Botrytis cinerea GalAR:

regulator of the D-galacturonic acid catabolic pathway

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Summary

The plant cell wall polysaccharide pectin consists of polymers that are rich in D-galacturonic acid. D-galacturonic acid is an important carbon source for saprotrophic and pathogenic organisms. The necrotrophic fungi *Botrytis cinerea* secretes many pectinases to degrade the plant cell wall for the release of monosaccharides or/and for the infiltration of plants. The D-galacturonic acid catabolic pathway in *B. cinerea* converts D-galacturonic acid in four catalytic steps into glycerol, involving the two non-homologues genes galacturonate reductase (*Bcgar*1 and *Bcgar*2), L-galactonate dehydratase (*Bclgd*1), 2-keto-3-deoxy-L-galactonate aldolase (*Bclga*1) and glyceraldehyde reductase (*Bcglr*1). The expression of the genes involved the D-galacturonic catabolic pathway are induced within 3 hours. Transcriptome analysis of *B. cinerea* grown in media containing glucose and pectate as sole carbon source revealed that 32 genes are induced by pectate. Eight of the 32 genes were specifically induced by D-galacturonic acid. Promoters of these genes, including bidirectional promoter of D-galacturonic acid catabolic pathway genes *Bcgar*2 and *Bclga*1, contained the conserved motif GAE1. The bidirectional promoter of *Bcgar*2-*Bclga*1 was functionally analysed and demonstrated that the GAE1 motif is required for expression. The regulator of the D-galacturonic acid catabolic pathway was previously unknown. Yeast-1-Hybrid screening with a library of predicted *B. cinerea* transcription factors revealed an interaction with the GAE1 motif. In this study, the role of the transcription factor, designated as *BcGalAR*, in the D-galacturonic acid catabolic pathway was analysed. Inactivation of *BcGalAR* and expression analyses in *B. cinerea* wild type and *ΔBcGalAR* mutant demonstrated the involvement of *BcGalAR* in the D-galacturonic acid pathway. Expression of *Bcgar*2, *Bclga*1 and *Bclgd*1 are induced by D-galacturonic acid in the wild type but were not induced in the *ΔBcGalAR* mutant when grown in media containing D-galacturonic acid as sole carbon source. In the *ΔBcGalAR* mutant, the expression of the hexose transporters *Bchxt*15 and *Bchxt*19 was also not induced by D-galacturonic acid. These data suggests that BcGalAR not only plays a role in the D-galacturonic acid catabolic pathway but also in D-galacturonic acid utilization. The virulence of the *ΔBcGalAR* mutant remained unaltered on tomato and *Nicotiana benthamiana* leaves. Revealing that D-galacturonic acid does no significantly contribute to the diet of *B. cinerea*.

1. Introduction

1.1 Plant cell wall

The function of the plant cell wall is to provide a strong structure to plant tissue and protection against microbes. Structural components of the plant cell wall are the polysaccharides cellulose, hemicellulose and pectin. Cellulose consists of β-1,4-linked D-glucose and can form a complex with hemicellulose that can coil around pectin. Most dicot primary cells are composed of ~30% cellulose, ~30% hemicellulose and ~35% pectin (Cosgrove, 1997). Pectin is built up of different types of polymers, including homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan. The major monosaccharide in pectin is D-galacturonic acid and it is an important carbon source for saprotrophs and pathogens. The cell wall is one of the first and most important barriers against invading pathogens (Reviewed by Vorwerk, 2004). Plants are capable to protect their cell wall in several ways from degradation and penetration by pathogens. The cell wall can be protected using cell wall degrading enzyme inhibitors, the formation of papillae at penetrated sites and the activation of the immune response when damage-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) are detected by plant immune receptors.

1.2 *Botrytis cinerea*

Grey mould is caused by the necrotrophic fungus *Botrytis cinerea* and can infect over 200 plant species, including many agricultural crops like apple, broccoli, gerbera, grape, rose, strawberry and tomato (Reviewed by Williamson *et al.*, 2007). Next to that, *B. cinerea* can also infect different plant organs like, flowers, fruits, leaves, shoots and even soil storage organs (Droby and Lichter, 2007). The wide host range and devastating damage caused by *B. cinerea* makes it a threat for agriculture and horticulture. Economic damage caused by *Botrytis* is difficult to estimate but it is estimated that the damage can reach over billions euro annually (Vrind, 2005; Elmer and Michailides, 2007; Dean *et al.,* 2012).

B. cinerea can survive as conidia, mycelia and sclerotia and can be spread mechanically or by wind. Conidia landing on leaves germinate and penetrate the tissue by creating an appressorium. After infestation/invasion of the tissue, *B. cinerea* starts decomposing plant tissue and uses the host-derived nutrients for its own growth. Within the arsenal of *B. cinerea* are enzymes to degrade the cell wall (pectinases, cellulases and hemicellulases) and phytotoxic metabolites and proteins, as well as oxalic acid and hydrogen peroxide (Reviewed by Choquer *et al.*, 2007; van Kan, 2006). Cell wall degrading enzymes contribute to the virulence of *B. cinerea* and are important for entering the plant. During infection *B. cinerea* secretes many pectinases to degrade the plant cell wall. Among the pectinases are pectin- and pectate-lyses, pectin methylesterases, exo-polygalacturonases (Exo-PG) and endo-polygalacturonases (endo-PG). The endo-PGs are the most studied cell wall degrading enzymes in *B. cinerea*. A total of six endo-PG genes were identified in the *B. cinerea* genome and these are all differentially expressed *in vitro* and *in planta* (ten Have, 2001; Kars *et al.,* 2005; Wubben *et al.,* 1999, 2000). *Bcpg*1 and *Bcpg2* have a basal constitutive expression (Wubben *et al.,* 2000). Knockout of *Bcpg1* and *Bcpg2* in *B. cinerea* B05.10 resulted in reduced virulence on tomato and broad bean leaves (ten Have *et al.*, 1998; Kars *et al.,* 2005). *Bcpg*3 is induced in low ambient pH. The expression of *Bcpg*4 and *Bcpg*6 is induced by D-galacturonic acid.

1.3 D-galacturonic acid catabolism pathway

In the fungal D-galacturonic acid catabolism, several enzymes are needed to convert to D-galacturonic acid to glycerol (Figure 1.1). These enzymes are galacturonate reductase (GAR1), L-galactonate dehydratase (LGD1), 2-keto-3-deoxy-L-galactonate aldolase (LGA1) and glyceraldehyde reductase (GLR1) and were identified in *Aspergillus niger* (Reviewed by Richard and Hilditch, 2009). Orthologues of these catabolic enzymes were also identified in *Hypocrea jecorina* and *B. cinerea* (Kuarelahti *et al.,* 2005, 2006, Zhang *et al.,* 2011). In *A. niger, H. jecorina* and *B. cinerea*, another gene encodes for an active galacturonate reductase, non-homologous to GAR1 and is designated as galacturonate reductase (GAR2) (Martens-Uzunova and Schaap, 2008; Mojzita *et al.,*2010; Zhang *et al.,* 2011). In *B. cinerea* it was shown that GAR2 has a similar function as GAR1 and has a higher enzymatic activity than GAR1 (Zhang *et al.,* 2011).

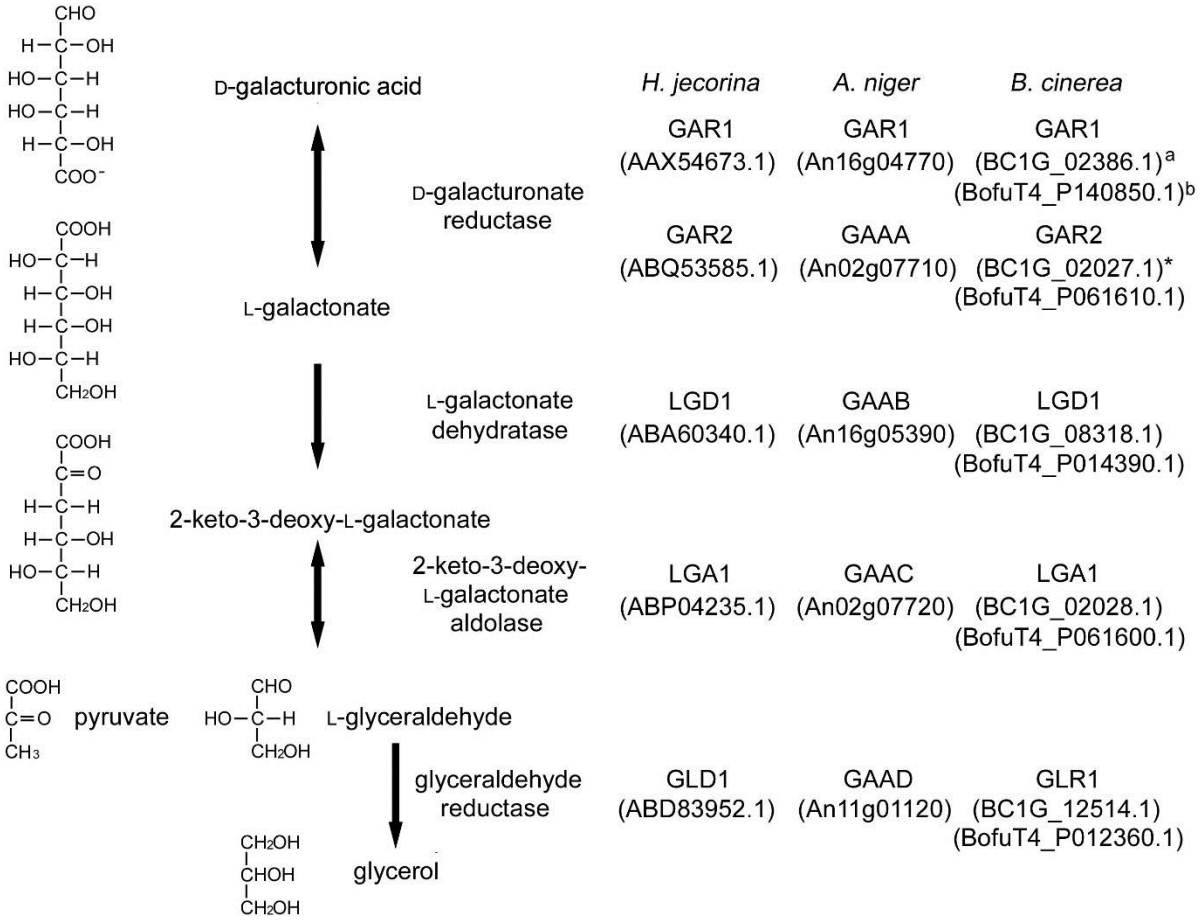

Figure 1.1. D-galacturonic acid catabolic pathway in the fungi *Hypocrea jecorina, Aspergillus niger* and *Botrytis cinerea.* For each step in pathway homologues are listed (Zhang *et al.,* 2011).

In *B. cinerea, Gar*1/*Gar*2, *Lgd*1 and *Lga*1 were replaced with the hygromycin phosphotransferase (hph) resistance gene. These mutants were unable to grow on pectate and D-galacturonic acid as sole carbon sources (Zhang *et al.,* 2011). When these mutants were grown on apple pectin and citrus pectin substrates, the growth was reduced. Inoculation of *ΔBcgar1/Bcgar2*, *ΔBclgd1* and *ΔBclga*1 mutants on *Nicotiana benthamiana* and *Arabidopsis thaliana* showed that the lesion size and the fungal biomass were reduced compared to the wild type (Zhang and van Kan*,* 2013). However, this was not observed on tomato leaves, peppers and apples (Zhang *et al.,* 2011 and Zhang and van Kan, 2013). Growing these mutants on 10 mM fructose with 0.3 mM galacturonic acid strongly reduced the growth compared with

the wild type, indicating that the intermediates of the D-galacturonic acid catabolic pathway can have a growth-reducing effect.

1.4 Transcription factors in *B. cinerea*

Environmental changes, such as ambient temperature, light or nutrient availability, make it necessary for fungi to respond rapidly. Signalling molecules respond to these environmental cues and activate transcription factors which induce the expression of genes (Reviewed by Weake and Workman, 2010). The transcribed genes encode proteins that will act on the stimulus. These proteins can be involved, for example, in the development of spores, the uptake of nutrients or the activation of immune responses. Whenever the stimulus is removed, the transcription of the genes will return to a basal expression. The transcription factor LTF1 (Light-responsive Transcription Factor 1) in *B. cinerea*, is strongly induced by light and influences the expression pattern of light-responsive genes (Schumacher *et al*., 2014). Deletion of LTF1 in *B. cinerea* results in hyperconidiation and reduced virulence. Another *B. cinerea* transcription factor is CRZ1 (Calcineurin-responsive zinc finger) that is involved in the induction of calcineurin which is involved in the uptake of Ca^{2+} (Schumacher *et al.,* 2008).

