmed cell death (PCD) called hypersensitive response (HR) (Garvey *et al.* 1986), a protection strategy of plant from pathogen. As a pathogen

living in necrotic plant tissue, *B. cinerea* is continuously exposed to high levels of AOS and plant defense metabolites, which would be potentially stressful for the pathogen. We considered the possibility that the *Bclysm* genes may be important for a necrotroph to survive in such a chemically aggressive environment. To test this proposition it was interesting to study the effect of different concentrations of AOS by using H₂O₂ on *Bclysm* mutants and wild type. All of the mutants are gradually decreased in growth with the increasing concentration of H₂O₂. In this experiment on 30 mM of H₂O₂ growth of the wild type strain was slower than all mutants and on 50 mM the wild type failed to grow. Possibly, there was a difference in the agar plugs used as inoculum. The experiment needs to be repeated to draw meaningful conclusions.

LysM is a sugar binding domain, and LysM domain containing fungal effector proteins can bind with carbohydrates (Bolton *et al.*, 2008; de Jonge *et al.*, 2010). It was interesting to compare growth of *B. cinerea* and the *Bclysm* mutants on different mono and polysaccharide containing medium plates. In addition as plant infection assay shows that mutants and wild type caused equal lesion size both in tomato and tobacco plant (Fig. 6) then tomato and tobacco plant leaf extract also use to test the growing preference. All mutants and wild type grew equally in galactose and fructose medium, as well as in 0.1% and 1% of cellulose and pectin. Mutants grew better than the wild type in 1% xylan compared to 0.1% xylan. It seems that single *Bclysm* genes are not essential for growing in different carbon source and nutrient medium.

As individual *Bclysm* mutants did not show any significant phenotype in plant infection assays, growth in stress condition and different nutrients, the next step was to determine the sensitivity to chitinase. Since *C. fulvum* Ecp6 has homology with *Bclysm2*, it was assumed that the wild type as well as all of the mutant would be sensitive to chitinase. The result shows that wild type *B. cinerea* is not at all sensitive to chitinase, and also the mutants do not show an altered sensitivity as compared to the wild type. Remarkably, it was observed that *B. cinerea* germination is better in combination of chitinase and fructose, as compared to germination only in fructose (Fig. 9B). *B. cinerea* needs nutrients for their spore germination and fructose can provide that nutrients along with B5 medium. Results shows that without fructose, mostly no germination occurs. Chitinase is not a nutrient but its presence stimulates germination along with fructose. It can be assume that *B. cinerea* is not sensitive to chitinase furthermore chitinase is seems to be an aid for spore germination. Possibly the presence of *B. cinerea* spores as an inoculum on host plant may induce to secrete chitinase, which may influence *B. cinerea* for

better germination. It is also important to say that germination and infection may be influenced by the amount of chitinase released by the host plant, however the germination of *B. cinerea* spores, lesion starting time and lesion size expansion with day post inoculation (dpi) may vary during infection in different plants. The amount of chitinase used in vitro experiment may not be complementary with natural situation. Therefore this result may not be the real picture of the nature.

The Ecp6 has 3 LysM domains and it shows binding ability with chitin oligosaccharides (de Jonge *et al.*, 2010). It has been said before that only BcLysM2 has homology with Ecp6 while other BcLysM proteins have no homology. Hence it was expected that at least BcLysM2, and possibly the other proteins would show affinity for chitin. As *Bclysm1* and *Bclysm2* both have a signal peptide and the other two don't, we decided to express only *Bclysm1* and *Bclysm2* in *Pichia pastoris* to obtain these proteins from the culture. Only BcLysM2 gave a signal on western blot. BcLysM1 probably is not stable enough during fermentation and thereby unable to show any signal in western blot.