It was shown that the expression of some genes encoding transcription factors involved in the catabolism of monosaccharides are induced by the corresponding monosaccharide. In *Aspergillus* species, the monosaccharide rhamnose induces the expression of the transcription factor RhaR which regulates the expression of genes involved in rhamnose utilization (Gruben, 2012). Another example of transcription factors induced by monosaccharides are AraR and XlnR and regulates the pentose catabolic pathway and pentose phosphate pathway in *A. niger* (Battaglia *et al.,* 2014). If the D-galacturonic acid catabolic pathway is activated by an transcription factor induced by D-galacturonic acid remains unknown. In *Aspergillus* and *B. cinerea*, transcription factors induced by D-galacturonic acid were not identified.

1.5 Identification of candidate transcription factor involved in the activation of D-galacturonic acid catabolic pathway

RNA sequencing technology was performed on *B. cinerea* grown on glucose and pectate to identify genes involved in pectate decomposition (Zhang *et al.*, 2014). 32 genes were significantly (q<0.05) up-regulated (>2 fold) in pectate-containing media compared to glucose-containing media. From these 32 genes, 14 encoded Carbohydrate Active enZymes (CAZymes). Using qRT-PCR, the reliability of the RNAseq data was tested. The expression data derived from qRT-PCR was in agreement with RNAseq for 23 of the 32 genes. To investigate if these genes are specifically up-regulated by D-galacturonic acid, the transcript levels of these genes were determined with qRT-PCR under different carbon sources: glucose, D-galacturonic acid, arabinose, rhamnose, galactose and xylose. From the 32 genes, 9 genes were up-regulated specifically for D-galacturonic acid and were used for transcriptional elements analysis.

The promoters of 8 genes that were specifically up-regulated in D-galacturonic acid were used for cis-regulatory element analysis (Zhang, 2013). Eight different potential regulatory motifs were identified, three of which are known from literature: the binding site for the carbon catabolism repressor CreA 5'-SYGGRG-3' (Cubero and Scazzocchio; 1994, Portnoy *et al.,* 2011), the binding site for the pH regulator PacC 5'-GCCARG-3' (Caracuel *et al.,* 2003; Tilburn *et al.,* 1995) and the regulatory element GAE1 5'-CCNCCAA-3' (Martens-Uzunova and Schaap, 2008). The seven analysed promoters regions of the 8 genes (*Bcgar2* and *Bcgla1* share a bidirectional promoter) have the GAE1 motif. The motif GAE1 in the *Bcgar2-Bcgla1* gene cluster is conserved in other fungi like *Aspergillus niger, A. fischerianus, A. nidulans,*

A. terreus, A. flavus, A. fumigatus, A. oryzae, A. clavatus, Penicillium chrysgemun, Phaeosphaeria nodorum, Magnaporthe grisea and *Sclerotinia sclerotiorum* (Martens-Uzunova and Schaap, 2008). In the promoter region of *Bcgar2* and *Bclga1*, one PacC and one GAE1 and eight CreA motifs are predicted. The promoter cluster of *Bcgar2* and *Bclga1* were fused to GFP (Full Length (FL) construct) and constructs with deletions in the motifs were made (Zhang, 2013). These constructs were transformed in B05.10 protoplasts. Conidia of the transformants were germinated and grown on glucose and D-galacturonic acid media. Transformants growing on the glucose media, did not activate the promoter of *Bcgar*2/*Bclga*1 and were not detected with the fluorescence microscope. The FL construct gave a fluorescence signal when growing on D-galacturonic acid medium. Fluorescence signal was not detected when PacC and GAE1 were deleted. The GAE1 motif occurs in 323 genes over the *B. cinerea* genome. However, it is still unknown which transcription factor binds to the GAE1 binding motif. Yeast one-hybrid (Y1H) was shown to be successful to identify the transcription factor involved in the regulation of *BcBOT* genes (Simon *et al.,* 2013). Using Y1H with a partial promoter sequence of *Bcgar*2/*Bclgd*1, three transcription factor candidates were identified (Vargas, 2014). Two of the transcription factor candidates were also found during different screenings and were considered false positives (Personal communication, A. Simon). The remaining candidate, with the gene identifier BofuT4_P100290.1 (called D-galacturonic Acid Regulator (*BcGalAR*) in this thesis), is similar to Cys_6 transcription factors and was not found in other Y1H screenings. Protein structure predictions propose that *BcGalAR* contains a Zn/Cys6 domain and a fungal-specific transcription factor domain.

1.6 Aim of this thesis

The aim of this thesis was to identify and investigate the role of BcGalAR in the D-galacturonic acid pathway. To confirm that BcGalAR specifically binds the GAE1 motif, Y1H was performed with BcGalAR and the GAE1 motif or mutated GAE1 motif. Furthermore, *BcGalAR* was deleted from the *B. cinerea* genome by replacing the coding sequence with the hygromycin resistance gene and mutants were phenotypically characterized, by growth on media containing different carbon sources, by infection on tobacco and tomato leaves, and by analysing the expression of genes that, in wild type *B. cinerea,* are induced by D-galacturonic acid.

2. Materials and methods

2.1 GAE1 mutated promoter construct

Plasmid pINT1-HIS3NB was used for making the GAE1 mutated promoter construct. The mutated GAE1 construct was made by using a tandem repeat of 15 nucleotides of the GAE1 motif (ATTGGTCCAAAAATA) with a CACACA spacer. The two cytosine of the GAE motif were replaced by two guanines (ATTGGTGGAAAAATA). The promoter of pINT1-HIS3NB was digested with SpeI and NotI. The digestion reaction contained; 2 µl 10x restriction enzyme buffer E (Promega), 1 µl SpeI (Promega), 1 µl NotI (Promega) and 1 µg pINT1-HIS3NB with a final volume of 20 µl. The reaction mix was incubated for 1h at 37°C. The mutated promoter was made by annealing the primers Mut GAE1 F and Mut GAE1 R with each other. Thereafter, the mutated promoter was ligated into the digested pINT1-HIS3NB using the following ligation reaction; 100 ng digested pINT1-HIS3NB, 17 ng mutated promoter, 1 µl 10x ligase buffer, 1 unit T4 DNA ligase with a final volume of 10 µl. The ligation mix was incubated for 3 hours at 37°C. After incubation, the ligated plasmid was transformed to electro competent *Escherichia coli* (*E.coli*) *DH5α* cells. For the transformation, the BioRad MicroPulser™ electroporator was used. 50 µL of *DH5α* cells with 2 µL ligation mixture was added into an 0.2 cm electroporation cuvette and subsequently transformed with the EC 2 (2.5 kV) program. Directly after the electroshock, 300 µL of LB broth was added to the cells and they were incubated for 1h at 37^{0}C. After the incubation, the transformed cells were selected by growing the cells on LB agar containing 100 µL/mg ampicillin. Growing colonies were selected and analysed with colony PCR. The colony PCR reaction contained 5 µl 5x GoTaq® Buffer with 25mM Mg_2Cl_2 (Promega), 1 µl 10µM dNTPs, 0.5 µl 10µM pINT1 screen F, 0.5 µl 10µM pINT1 screen R, 0.2 µl GoTaq® G2 DNA polymerase (Promega) in a final volume of 25 µl. The used PCR program was, 3 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C, 45 seconds at 72°C and followed by a final extension of 5 minutes at 72°C. When a band of the expected size in the colony PCR was observed, the band was cut from the gel and purified with NucleoSpin® Gel and PCR Clean-up' kit (Macherey-Nagel). To analyse if the plasmid contained the mutated promoter, isolated plasmids were sent for sequencing together with the primer pINT1 Screen R to Macrogen Europe, Amsterdam. Colonies verified with the correct insert were digested with NcoI and SacI.

2.2 Yeast transformation

Saccharomyces cerevisiae strain FY834 (Genotype: *MATα his3Δ200 ura3-52 leu2Δ1 lys2Δ202 trplΔ63*) was grown in 10 ml YPD medium and incubated o/n at 30°C 200 rpm. After o/n incubation, 12 ml YPD was inoculated with the overnight culture to an OD_{600} of 0.2 and incubated at 30°C until an OD_{600} ~0.8 was reached. Then, the culture was centrifuged at 4000 rpm for 3 minutes. The supernatant was discarded, the pellet was resuspended in 5 ml sterile MQ and centrifuged at 4000 rpm for 3 minutes. Again, the supernatant was discarded, the pellet was resuspended in 2.5 ml 0.1M LiAc and centrifuged at 4000 rpm for 2 minutes. Finally the supernatant was discarded, the pellet was resuspended in 200 µl 0.1M LiAc and transferred to an Eppendorf tube. The cells were centrifuged for 1 minute at 6500 rpm and supernatant was completely removed. Before the transformation, the transformation mix containing the DNA insert and carrier DNA was prepared. Carrier DNA was denatured for 5 minutes at 95°C and cooled for 5 minutes on ice. Then, 10 µl of denatured carrier DNA, 250 ng insert and 38 µl 1M LiAc in a final volume of 80 µl was mixed. Thereafter, 300 µl 40% Polyethylene glycol (PEG) 3350 was added to the transformation mix. The mix was transferred to the yeast suspension and incubated for 1 hour at 30°C and mixed every 30 minutes. Directly after incubation, the cells were incubated at 42°C for 30 minutes. Cell suspension was inoculated on selective media.

2.3 Amplification of flanking fragments and hygromycin cassette

For the amplification of the flanking fragments, genomic DNA of *B. cinerea* B05.10 was used. The primer combination 5.1 and 5.3 was used for the amplification of *BcGalAR* 5' flanking region and the combination 3.1 and 3.3 was used for the amplification of BcGalAR 3' flanking region (Table 1). The PCR reaction contained 1 µl genomic DNA, 5 µl 5x GoTaq® Buffer with 25mM Mg_2Cl_2 (Promega), 1 µl 10 µM dNTPs, 1 µl 10 µM of each primer, 0.5 µl GoTaq® G2 DNA polymerase (Promega) with a final volume of 25 µl. PCR program was 3 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 45 seconds at 55°C and 1 minute at 72°C and followed by a final extension of 5 minutes at 72°C. After the PCR, products were checked for nonspecific amplification and correctness of size with gel electrophoresis. The PCR reaction was purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The hygromycin cassette was obtained through digestion of pLOB7 with HindIII (Promega) and EcoRI (Promega). The digestion mix was purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). The concentration of the hygromycin (Hph) cassette and flanking fragments were measured with Nanodrop 1000. Concentrations were converted to pmol (using Promega Biomath calculator) and were diluted to 0.01 pmol/µl.

2.4 Overlap extension

The overlap extension was done with an adjusted protocol from Nelson and Fitch, 2011. The first PCR reaction contained 10 µl of 0.01 pmol Hph cassette, 10 µl of 0.01 pmol of each flanking fragment, 1 µl 10 mM dNTPs, 5 µl 10x Expand high fidelity buffer with $MgCl_2$ (Roche) and 0.5 µl Expand high fidelity enzyme mix (Roche) in a final volume of 50 µl. The used PCR program was; 2 minutes at 94°C, 13 cycles of 15 seconds at 94°C, 1 minute at 60°C and 2.5 minutes at 72°C and followed by a final extension of 7 minutes at 72°C. After the first PCR, PCR products were purified with NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and eluted in 50 µl MQ. In the second step, 10 µl of purified PCR product, 1 µl 10 µM primer 5.2, 1 µl 10 µM primer 3.2, 1µl 10 µM dNTPs, 5 µl 10x Expand high fidelity buffer with $MgCl_2$ (Roche) and 0.5 µl Expand high fidelity enzyme mix (Roche) in a total volume of 50 µl was used for the reaction. The following PCR program was used; 2 minutes at 94°C, 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2.5 minutes (+3 seconds/cycle) at 72°C and followed by a final extension of 7 minutes at 72°C. Overlap extension products were checked for the correct size. Also, a restriction digest analysis was performed to analyse if the flanking fragments and Hph cassette were present. The OE construct is then purified with ethanol precipitation and dissolved in KC buffer (0.6 M KCl and 50 mM $CaCl_2$).

2.5 Growth conditions of *B. cinerea*

B. cinerea was grown on Malt Extract Agar (MEA) in the dark at 20°C for 3-4 days. After incubation, plates were incubated for one night under near-UV light (350-400 nm) to promote sporulation and were afterwards returned to darkness. Conidia were harvested after 4-7 days in 10 ml sterile water. The conidia suspension was filtered over glass wool and centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded, the conidia were resuspended and diluted with sterile milli Q to the required concentration.

For the radial growth assays, identical conditions as Zhang *et al*., 2011 were used. Transformants were grown on Gamborg's B5 (Duchefa, Haarlem, The Netherlands) agarose medium supplemented with 10 mM $(NH_4)H_2PO_4$ and 30 mM fructose for two days. Agar plugs were transferred to Gamborg's B5 agarose medium with 10 mM $(NH_4)H_2PO_4$ and either 50 mM glucose, 50 mM D-galacturonic acid, sodium pectate

(1% w/v; Sigma), citrus pectin (1% w/v; Sigma) or apple pectin (1% w/v; Carl Roth KG) and were incubated at 20°C.