BcLysM2 produced in *P. pastoris* was used for a carbohydrate binding assay. As Ecp6 of *C. fulvum* shows binding ability with crab chitin, and chitin beads but not with chitosan, xylan and cellulose (de Jonge *et al.*, 2010), it was expected that BcLysM2 protein would also bind with crab chitin and chitin beads. In both repeats of this experiment, it was confirmed that BcLysM2 can bind with crab chitin. It was found that the protein also bound with xylan and cellulose in the first experiment but not in the second experiment. More repetitions of this experiment are needed to prove that BcLysM2 only binds with crab chitin but not with xylan and cellulose. As BcLysM2 shows crab chitin binding ability therefore it can be predicted that as a homologs BcLysM2 might have chitin binding function as well as Ecp6.

It might be interesting to study the double knockout mutants of *Bclysm* genes of *B. cinerea* in plant infection assay, chitinase sensitivity and fungal colony growth in different stress medium. Overexpression of single *Bclysmi* gene in *B. cinerea* would be another possibility to study the function of those genes. There are seven *Bclysm* genes were identified in *B. cinerea* genome. As the current study was only performed by four of those genes, those have only LysM-domains, still three of *Bclysm* genes are remind to study those have some other protein domains including LysM-domain. It would be quite interesting to study other three of seven *Bclysm* genes of *B. cinerea*.

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Supplementary files

Table 1: QPCR primers for 4 *Bclysm* genes.

Primer name	Sequence	Number of bp	Annealing tem (°c)
Q1F	5'-GGAAGTCTGCTGCAAATTGCACT- 3'	21	58
Q1R	5'-CGTACAACCAGCATCAATTTTCG- 3'	22	
Q2F	5'-CAATTCAACTGTTGGTCAACTGT- 3'	23	
Q2R	5'-AAGTGGCTTGCAGTTCATTGG- 3	21	
Q3F	5'-GACTGGGAGTGATGACGGTAG- 3'	21	
Q3R	5'-GATCTTCTCTCCCAGAAGTTCC - 3'	22	
Q4F	5'-GGGTGTAAAGTCTCTGAATACG- 3'	22	
Q4R	5'-GGATTTTCTTTCTCCCATCTCTC- 3'	23	

Table 2: Primers for knockout mutations.