2.6 Transformation of *B. cinerea* B05.10 protoplasts

For the transformation of B05.10, a spore suspension with an concentration of $5*10^7$ was added to 250 ml 1% Malt extract broth and incubated for 2h at RT. After incubation, the spore suspension was transferred to a round bottom flask and incubated o/n at 20°C 180 rpm. The germinated spores were filtered through 22.4 µm nylon filter and were washed three times with sterile KC buffer. 10 ml of germinated spores was transferred to a new 100 ml flask. 5 mg/ml filter sterile enzyme mix (Chitinase and Glucanase) was added to the flask and incubated for 2h at 25°C 80 rpm. Thereafter, protoplasts were filtered through 22.4 µm nylon filter and were collected into a sterile tube. Then, the protoplasts were centrifuged for 10 minutes at 1200 rpm. The supernatant was discarded and the pellet was resuspended and washed with KC buffer. Protoplast were centrifuged, resuspended and diluted with KC buffer to a concentration of $1*10^8$ protoplast/ml. 5 µl of 5mM spermidine was added to 95 µl OE construct in KC buffer and incubated for 5 minutes on ice. 100 µl of protoplasts together with 100 µl 25% PEG 3350 (dissolved in 12.5mM TRIS HCl and 65.5mM $CaCl_2$) was added to the OE construct with spermidine and incubated for 20 minutes at RT. After incubation, 500 µl 25% PEG was added and incubated for 10 minutes at RT. Thereafter, 200 µl of KC buffer was added. The protoplast mixture was transferred to 100 ml SH media. Ten plates were poured (10 ml each) and incubated o/n at 20°C. The next day, a 10 ml top layer of SH containing 100 µg/ml hygromycin or 150 µg/ml nourseothricin was added to the plates and incubated at 20°C. Plates were checked daily for growth and hyphae that emerged through the top layer was transferred to MEA with 100 µg/ml hygromycin. If the transformant was able to grow on the MEA media containing hygromycin, mycelium was transferred to MEA without hygromycin for spore production. To obtain homokaryotic transformants, spores were harvested, diluted to 200 spores/ml and 250 µl was plated on MEA with 50 µg/ml hygromycin. Three single germinated spores were transferred to MEA with 50 µg/ml hygromycin and grown until hyphae reached the end of the plate.

2.7 *Botrytis* DNA isolation

To extract DNA from *Botrytis*, mycelia was collected from MEA plates and the tissue was ground in Eppendorf tubes with metal balls. 600 µl of Cell lysis Solution (Qiagen) with 3 µl proteinase K (20 mg/ml) was added to the ground tissue and incubated at 55°C for 1 hour. After the incubation, 3 µl of RNAse A (5 mg/ml) was added and incubated at 37°C for 15 minutes. Proteins were precipitated by adding 200 µl protein precipitation solution (Qiagen) and incubated for 30 minutes on ice. After incubation, the samples were centrifuged for 3 minutes at 13000 rpm. Supernatant containing DNA was transferred to a new eppendorf tube. DNA was precipitated with 600 µl 100% isopropanol (2-propanol) and centrifuged at 13000 rpm for 1 minute. Supernatant was removed and the pellet was washed with 70% ethanol. After washing, DNA was centrifuged at 13000 rpm for 1 minute. The supernatant was discarded and the pellet was dried for 15 minutes using speedvac. Finally, the pellet was hydrated and resuspended in sterile MQ.

2.8 Screening for homokaryotic *Botrytis* transformants

To analyse whether single spore isolates were homo- or heterokaryotic, five primer combinations were used. The combinations are 5.1+22, 3.1+23, qBcGalAR F + qBcGalAR R, BcGalAR screen + 3.1 and LZ92 + LZ93. The PCR reaction contained 25 ng of transformant genomic DNA, 5 µl 5x GoTaq® Buffer with 25mM Mg_2Cl_2 (Promega), 1 µl 10 µM dNTPs, 1 µl 10 µM of each primer, 0.5 µl GoTaq® G2 DNA

polymerase (Promega) in a final volume of 25 µl. The used PCR program was; 3 minutes at 95°C, 10 cycles of 30 seconds at 95°C, 45 seconds at 55°C and 1 minute at 72°C, 20 cycles of 30 seconds at 95°C, 45 seconds at 55°C and 1 minute (increased with 3 s per cycle) at 72°C followed by a final extension of 5 minutes at 72°C.

2.9 Complementation construct in *ΔBcGalAR* mutant

Using yeast homologues recombination system, complementation constructs were made. The constructs were made using the protocol described by Schumacher, 2012. Three complementation constructs were made, two *BcGalAR* constructs containing the endogenous and constitutive pOliC promoter fused to codon optimized eGFP and one *BcGalAR* construct with only the endogenous promoter. Fragments containing BcGalAR with overlaps were made using the primer combinations C1+C2 (Tgluc-*BcGalAR*-ptrpC), C2+C3 (ptrpC-*BcGalAR*-eGFP) and C3+C4 (pOliC-*BcGalAR*-eGFP) (table 2.1). The following PCR program was used for the amplification of the complementation fragments; 2 minutes at 95°C, 30 cycles of 1 minute at 95°C, 40 seconds at 55°C and 9 minutes at 72°C, and followed by a final extension of 10 minutes at 72°C. The plasmid pNDN-OGG was used as template and was digested with NotI, NcoI or/and SpeI to obtain the overlap with the fragments (Figure 2.1). To obtain the complementation construct without GFP, pNDN-OGG was digested with NotI and SpeI. To obtain the constructs with GFP, pNDN-OGG was digested with NcoI (and SpeI for the endogenous promoter). Using the digested plasmid with the overlapping fragments, constructs were made through homologues recombination by transforming the yeast strain FY834 and selected on SD-ura.

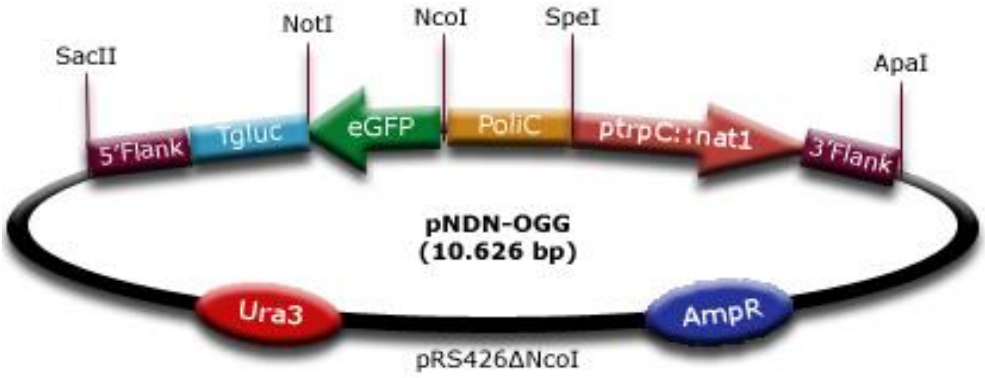

Figure 2.1. Schematic representation of pNDN-OGG. pNDN-OGG is derived from the shuttle vector pRS426 and is modified by the removal of the NcoI resitriction site in Ura3. The plasmid contains selection markers Ura3, AmpR and Nat1. The reporter gene eGFP is expressed by the constitutive promoter OliC of *A. nidulans* and terminated by Glucanase terminator of *B. cinerea*. The 5' and 3' flanks are identical to *BcniaD* (nitrate reductase) flanking regions (Adapted from Schumacher, 2012).

2.10 RNA extraction, cDNA synthesis and qRT-PCR

Spores of B05.10 and the *ΔBcGalAR* mutant were incubated in Gamborg's B5 supplemented with 30mM glucose and 10mM $(NH_4)H_2PO_4$ at 20°C, 150 rpm. After 16 hours of incubation, mycelium was harvested and divided into 3 portions. 1/3 of mycelia was transferred to an Eppendorf tube and used as 0 hour control. The other portions of mycelia were transferred and incubated for 3 hours in Gamborg's B5 supplemented with either 30mM glucose or D-galacturonic acid and 10mM $(NH_4)H_2PO_4$ at 20°C, 150 rpm**.** Thereafter, mycelium was harvested and freeze-dried. RNA was extracted from these samples using the zymoresearch RNA isolation kit, according to the manufacturer's instructions. First strand cDNA was synthesised from 500ng total RNA with M-MLV (Promega), according to the manufacturer's instructions.

To detect the transcripts of B05.10 and the *ΔBcGalAR* mutant grown in glucose or D-galacturonic acid, qRT-PCR was used. qRT-PCR was performed using ABI7300 PCR machine (Applied Biosystems, Foster city U.S.A.) with qPCR SensiMix kit (Bioline, London, U.K.). Primers to detect genes are described in table 2.1. The following qRT-PCR conditions are used; 95^{o}C for 10 minutes followed by 40 cycles of 95^{o}C for 15 seconds and annealing/extension at 60^{o}C for 45 seconds. Using the 2-*ΔΔCT* method, transcript levels of the target genes were normalized to the references gene *Bcrpl*5.

Table 2.1: list of primers used in this study

Primers	Target	Sequence (5’ -> 3’)
5.1	*BcGalAR*	CCAGAATACGTCAACCGTCA
5.2		AGCCAAAAACCGAAGAGGAT
5.3		CCCCGGGTACCGAGCTCGAATTCATTCTCGTGGTTCGTTGCTCA
3.1		GAACTTTGCACGGTCTGACA
3.2		CGGAAAAGCAAGGTACAAGC
3.3		AACTCGGCGCGCCGAAGCTTAGTACCGGAGTTTGAGTTGG
22	Hph cassette	GTAACCATGCATGGTTGCCT
23		GGGTACCGAGCTCGAATTC
Mut GAE1 F	GAE1	pGGCC'ATTGGTGGAAAAATACACACAATTGGTGGAAAAATA
Mut GAE1 R		TAACCACCTTTTTATGTGTGTTAACCACCTTTTTAT’GATCp
pINT1 screen F	pINT1	GGCTTATTGTTTGGCATTTGTAG
pINT1 screen R		TCTTTGCCTTCGTTTATCTTGC
AS41	pACTII	GCGTTTGGAATCACTACAGG
AS42		CACGATGCACAGTTGAAGTG
LZ80	*Rpl*5	GATGAGACCGTCAAATGGTTC
LZ81		CAGAAGCCCACGTTACGACA
LZ35	*Bcgar*2	CCCAGCTATCCGTGAACATC
LZ36		CACCTGGGGAAAGCGCATC
LZ37	*Bclgd*1	TGGTCATGGCATGACTTTCAC
LZ38		GTTGCGAATCGGAAACGAGATA
LZ39	*Bclga*1	CAAGGTTTGGGAATTGTACAGAG
LZ40		GTATCCTCCATATCCATAGGATG
LZ41	*Bcglr*1	GACAGGAAAGACATATTTATCAC
LZ42		CAGTGGATAAGATAAGGTCAAG
LZ92	hph	
LZ93		
LZ103	*Bchxt*15	CCCAGGATGTAGAAGCAGTG
LZ104		TTTCAGGACTGTCCTCAACTC
LZ310	*Bchxt*19	GGCCAAGTGCAGTTTCTACAC
LZ311		AACATGGTTTACGCCTCCGAAG
LZ302	B0510_2787	GCTATTGCGAGGACTTTCAC
LZ303		TAATCGTTCCATTCACACAAGTG
qBcGalAR F	*BcGalAR*	CCAAAGCGGATCAAATCTTC
qBcGalAR R		CTCTCATTTCCCATGGCACT
BcGalAR screen		GTACACGATAGCTTATGCACG
C1 TGluc	*BcGalAR*	CATACATCTTATCTACATACGCTAAGCGGCCGCTCAACCCTGCCCACCACCCAAG
C2 PtrpC		GCCCAAAAAATGCTCCTTCAATATCACTAGTGGATGAGGAGTGGAGATGTAAGCTC
C3 GFP		CCTCACCCTTGGAAACCATGGAACCCTGCCCACCACCCAAG
C4 oliC		CCATCACATCACAATCGATCCAACCATGTCCGGAAGGCCAGCGCAAGTTGG

3. Results

At the start of the research, a plasmid was available that was isolated during the Y1H screen. The plasmid contained a fragment of the DNA binding domain of the transcription factor-encoding gene BofuT4_P100290.1 (designated *BcGalAR* in this thesis).

3.1 BcGalAR binds the transcription binding site motif GAE1

To determine if BcGalAR binds to the GAE1 motif, two Y1H constructs were made: pINT1-HIS3-NB GAE1 which contains the GAE1 motif and pINT1-HIS3-NB Mutant GAE1 which contains a mutated GAE1 motif. Both pINT1-HIS3-NB GAE1 and pINT1-HIS3-NB Mutant GAE1 were inserted in yeast strain FY834 through homologous recombination and selected on YPD with G418 (geneticin). Growing colonies were analysed for the presence of the pINT1 insert with colony PCR (Supplemental figure 1). Positive colonies were transformed with the pACTII plasmid containing BcGalAR and the selection marker Leu2. Transformed colonies were selected on SD-Leu plates and growing colonies were transferred to SD-Leu-His with and without 3-AT. The pINT1-HIS3 transformants containing the pACTII-Leu2-BcGalAR plasmid were able to grow on SD-Leu whereas the pINT1-HIS3 transformants without the pACTII-Leu2-BcGalAR plasmid were not (Figure 3.1). The transformant pINT1-HIS3-BS-GAE1 + pACTII-Leu2-BcGalAR was able to grow on SD-Leu-His, indicating that the BcGalAR binds to the GAE1 motif. To ensure that the growth was not caused by the leaky selection that was reported to occur with HIS3, 5mM 3-AT was supplemented to SD-Leu-His medium (data not shown). Growth was observed on SD-Leu-His + 5mM 3-AT with colonies containing the GAE1 motif and pACTII-LEU2-BcGalAR construct. Transformants containing the mutated GAE motif were not able to grow on SD-Leu-His medium which confirms that BcGalAR binding to the GAE motif is specific.

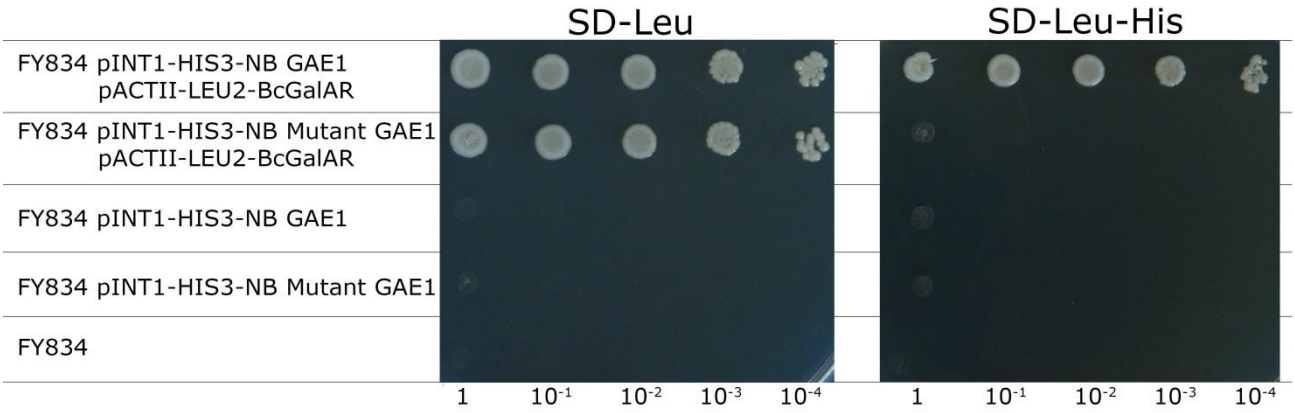

Figure 3.1. Y1H screening for BcGalAR interaction on GAE1 motif. Yeast transformants were diluted and grown on SD-Leu as growth control and SD-Leu-His for the interaction between the GAE1 motif and BcGalAR. FY834 with pINT1:HIS3-NB GAE1, pINT1:HIS3-NB mutant GAE1 and the wild type strain FY834 were used as control.

3.2 Expression of *BcGalAR* in presence of D-galacturonic acid

To analyse if the expression of *BcGalAR* is up regulated by D-galacturonic acid, B05.10 was grown in Gamborg B5 with glucose or D-galacturonic acid as carbon source. RNA was isolated during different time points and synthesize to cDNA for qRT-PCR. The expression of *BcGalAR* in glucose remained below a fold change of 2, which was considered as background noise. The expression of *BcGalAR* in D-galacturonic

acid was higher compared to glucose (Figure 3.10). However, the expression of *BcGalAR* in D-galacturonic acid remains below 5 fold.

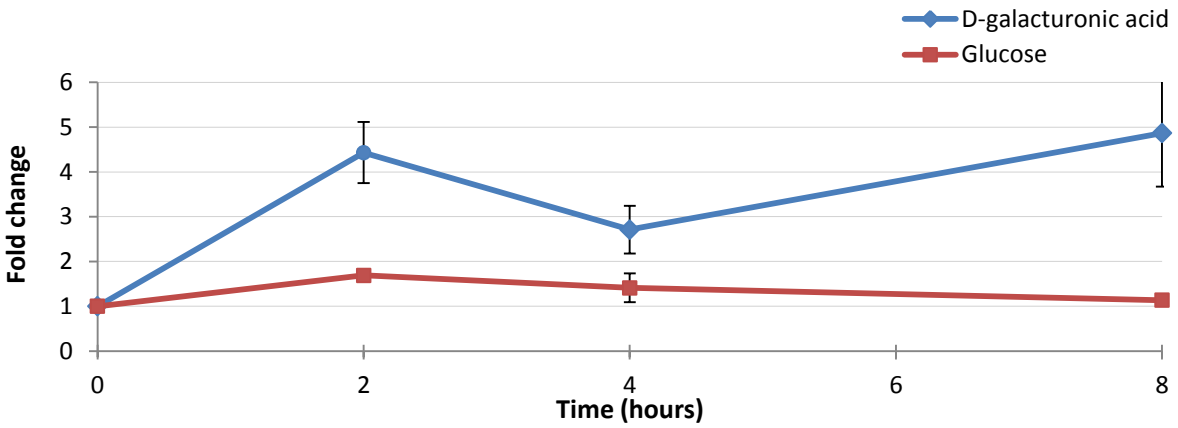

Figure 3.2. Expression of *BcGalAR* in B05.10 in glucose and D-galacturonic acid. Expression levels were normalized against *BcRpl5* according to $2^{-\Delta\Delta CT}$ method. Data is represented as average from 2 biological repeat with each three technical replicates. For time point 2 and 4, a second independent repeat was performed. Error bars represent the standard error between the biological repeats.

3.3 Deletion of BcGalAR in *B. cinerea*

For the deletion of *BcGalAR* in *B. cinerea* B05.10, a replacement strategy was used. This strategy uses flanking fragments of the target gene that are fused to the hygromycin resistance gene. The target gene is replaced in B05.10 protoplasts with the hybrid gene through homologous recombination. The flanking fragments were amplified with PCR and checked with gel electrophoreses (Figure 3.3a). Expected sizes for the 5' flanking fragment and the 3' flanking fragment were 529 bp and 486 bp, respectively. The hygromycin cassette was obtained through amplification and digestion of pLOB7 or pNR3. The cassettes were digested with EcoRI and HindIII to check if the hygromycin gene had the correct size and correct molecular pattern (Figure 3.3b). The hygromycin cassette derived from digestion had the correct size and molecular pattern.

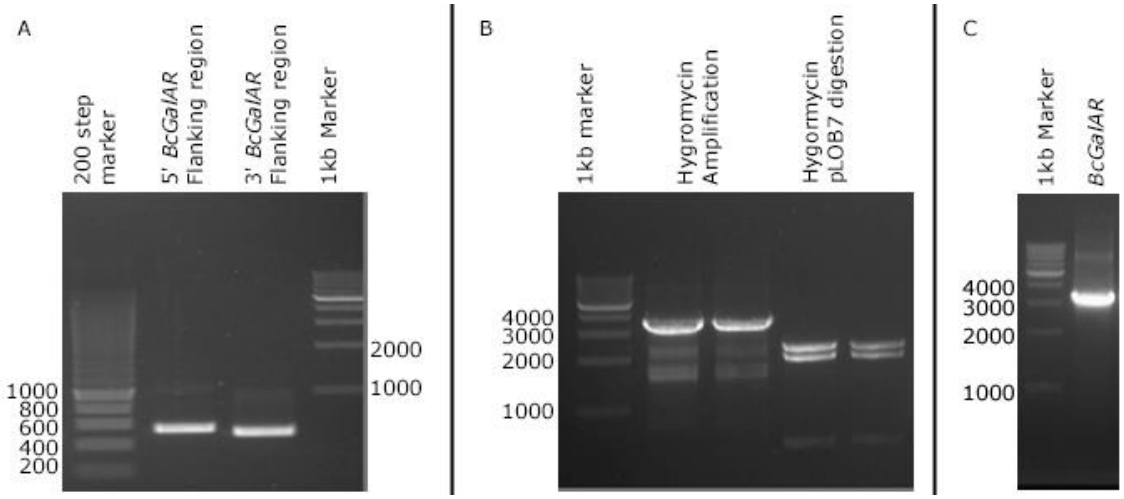

Figure 3.3. Amplification of flanking fragments, restriction enzyme digestion of hygromycin the overlap extension. A. Amplification of the *BcGalAR* flanking fragment. The 5' and 3' flanking fragment matches the expected molecular size of 529 bp and 486 bp. **B.** Digestion of the hygromycin with EcoRI and HindIII. The amplification of hygromycin did not match the expected molecular pattern. Hygromycin obtained through digestion matched the expected molecular pattern. **C.** The gene fusion of hygromycin with the flanking fragments was successful and resulted in a product of 3.2kb. The numbers next to the marker represent the size of the fragment in bp.

The overlap extension with the flanking fragments and hygromycin gene was checked on gel and matches the expected size of 3.2 kb (Figure 3.3c). To analyse if the hybrid gene was correctly fused, two restriction enzyme analyses were done. The digestion of the hybrid gene was done with the enzymes SacII and EcoRI or HindIII. The expected molecular pattern of SacII and EcoRI was 205bp, 324bp, 1.25 kb and 1.65 kb and the expected molecular pattern of SacII and HindIII was 486 bp, 750 bp and 2.15 kb. The expected molecular pattern matches with the restriction analysis and indicates that hybrid gene was correctly fused (Supplemental figure 2). B05.10 protoplasts were transformed with the *BcGalAR*-Hph hybrid gene and selected on medium containing hygromycin. Hyphae that were growing through the top layer were transferred to new plates of MEA with hygromycin. Transferred hyphae that were able to grow were transferred to MEA without hygromycin for spore harvesting.

3.4 Screening of *ΔBcGalAR* mutants

To confirm that *BcGalAR* was replaced with the hybrid gene, genomic DNA was isolated from mycelium and analysed for the presence of the flanking fragments with hygromycin (Figure 3.4). The transformants that contained both the 5' and 3' flanking region were transferred to Gamborg's B5 media containing glucose or galacturonic acid as sole carbon source. It is expected that homokaryotic transformants are not able or will have reduced growth on Gamborg's B5 containing galacturonic acid as sole carbon source. Three days after transfer, four transformants had growth reduction compared to a transformant in a different gene, unrelated to pectate degradation or catabolism (Supplemental figure 3). After 7 days of growth on D-galacturonic acid media, colonies of TF5, TF9, TF14 and TF16 showed symptoms of stress *e.g.* irregular colony shape, premature sporulation and thin hyphae (data not shown).

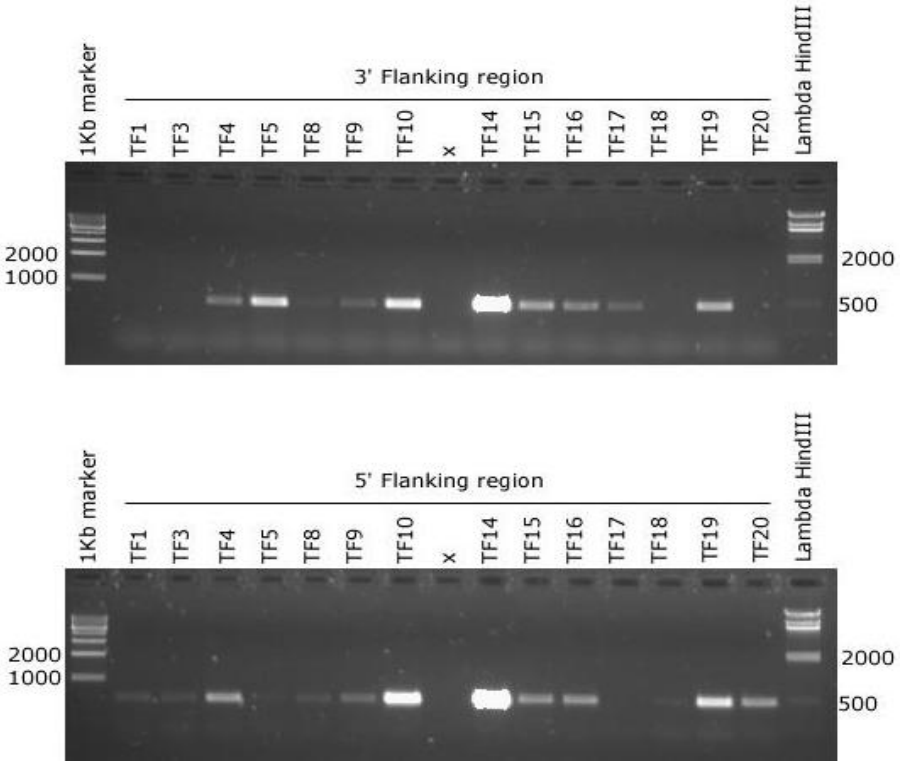

Figure 3.4. Amplification of 3' and 5' flanking region in *ΔBcGalAR* transformants. The 3' flanking region was amplified in transformant TF4, 5, 8, 9, 10, 14, 15, 16, 17 and 19. The 5' flanking region was amplified in all transformants except TF17. TF4, 5, 8, 9, 10, 14, 15, 16 and 19 had both of the flanking regions and were selected for single spore isolation. X is the mastermix without DNA template. The numbers next to the marker represent the size of the fragment in bp.