Primer's Name	Sequence (bold font for hygromycine primer)	Number of bp	Annealing temp	Name for PCR	Remark
LysM 1- 5.1 F	5'-GGCAGTGCAATCACCCTCC- 3'	20	58	K2-5.1F	
LysM 1- 5.2 F	5'-TCTGTACCTCACCTCACCTG- 3'	20		K2-5.2F	
LysM 1- 5.3 R	5'- GGGTACCGAGCTCGAATTC GCCAAGATGGCGAGCGTGAG- 3'	39(20)		K2-5.3R	RC
LysM 1- 3.3 F	5'- CTCGGCGCGCCGAAGCTT GACACGTGTGATAGTTGAC- 3'	39 (21)		K2-3.3F	
LysM 1- 3.2 R	5'-GCATGTACGAAGTACAGCATC- 3'	21		K2-3.2R	RC
LysM 1- 3.1 R	5'-GGCTGTTGATTATGTGGATGG- 3'	22		K2-3.1R	RC
LysM 2- 5.1 F	5'-GACTCGACTGTGAGTTGACC- 3'	20	58	K4-5.1F	
LysM 2- 5.2 F	5'-GTCCCTTGTGGATTATCTGTC- 3'	21		K4-5.2F	
LysM 2- 5.3 R	5'- GGGTACCGAGCTCGAATTC GGTAGCGGCAGCAATGCTG- 3'	38 (19)		K4-5.3R	RC
LysM 2- 3.3 F	5'- CTCGGCGCGCCGAAGCTT GTTATCGCTACAGTTGTCG- 3'	39 (21)		K4-3.3F	
LysM 2- 3.2 R	5'-GCGCTTCCACTGCGCCAAC- 3'	19		K4-3.2R	RC
LysM 2- 3.1 R	5'-GCAAGTCCCAGTTTCGATCTG- 3'	21		K4-3.1R	RC
LysM 3- 5.1 F	5'-CTTGACGAGCTATTCCTCG -3'	20	58	K1-5.1F	
LysM 3- 5.2 F	5'-GAGCGGTAGGTGCTCATGC-3'	20		K1-5.2F	
LysM 3- 5.3 R	5'- GGGTACCGAGCTCGAATTC GTAGAGGAGGTTGCTGCACTG- 3'	40 (21)		K1-5.3R	RC
LysM 3- 3.3 F	5'- CTCGGCGCGCCGAAGCTT ACTGGGAGTGATGACGGTAG- 3'	38 (20)		K1-3.3F	
LysM 3- 3.2 R	5'-CCACCCACAGCCCAATCAGC-3'	20		K1-3.2R	RC
LysM 3- 3.1 R	5'-CACTCTCTTACCACTACTC-3'	21		K1-3.1R	RC
LysM 4- 5.1 F	5'-GGTCGGTGGATGTCGATTGTG- 3'	21	58	K3-5.1F	
LysM 4- 5.2 F	5'-CTACGTCACCCGAGGTCAC- 3'	20		K3-5.2F	
LysM 4- 5.3 R	5'- GGGTACCGAGCTCGAATTC TCTGCACTCCTGACCTGACAG- 3'	39 (20)		K3-5.3R	RC
LysM 4- 3.3 F	5'- CTCGGCGCGCCGAAGCTT GCAGTCCTGTACCTCGAAC- 3'	38 (20)		K3-3.3F	
LysM 4- 3.2 R	5'-CCGCCTCTGGCTGGAAGAG- 3'	19		K3-3.2R	RC
LysM 4- 3.1 R	5'-CTTCCAGACATTAGAACAACACC- 3'	23		K3-3.1R	RC

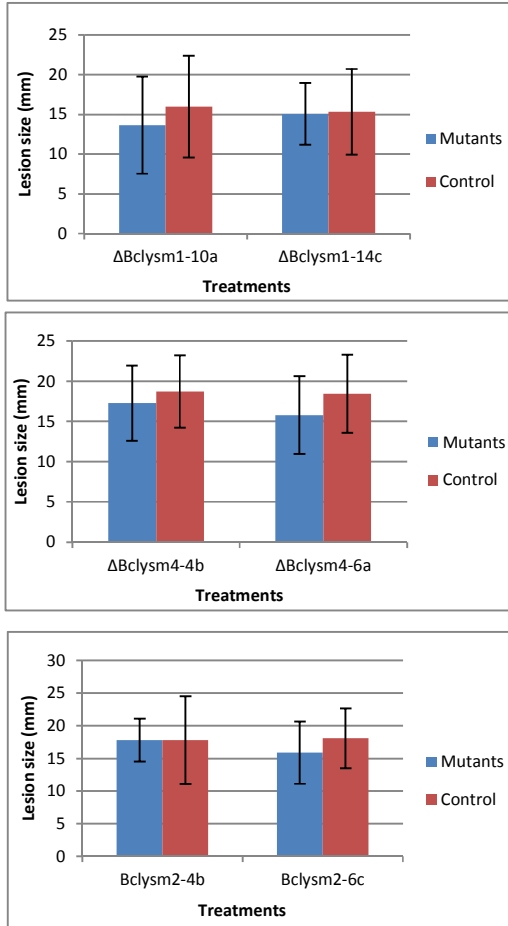
RC- Reverse Complement of the sequence.