The transformants that contained both the 5′ and 3′ flanking region and had reduced growth in galacturonic acid were used for single spore isolation. To analyse if the transformants are heterokaryotic or homokaryotic, the 5′ flanking region + hygromycin, 3′ flanking region + hygromycin, the end of *BcGalAR* + 3′ flanking region and a fragment of *BcGalAR* were amplified (Supplemental figure 3). Amplification of *BcGalAR* + 3′ flanking region and *BcGalAR* indicated that the B05.10 wild type nuclei are present and the amplification of the 3′ flanking fragment indicates that the hygromycin gene is present.

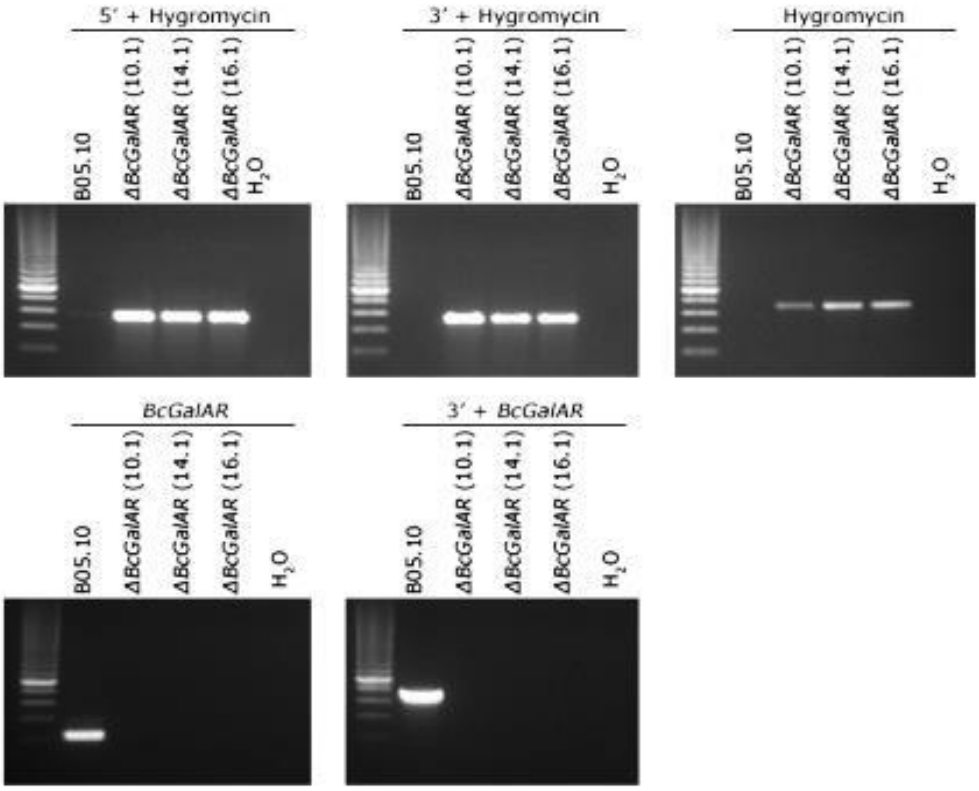

Figure 3.5. Deletion of *BcGalAR* by targeted gene replacement. To verify the targeted gene replacement of *BcGalAR* in B05.10, genomic DNA of each the individual transformants was used. The primer combinations 5.1/23, 3.1/22 and LZ92/LZ93 was used to verify homologues recombination and primer combinations qRT-*BcGalAR* F/R and 3.1/*BcGalAR*-screen were used to verify the absent of *BcGalAR*. 200 bp marker was used as molecular size reference.

Growth of the transformants was reduced on D-galacturonic acid media compared to glucose media. The growth reduction was an indication that the transformants were homokaryotic, as was shown with PCR (Supplemental figure 4). However, TF5 and TF9 showed reduced growth on D-Galacturonic acid but were identified as heterokaryotic transformants. Single spores of TF14 and TF16 were isolated and screened (Supplemental figure 5). The *BcGalAR* knockout mutants TF10.1, TF14.1 and TF16.1 were used in following experiments (Figure 3.5).

3.5 BcGalAR is involved in the utilisation of D-galacturonic acid

To investigate whether the deletion of *BcGalAR* results in the same phenotype as Δ*Bcgar1/Bcgar*2, Δ*Bclgd*1 and Δ*Bclga*1, growth experiments were performed as described by Zhang *et al.,* 2011. B05.10 and *ΔBcGalAR* mutants were grown on Gamborg's B5 containing 30mM fructose. Agar plugs were transferred to Gamborg's B5 media containing glucose, D-galacturonic acid, sodium polygalacturonate (Pectate), apple pectin (consisting of 61% D-galacturonic acid) or citrus pectin (consisting of 78% D-

galacturonic acid) as sole carbon source. The radial growth of B05.10, TF10.1, TF14.1 and TF16.1 was measured on 3 and 4 days past inoculation (dpi). Deletion of *BcGalAR* had no influence on the growth rate on glucose and apple pectin (Figure 3.6). *ΔBcGalAR* mutants had reduced growth on D-galacturonic acid, pectate and citrus pectin.

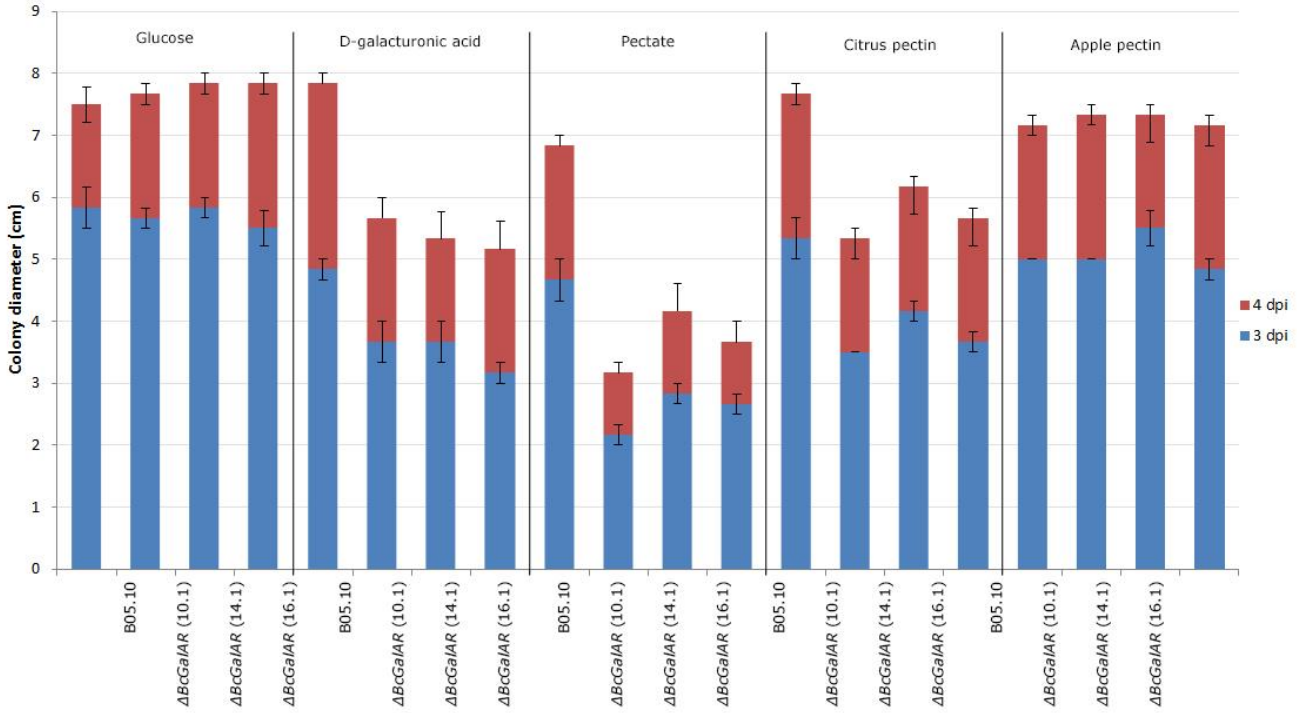

Figure 3.6 Radial growth of *ΔBcGalAR* mutants on agar medium containing glucose, D-galacturonic acid, pectate, citrus pectin and apple pectin. Agar media plates were inoculated with 30mM fructose agar plugs. Colony diameter were measured on 3 and 4 dpi. Maximum radial growth on media plates was 8.5 cm. Presented data are the means with error bars from three technical replicates.

To investigate if transformants were able to grow on the media using the fructose from the agar plugs, the experiment was repeated with spores (Figure 3.7). Also, D-galacturonic acid catabolic mutants were grown to observe if there are similar effects. Growth of the *ΔBcGalAR* mutants was reduced compared to B05.10 but was less reduced than the catabolic mutants. Nevertheless, the growth of the ΔBcGalAR mutants was significantly different from B05.10 after 5 dpi on D-galacturonic acid, pectate and citrus pectin. Interesting was that the *ΔBcGalAR* mutants were still able to grow and that the effect was less severe than incubating them on agar plugs. Another observation was that the mycelium of the *ΔBcGalAR* and catabolic mutants appeared to be thinner and less dense (Supplemental figure 6). This indicates that less biomass is produced and that the mutant is growing in search of nutrients. Biomass assay was performed to confirm this observation (Figure 3.8). The biomass of the mutants was strongly reduced in D-galacturonic acid as compared to the wild type. Another observation was that the catabolic mutant *ΔBclgd*1 appeared smaller in all D-galacturonic acid containing media.

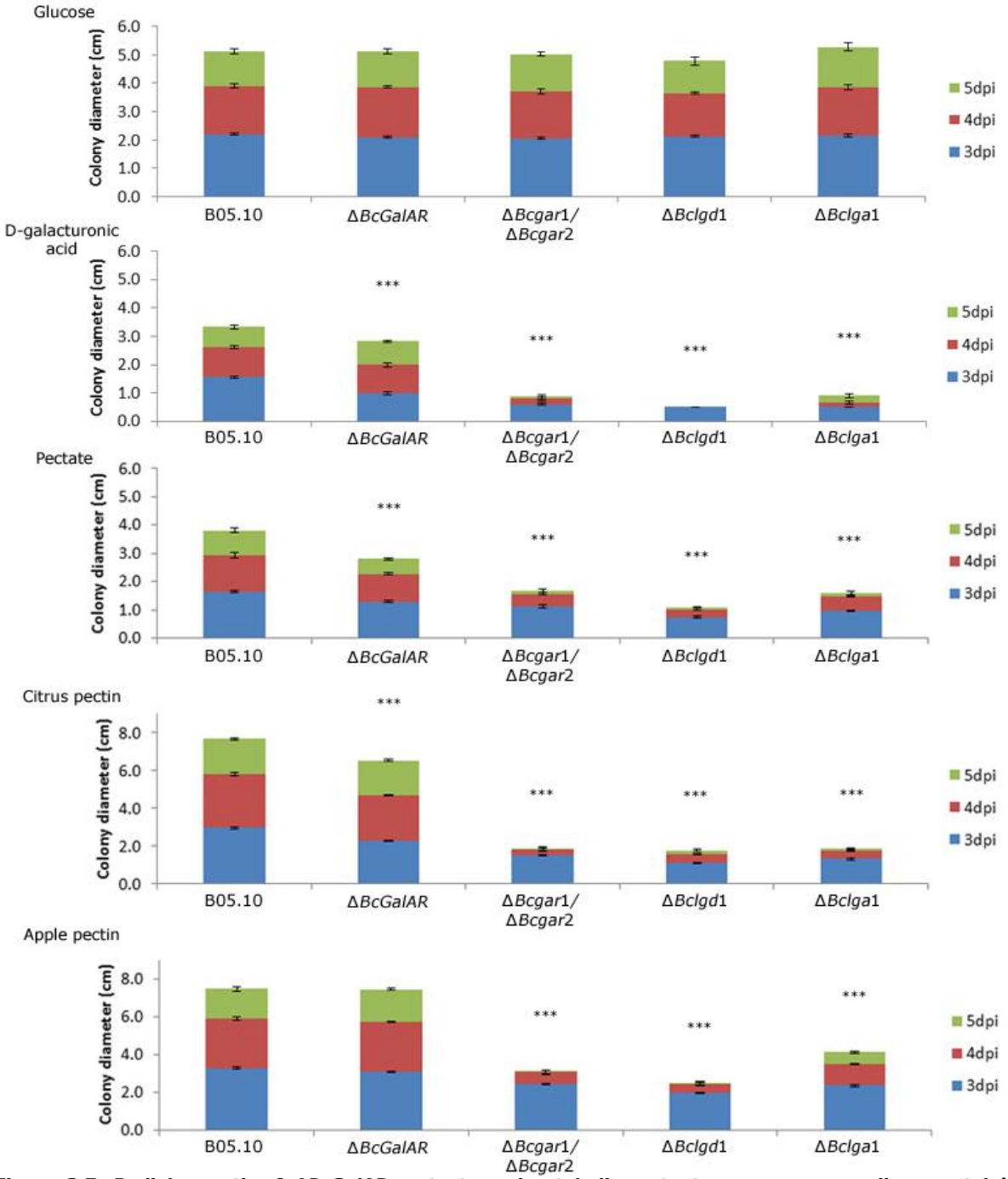

Figure 3.7. Radial growth of *ΔBcGalAR* mutants and catabolic mutants on agarose medium containing glucose, D-galacturonic acid, pectate, citrus pectin and apple pectin as sole carbon source. Agarose media plates were inoculated with spores incubated for 2 hours in 10mM Fructose. Colony diameter were measured from 3 to 5 days past incubation (dpi). Presented data are the means with error bars from four technical replicates. The *ΔBcGalAR* mutant is the average of three biological replicates containing each 4 technical repeats. Asterisks indicate a significant difference to the wild type strain B05.10 on 5 dpi based on Fisher's LSD test (*** P < 0.001).

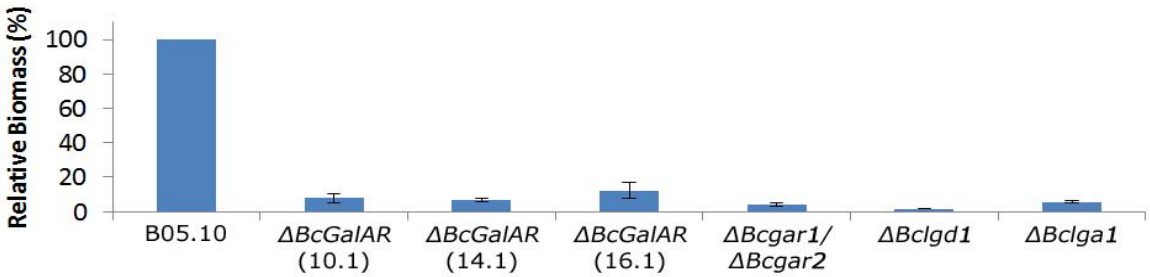

Figure 3.8. Biomass assay on B05.10, *ΔBcGalAR* and D-galacturonic acid catabolic mutants. Liquid cultures of Gamborg's B5 medium containing 50mM D-galacturonic acid were inoculated with spores from the mutants and B05.10. Represented data is the means with error bars from three technical repeats compared to the wild type B05.10.

Zhang and van Kan, 2013, described that the intermediates of the D-galacturonic acid catabolic pathway have growth inhibiting effects. To investigate that the intermediate L-galactonate of the *ΔBclgd*1 mutant inhibits the growth, the wild type B05.10 and the mutants *ΔBclgd1* and *ΔBcGalAR* were grown on medium containing fructose and medium containing fructose and D-galacturonic acid. The *ΔBclgd*1 mutant was chosen because it had the strongest reduced growth when D-galacturonic acid was present. The effect of D-galacturonic acid on the *ΔBclgd*1 mutant shows that the growth is clearly inhibited compared the media containing only fructose (Figure 3.9) The radial growth of the wild type and *ΔBcGalAR* mutant remained unaffected and indicates that the intermediate L-galactonate has growth inhibiting effects.

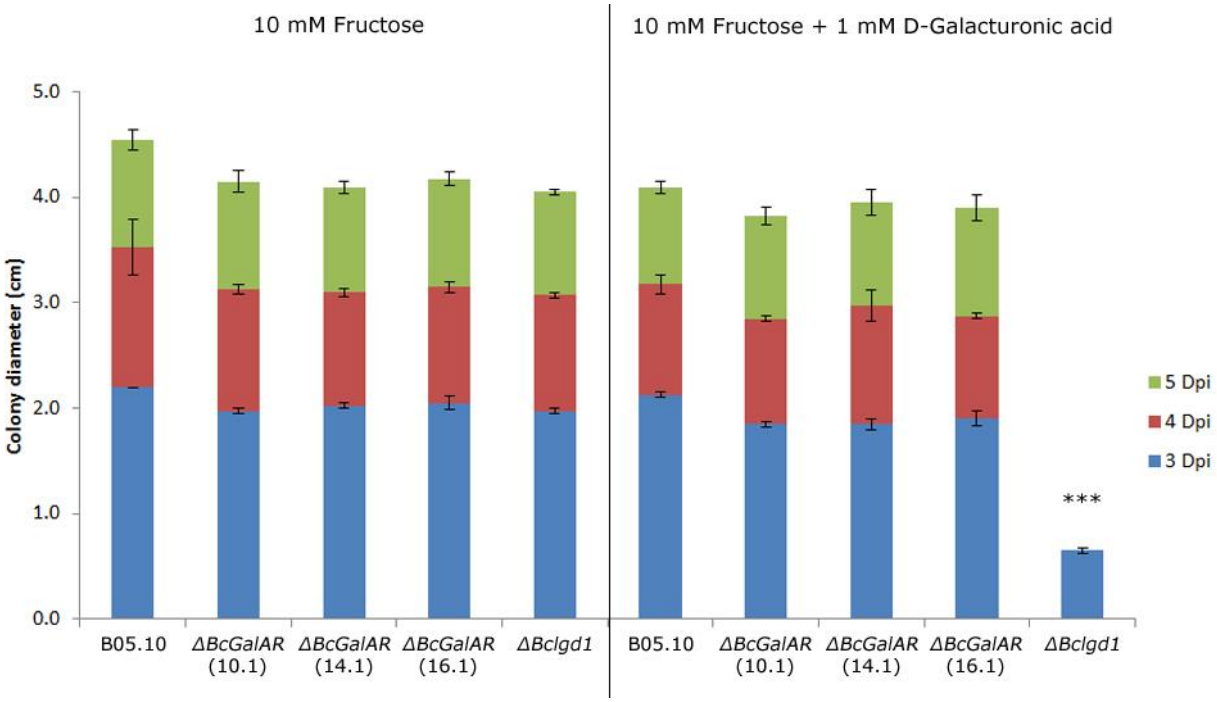

Figure 3.9. **Radial growth of *ΔBclgd*1 and *ΔBcGalAR* mutants on agarose medium containing fructose with or without D-galacturonic acid.** Colony diameter was measured on 3, 4, and 5 days after inoculation. Data represented are the means with error bars of 4 technical replicates. Asterisks indicate a significant difference to the wild type strain B05.10 and *ΔBcGalAR* mutants on 3, 4 and 5 dpi based on Fisher's LSD test (*** $P < 0.001$).

3.6 Virulence assay of *ΔBcGalAR* on tomato and *N. benthamiana*

To demonstrate that the virulence is not effected by the inability to utilize D-galacturonic acid but by the growth inhibiting effects of the intermediates, the wild type B05.10 and the mutants *ΔBcGalAR* and *ΔBcLgd*1 were inoculated on tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* (Figure 3.10A and 3.10B). The virulence of the wild type and the mutants were determined by the lesion size, that were measured after 3 dpi. The lesion size of *ΔBclgd*1 is significant different from the lesion size of B05.10 and *ΔBcGalAR* mutant. This indicates that the virulence is not effected by the deletion of *BcGalAR*.

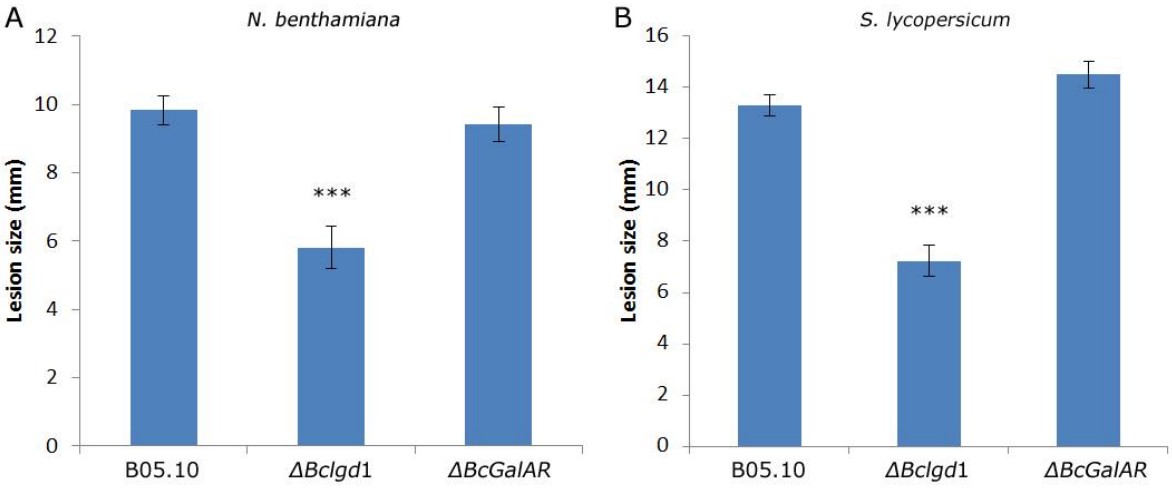

Figure 3.10. **Virulence assay of *ΔBcGalAR* and *ΔBclgd*1 mutants on *N. benthamiana* and *S. lycopersicum*. A.** Lesions size of B05.10 (N=129), *ΔBcGalAR* (N=85) and *ΔBclgd*1 (N=44) on *N. benthamiana* leaves were measured after 3 dpi. **B.** Lesions size of B05.10 (N=52), *ΔBcGalAR* (N=24) and *ΔBclgd*1 (N=24) on *S. lycopersicum* leaves were measured after 3 dpi. Represented data is the mean of the lesion size and the error bars represent the standard error. Asterisks indicate a significant difference to the wild type strain B05.10 on 3 dpi based on Fisher's LSD test (*** P < 0.001).

3.7 BcGalAR regulates D-galacturonic catabolic pathway genes

With the radial growth assay of *ΔBcGalAR* mutants on D-galacturonic acid, we observed that growth was reduced and not stopped like the D-galacturonic acid catabolic mutants (Zhang *et al.,* 2011). B05.10 and *ΔBcGalAR* were grown for 3 hours in Gamborg's B5 with glucose or D-galacturonic acid as sole carbon source. From the mycelia, total RNA was isolated and synthesized to cDNA. To determine the expression levels of the D-galacturonic catabolic genes, *Bchxt*15*, Bchxt*19 and B0510_2787, qRT-PCR was used. The relative expression of the selected genes was compared between B05.10 grown in glucose and D-galacturonic acid and *ΔBcGalAR* grown in glucose and D-galacturonic acid. The expression of the catabolic genes in B05.10 were upregulated when grown in D-galacturonic acid (Figure 3.11). Strikingly is that these genes were not upregulated when the *ΔBcGalAR* mutant was grown in D-galacturonic acid. This indicates that BcGalAR is involved in regulation of the D-galacturonic catabolic genes. The genes *Bchxt15, Bchxt19* and B0510_2787 were also upregulated by B05.10 when grown in D-galacturonic acid. These genes were not up regulated in the *ΔBcGalAR* mutant. *BcGalAR* was detected in the cDNA samples of the wild type and *ΔBcGalAR* mutant and in the no-template control (data not shown).