Table 3: Primers for *Bclysm1* and *Bclysm2* gene sequences amplification

Name	Sequence with	Tm (°C)	Rem-ark
Bcl1 F	3'-CGGTATGAATTCcatcatcatcatcatcatcccgactacaaggacgacgatgacaag TCTCCAATTCAATTGGAGACGAG- 5'	53	With restriction site and his tag
Bcl1 R	3'-GCGGCCGC CTAAGTCTTAACACAAAGCGCTTG TC-5'	53	RC
Bcl1 LF	TCTCCAATTCAATTGGAGACGAGAGATATAATAACAATCGCCGATCCAAAC	67.8	
Bcl-2 F	CGGTATGAATTCcatcatcatcatcatcatcccgactacaaggacgacgatgacaagCAAGACA CAAACGTAGCACCG	54.8	With restriction site and his tag
Bcl-2 R	CGGTATGCGGCCGC TTAAGAGATAATAGTGATATTATCCGA AAG	53.4	RC

RC- Reverse compliment

A



B

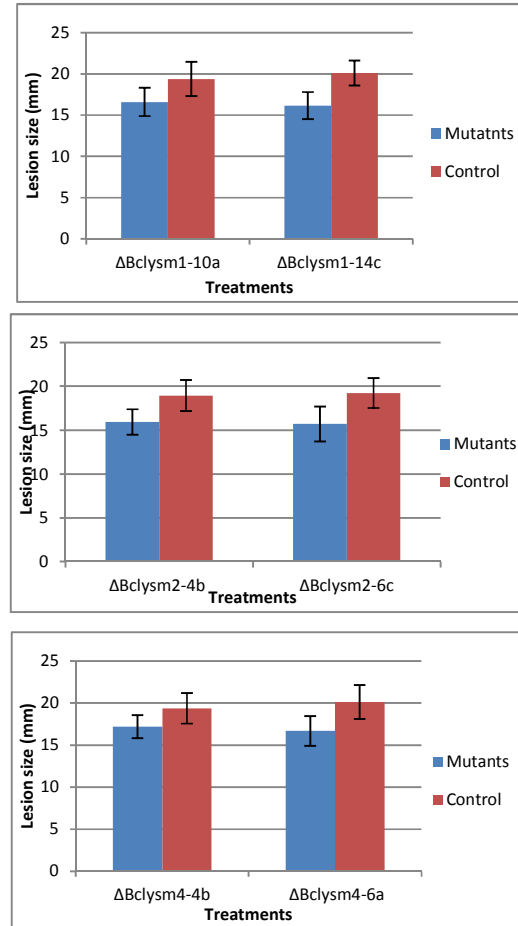
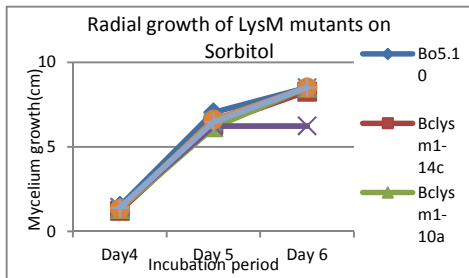
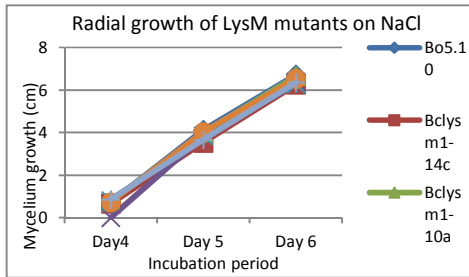
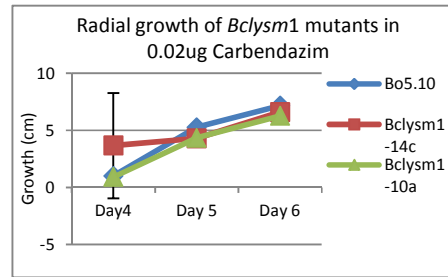


Fig 1. In experiment 1: Two individual mutants of each of three knockout mutants induced lesion size average in millimeter. Two individual clusters in each of the graph shows individual mutants and corresponding wild type lesion size as a control. Results shows the average lesion size from tobacco (A) and tomato (B) plants.