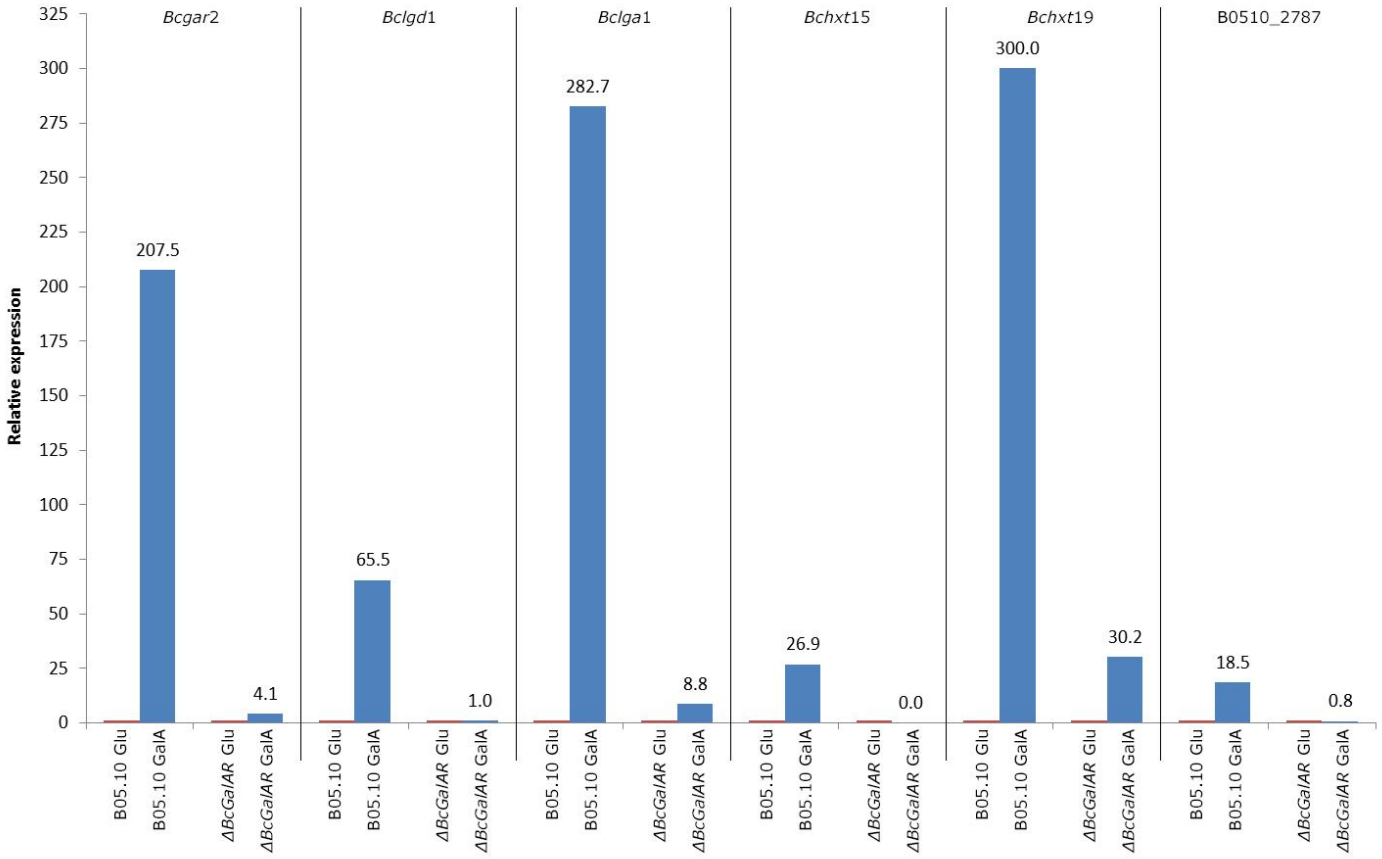

Figure 3.11. **Expression of D-galacturonic acid catabolic pathway genes in B05.10 and *ΔBcGalAR* mutant grown in liquid media containing glucose and D-galacturonic acid.** B05.10 and *ΔBcGalAR* cultures were sampled for RNA extraction at 3h after transfer with glucose as carbon source. Expression levels were normalized against *BcRpl5* and calibrated to the levels of the pre-culture containing media with glucose as sole carbon source (Set as 1), according to $2^{-\Delta\Delta CT}$ method. Relative expression was calculated by dividing the ΔΔCT of GalA 3h samples with ΔΔCT of Glu 3h samples. Data is represented as average from 1 biological repeat with three technical replicates.

4. Discussion

One of the main questions of this thesis was if the candidate transcription factor BcGalAR is involved in the activation of the D-galacturonic acid catabolic enzymes in *B. cinerea*. With Y1H, the interaction between the promoter of the selectable marker containing the GAE1 motif and BcGalAR was confirmed (Figure 3.1). Mutating the GAE1 motif in the promoter of the selectable marker revealed that the interaction with BcGalAR was abolished. Concluding that the interaction of BcGalAR with the GAE1 motif is specific. Radial growth assays with B05.10 and the *ΔBcGalAR* mutants showed that there was a significant difference between the radial growth on D-galacturonic acid, pectate and citrus pectin between B05.10 and the *ΔBcGalAR* mutant (Figure 3.6 and 3.7). The radial growth was less severely affected with the *ΔBcGalAR* mutants on D-galacturonic acid as compared to catabolic pathway mutants. The colonies of the catabolic mutants and *ΔBcGalAR* mutants appeared both less dense and had thinner mycelia then the wild-type when growing on pectate and D-galacturonic acid (Zhang *et al.,* 2011) (Supplemental figure 6). Indeed the biomass of the *ΔBcGalAR* mutants in a liquid culture was 11 times lower compared with the wild type whereas the catabolic pathway mutants produced even less biomass then the *ΔBcGalAR* mutants (Figure 3.8). This observation suggests that *Bcgar*1, *Bcgar*2, *Bclgd*1 and *Bclga*1 are still expressed in the *ΔBcGalAR* mutants. Indeed, *Bcgar*2, *Bclgd*1 and *Bclga*1 were still expressed in the *ΔBcGalAR* mutant (Figure 3.11).

The expression level of *BcGalAR* was analysed when B05.10 is grown liquid media containing glucose or D-galacturonic acid as sole carbon source (Figure 3.2, Supplemental figure 7). The expression level of *BcGalAR* increases when grown in D-galacturonic acid however the expression level of *BcGalAR* remained below 5 fold. The up-regulation of *BcGalAR* in D-galacturonic acid is low compared to the L-rhamnose catabolic regulator RhaR which is upregulated 400 fold when grown in L-rhamnose (Gruben *et al.,* 2012). The expression level of *BcGalAR* does not match the expression pattern of the D-galacturonic acid catabolic pathway genes which are highly upregulated within 3 hours. We assume that BcGalAR is already present before D-galacturonic acid exposure, presumably in an inactive state and is activated when D-galacturonic acid is present. At this point, it remains unknown how BcGalAR is regulated.

It was shown before that the expression of *Bcgar*2, *Bclga*1 and *Bclgd*1 is upregulated when B05.10 is grown in D-galacturonic acid (Zhang *et al.,* 2011). However it was previously unknown how the D-galacturonic acid catabolic genes were regulated since no transcription factor or DNA binding protein was upregulated in D-galacturonic acid. Deletion of *BcGalAR* in *B. cinerea*, resulted in reduced expression of the D-galacturonic acid catabolic pathway genes (Figure 3.11). However, transcription analysis of *Bcgar*2, *Bclgd*1 and *Bclga*1 in the *ΔBcGalAR* mutant shows that there is a basal expression of these genes when D-galacturonic acid is present. This explains why the *ΔBcGalAR* mutants had a better radial growth then the catabolic pathway mutants. The expression levels of *Bcgar*1 were not analysed due to the lacking of the GAE1 motif in the promoter. Transcripts of *BcGalAR* were detected in the cDNA of the *ΔBcGalAR* mutant and no template control. The *ΔBcGalAR* mutant contained the inserted 5' flanking region, 3' flanking region and hygromycin gene and *BcGalAR* was not amplified (Figure 3.5). cDNA samples were analysed with PCR and genomic DNA was detected. The samples were contaminated after the incubation in glucose and D-galacturonic acid since the expression pattern of the D-galacturonic acid catabolic genes in the *ΔBcGalAR* mutant are not similar as the wild type. The origin of the contamination of the cDNA samples remains unknown and the experiment has to be repeated.

Next to the regulation of the D-galacturonic acid catabolic genes, BcGalAR also regulates the expression of *Bchxt*15*, Bchxt*19 and B0510_2787. The expression of the hexose transporters *Bchxt*15 and *Bchxt*19 is upregulated when grown in D-galacturonic acid (Zhang, 2013). It was shown that both *Bchxt*15 and *Bchxt*19 are involved in the uptake of D-galacturonic acid (Zhang *et al.,* 2014). B0510_2787 encodes an putative exo-pg. Alike the promoters of the D-galacturonic acid catabolic pathway genes, *Bchxt*15, *Bchxt*19 and B0510_2787 contain the GAE1 motif and are regulated by BcGalAR (Figure 3.11). I purpose that BcGalAR regulates all the GAE1 motif containing genes involved in the pectin degradation and D-galacturonic acid utilization. In the *B. cinerea* genome, there are 323 GAE1 motif containing genes identified (Zhang and van Kan, 2013). The GAE1 motif was first identified in the promoters of pectinolytic genes and in the bidirectional gene cluster of *gar2-lga1* in several filamentous fungi (Martens-Uzunova and Schaap, 2008, Zhang, 2013). Blasting the sequence of *BcGalAR*, homologues were identified in *A. niger, A. nidulans, F. oxosporum, P. chrysgemun, P. nodorum, M. grisea* and *S. sclerotiorum*. In future experiments, the deletion *BcGalAR* in other fungi, like *A. niger,* shows if the function of BcGalAR in the D-galacturonic acid pathway is conserved.

Another question of this thesis was if the virulence of *B. cinerea* was effected by the deletion of the *BcGalAR*. The catabolic mutants on *A. thaliana* and *N. benthamiana* showed reduced lesion diameter compared to B05.10 (Zhang *et al.,* 2011, Zhang and van Kan, 2013). However, it was not possible to conclude if the virulence was reduced or that the growth was inhibited by the D-galacturonic pathway intermediates. The catabolic mutants had less growth on media containing 10mM fructose with 0.03 and 1mM D-galacturonic acid (Zhang and van Kan, 2013). Indicating that the intermediates can have a growth inhibiting effect. To investigate if the intermediate has an growth reducing effect, B05.10 and the *ΔBcGalAR* and *ΔBclgd*1 mutants were grown on 10mM fructose with and without 1mM D-galacturonic acid. Growth was not reduced on 10mM fructose without D-galacturonic acid. The radial growth of the *ΔBcGalAR* mutant on 10mM fructose with D-galacturonic acid was not reduced. However, *ΔBclgd*1 was not able to grow larger after 3 dpi indicating that the intermediate L-galactonate has a growth inhibiting effect. In *A. niger* and *H. jecorina*, no growth inhibition was observed when the catabolic genes were deleted (Kuivanen *et al.,* 2012, Wiebe *et al.,* 2010). It is possible that L-galactonate is not toxic for these fungi. Another possibility is that *A. niger* and *H. jecorina* are able to convert the intermediates to other metabolites. It is likely that the virulence of the catabolic mutants on plants is not reduced through the inability to utilize D-galacturonic acid but rather to the inhibitory effect of the intermediates. The lesion size of the *ΔBcGalAR* mutant on *N. benthamiana* and *S. lycopersicum* leaves showed that there was no difference from B05.10. However, the lesion size of *ΔBclgd*1 mutant was smaller than B05.10 and *ΔBcGalAR* on both *N. benthamiana* and *S. lycopersicum* (Figure 3.10). The lesion size of the *ΔBcGalAR* mutant was not reduced which means that the D-galacturonic acid pathway is not needed for virulence. It is apparent that there are enough nutrients available in the plant and that D-galacturonic acid does no significantly contribute to the diet of *B. cinerea*.

In future experiments, *BcGalAR* can be reintroduced in the *ΔBcGalAR* mutant which reveals if *BcGalAR* can restore the impaired growth on media containing D-galacturonic acid, pectate and citrus pectin as sole carbon source. Complementation with a homologue of another fungi will reveal if the homologue has a conserved function. For localisation studies, the *ΔBcGalAR* mutant can be complemented with constructs of *BcGalAR* fused to GFP with the native or a constitutive promoter. To investigate if BcGalAR is present before exposure to D-galacturonic acid, spores of the complemented *ΔBcGalAR*/*BcGalAR* mutant can be germinated in fructose and analysed for fluorescence with the fluorescence microscope.

To investigate if BcGalAR is transported through the cell, D-galacturonic acid can be supplemented to the germinated *ΔBcGalAR*/*BcGalAR* spores and monitored for protein movement. This method can also reveal if BcGalAR is expressed or stabilized when D-galacturonic acid is introduced. The qRT-PCR analysis has to be repeated to confirm that *BcGalAR* is deleted in the *ΔBcGalAR* mutant. Transcription analysis of *Bcglr1* and *Bcgar1* can reveal if BcGalAR regulates the complete D-galacturonic acid catabolic pathway. This analysis can also shed light on the role of *BcGalAR* in the regulation of D-galacturonic acid utilization. For example, the endo-PG *Bcpg2* also contains the GAE1 motif in the promoter and is induced by D-galacturonic acid (Zhang, 2013). Altogether will display the role and regulation of *BcGalAR* in the D-galacturonic acid catabolic pathway and utilization.