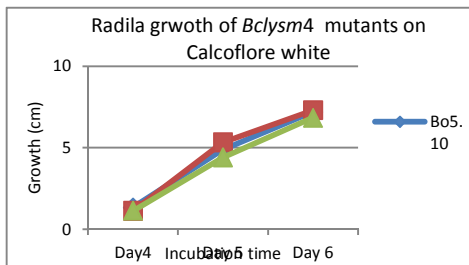
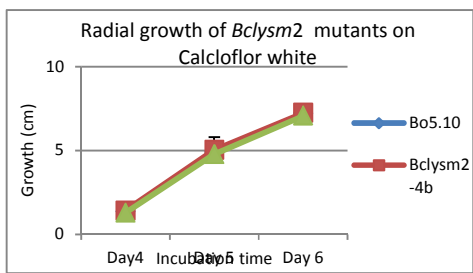
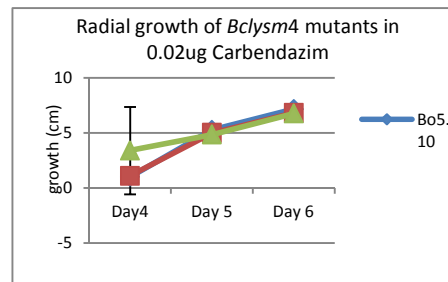
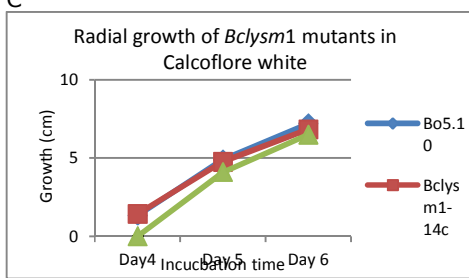
A



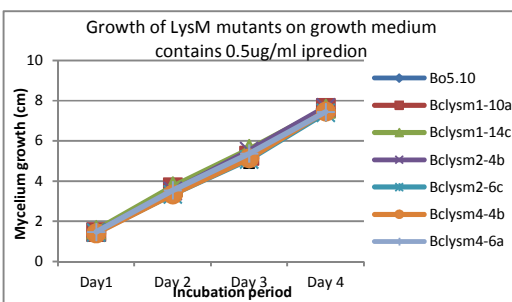
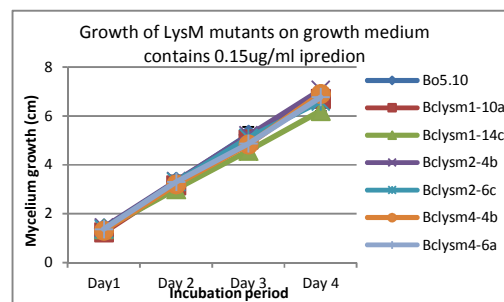
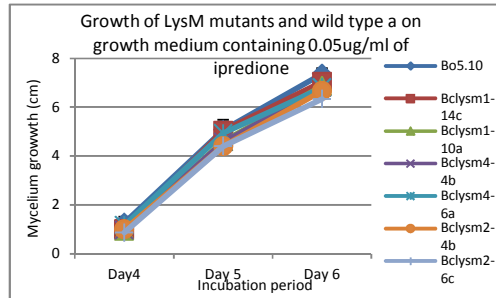
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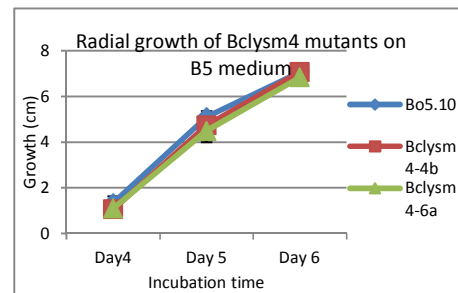
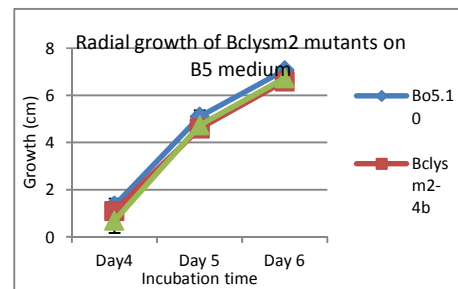
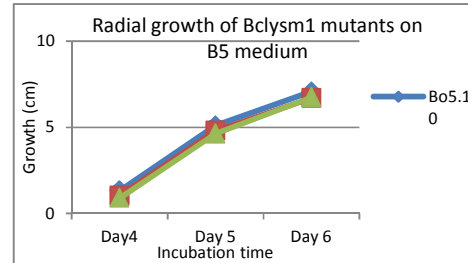
C



D



E



F

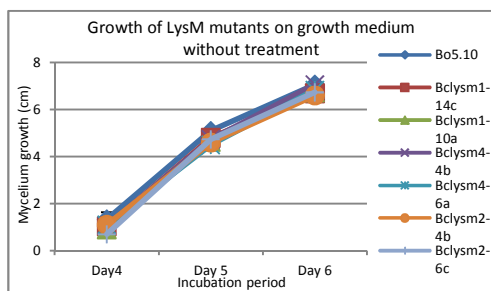


Fig2. A-E are the radial growth in centimeter (cm) of two individual mutants of each of the *Bclysm* mutants. F is the control without treatment. Value of each of the day are the average of three replications.

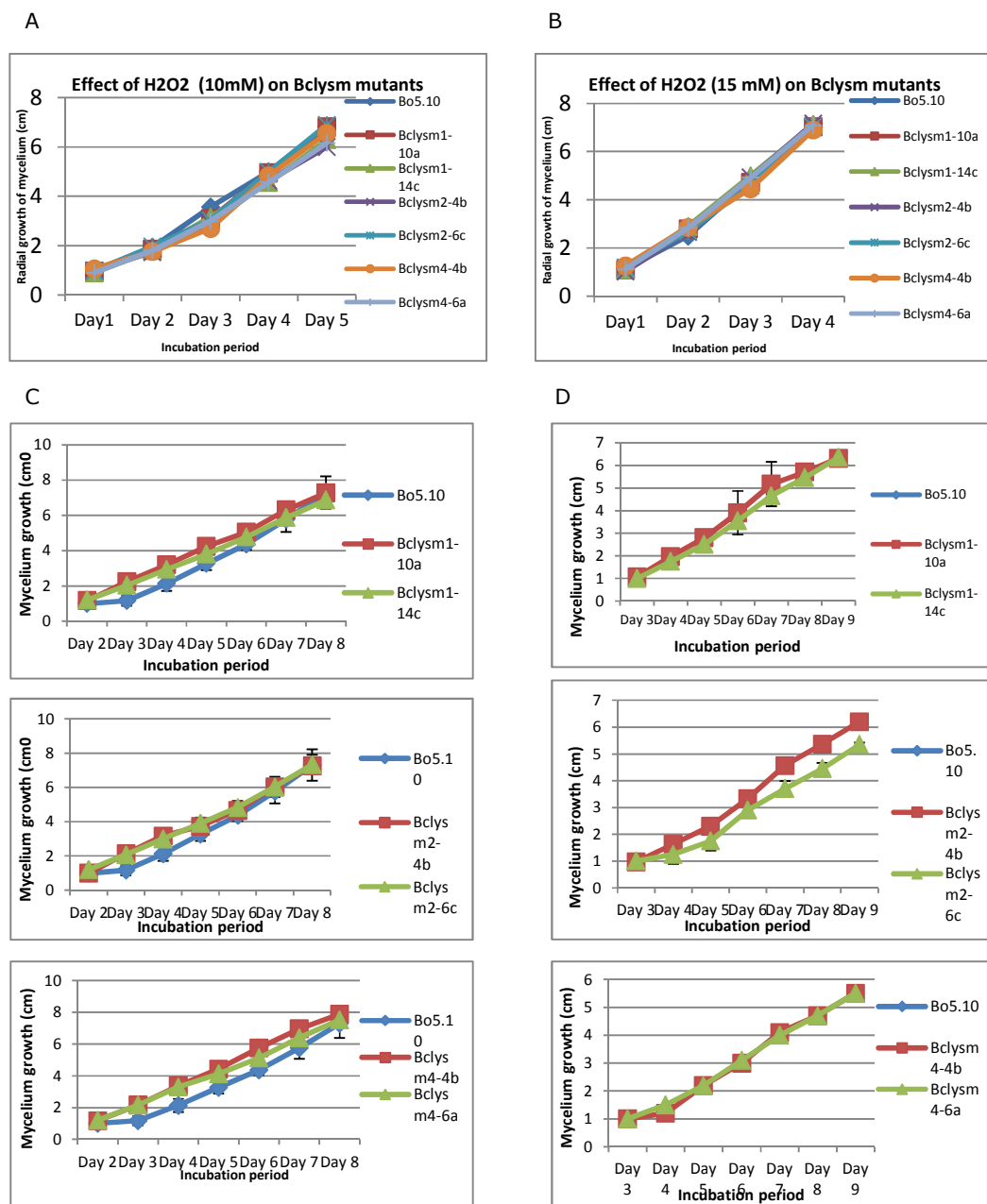


Fig 3. Radial growth of *Bclysm* mutants (two individual mutants of each *Bclysm* mutants) and wild type (Bo5.10) of *Botrytis cinerea* on B5 agar medium containing 10 mM (A), 15 mM (B), 30mM (C) and 50mM (D) of H₂O₂. Each of the value is the average of three replicates. Data were collected up to 8th day. B- wild type not included as it was unable to grow. Most of the replication unable to grow therefore standard error bar not included.

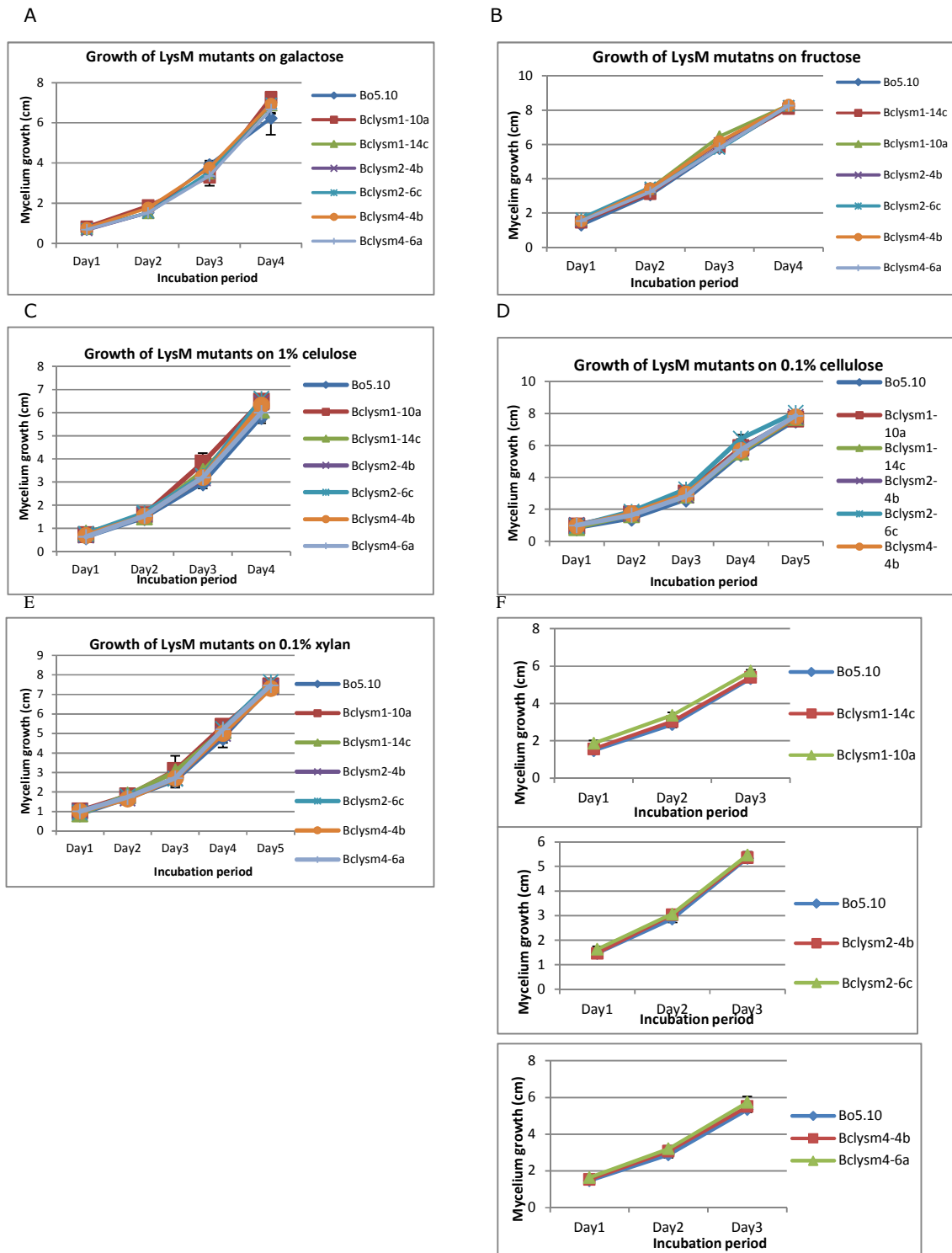


Fig. 4 Mycelium growth of two individual mutants of each of three *Bclysm* mutants of *Botrytis cinerea* and wild type (B05.10) on Galactose (A), Fructose (B), 1% cellulose (C), 0.1% cellulose (D), 0.1% xylan (E), 0.1% pectin (F) containing B5 agar medium. Each value shows the average of three replications

Appendix 1

a. Overlap PCR

94°C	5.00 minutes	
58°C	2.00 minutes	
72°C	8.00 minutes	
92°C	30 seconds	
Ramp 1.2°C/S to 58°C		} 30 cycles
58°C	2 minutes	
Ramp 0.1°C/S to 72°C		
72°C	7 minutes	
Ramp 0.2°C/S to 92°C		
Go to 4,	29 times	
72°C	8 minutes	
10°C	for ever	

b. PCR- Expand

94°C	2.00 minutes	
94°C	15 Seconds	} 10 cycles
58°C	30 Seconds	
68°C	3.00 minutes	
94°C	5 Seconds	} 20 cycle
58°C	30 Seconds	
68°C	3.00 minutes+5 Seconds/cycle	
72°C	7.00 minutes	
10°C	for ever	

c. Colony PCR

95°C	10.00 minutes	
94°C	30 seconds	} 30 cycles
58°C	30 seconds	
72°C	1.00 minutes	
Go to 2-29 times		
72°C	5.00 minutes	
10°C	for ever	

e. Gradient PCR

95°C	5.00 minutes
94°C	30 seconds
56°C to 60°C	for 30 seconds
72°C for	1.00 minutes
Go to 2, 29 times	
72°C	5.00 minutes
10°C	for ever

Appendix 2

Appendix 2

BMGY (Buffered Glycerol-complex Medium)

1% of yeast extract

2% of peptone

100 mM of potassium phosphate,

1.34% YNB (Yeast Nitrogen Base)

4×10^{-5} of biotin

1% of glycerol or 0.5% of methanol

MilliQ water (according to the expected volume)