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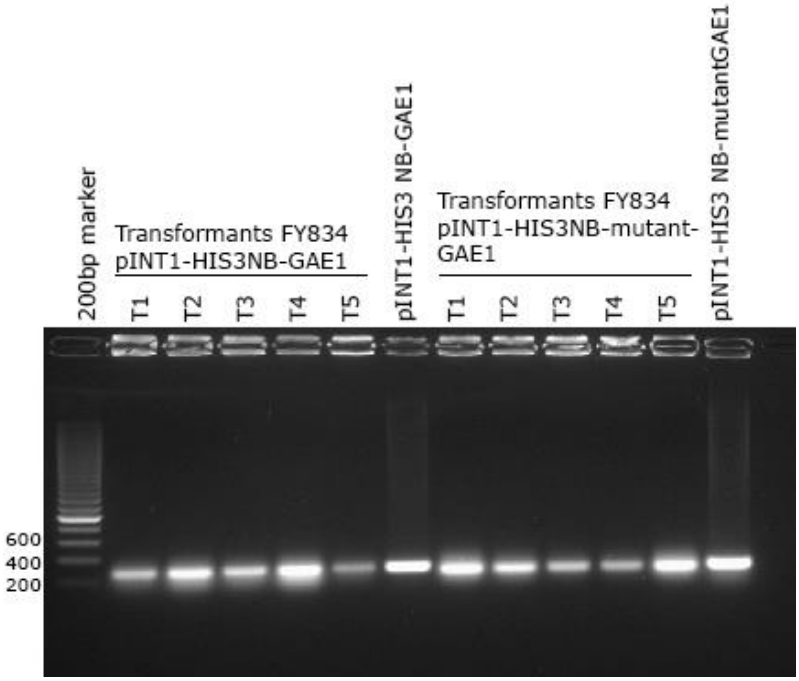
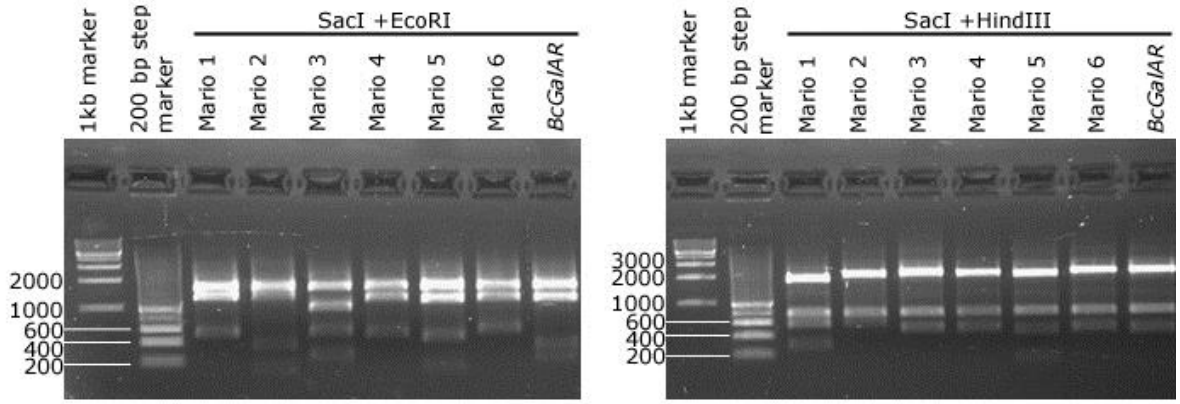
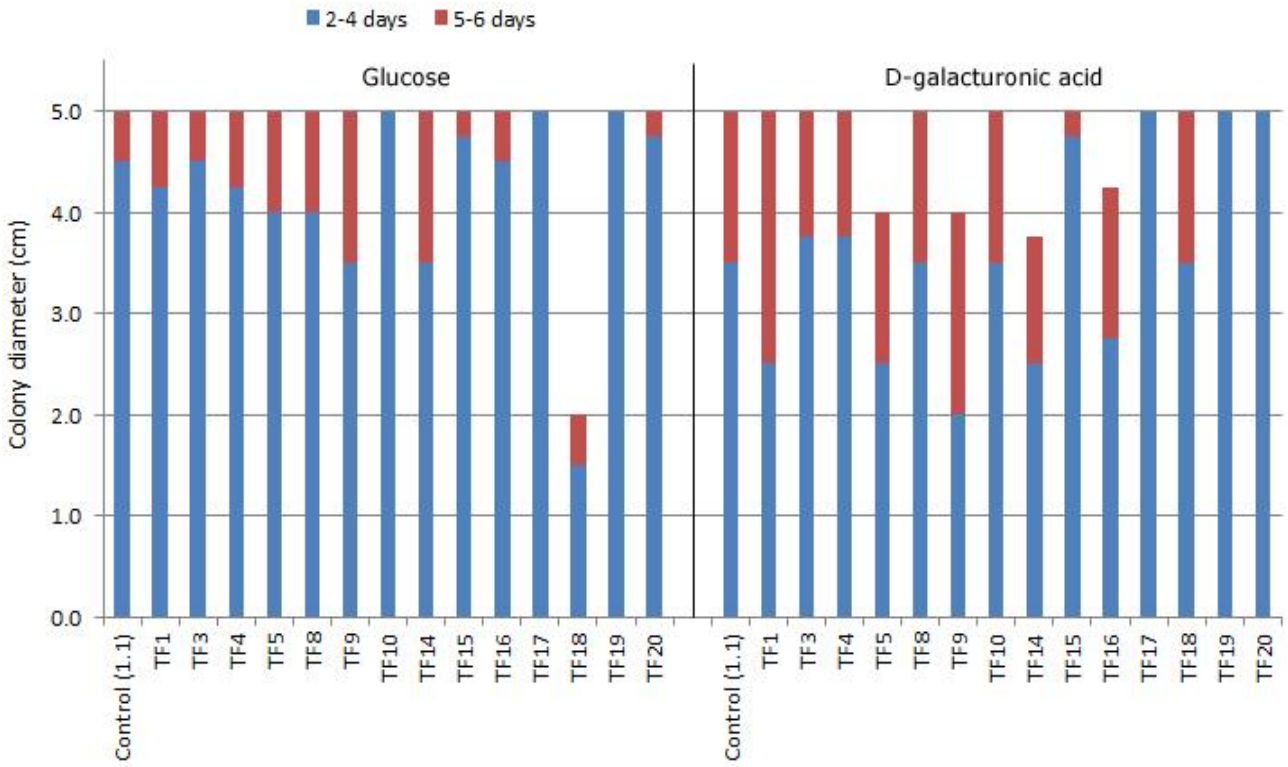
Supplemental data



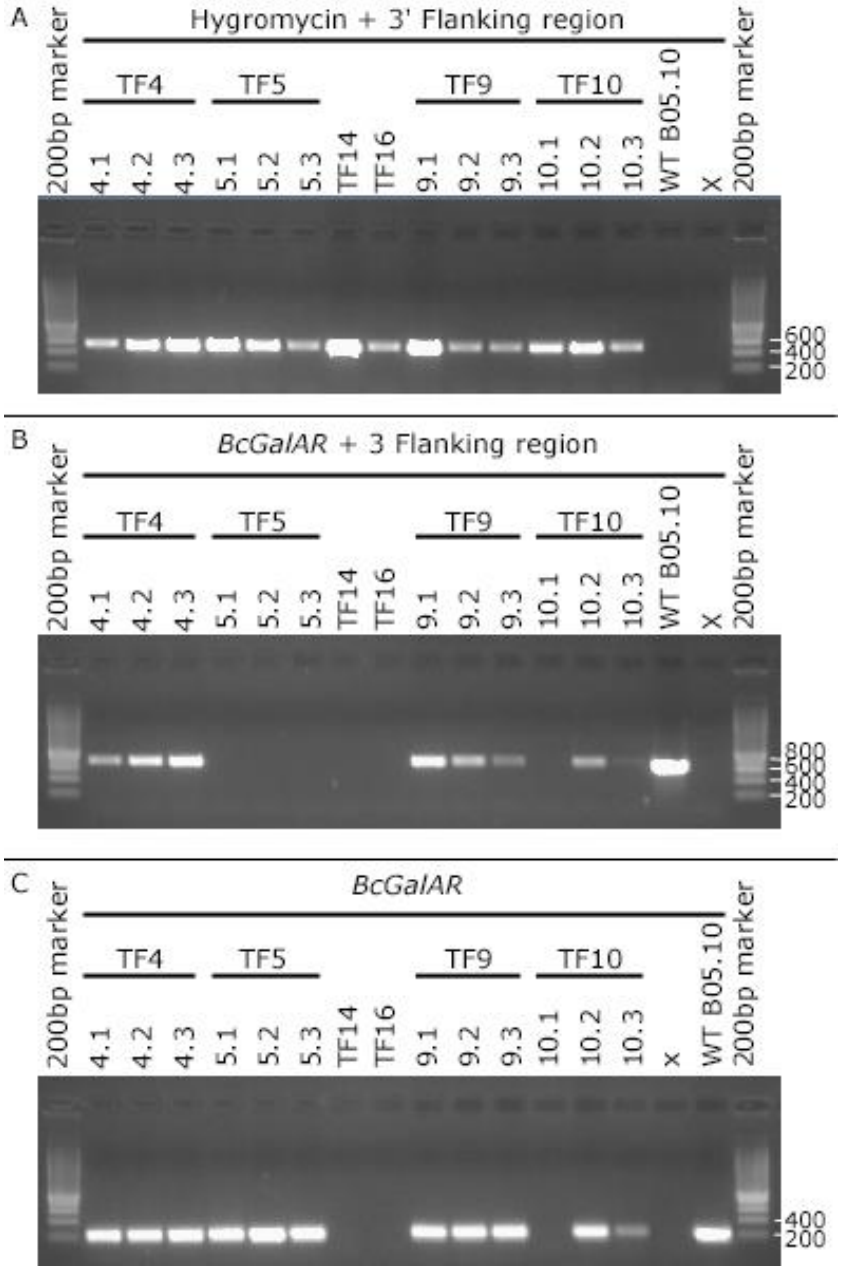
Supplemental figure 1. Colony PCR on FY834 pINT-HIS3NB GAE1 and mutant GAE1 screen. Colony PCR was performed on FY834 colonies selected on YPD with G418 using the primers pINT1-screen F and R. pINT-HIS3 NB plasmids isolated from *E.coli* were used as control. The numbers next to the marker represent the size of the fragment in bp.



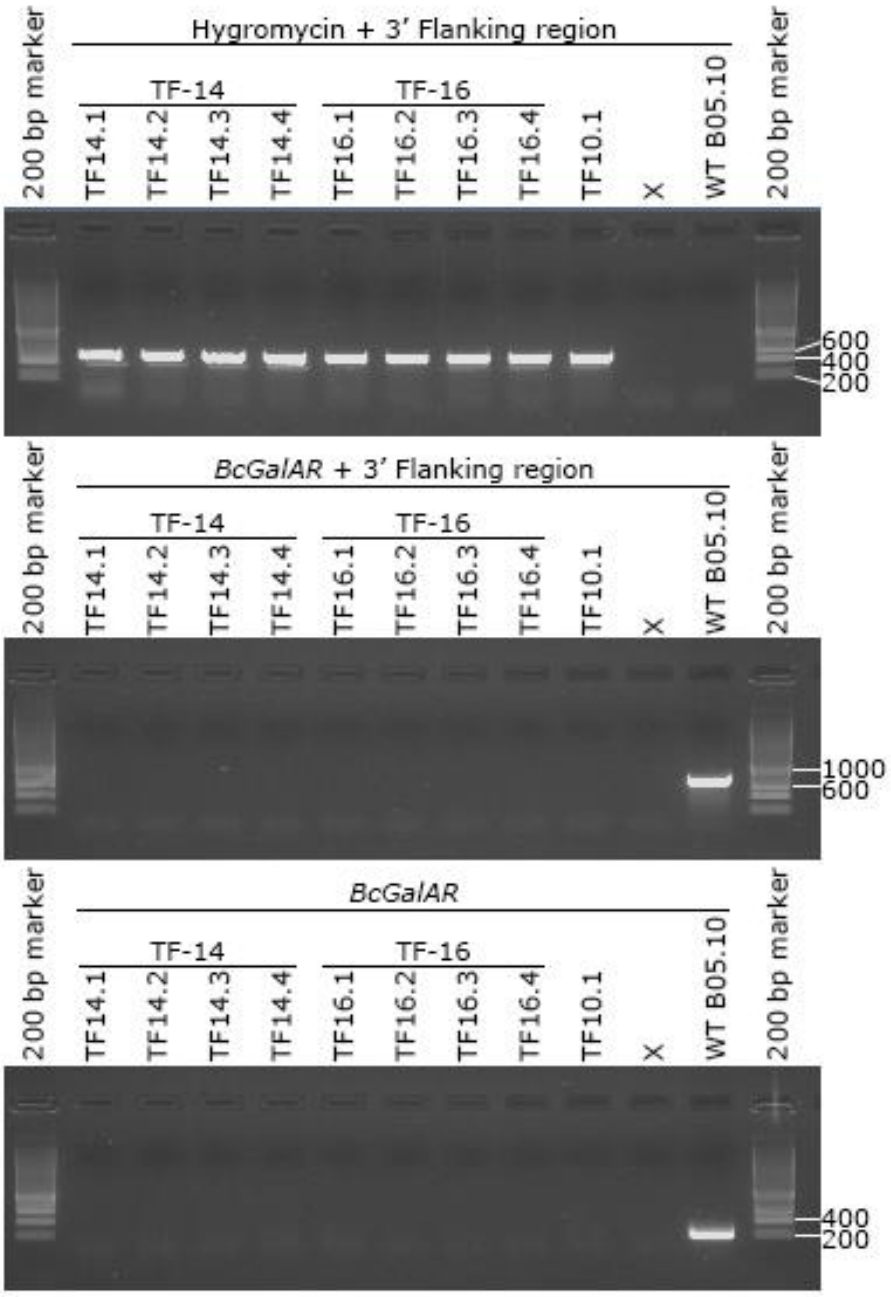
Supplemental figure 2. Digestion of the overlap extension products. For the confirmation of the overlap extension, two digestions were done. Expected molecular patterns for *BcGalAR* were The digestion of the *BcGalAR* hybrid gene matched both the expected molecular patterns 205bp, 324bp, 1.25 kb and 1.65 kb for SacI + EcoRI and 486 bp, 750 bp and 2.15 kb for SacI + HindIII. The samples Mario 1 to 6 are for illustration and shows that the used program was successful with different primers. All hybrid genes of Mario matched with the expected molecular patterns and shows that the used program was efficient. The numbers next to the marker represent the size of the fragment in bp.



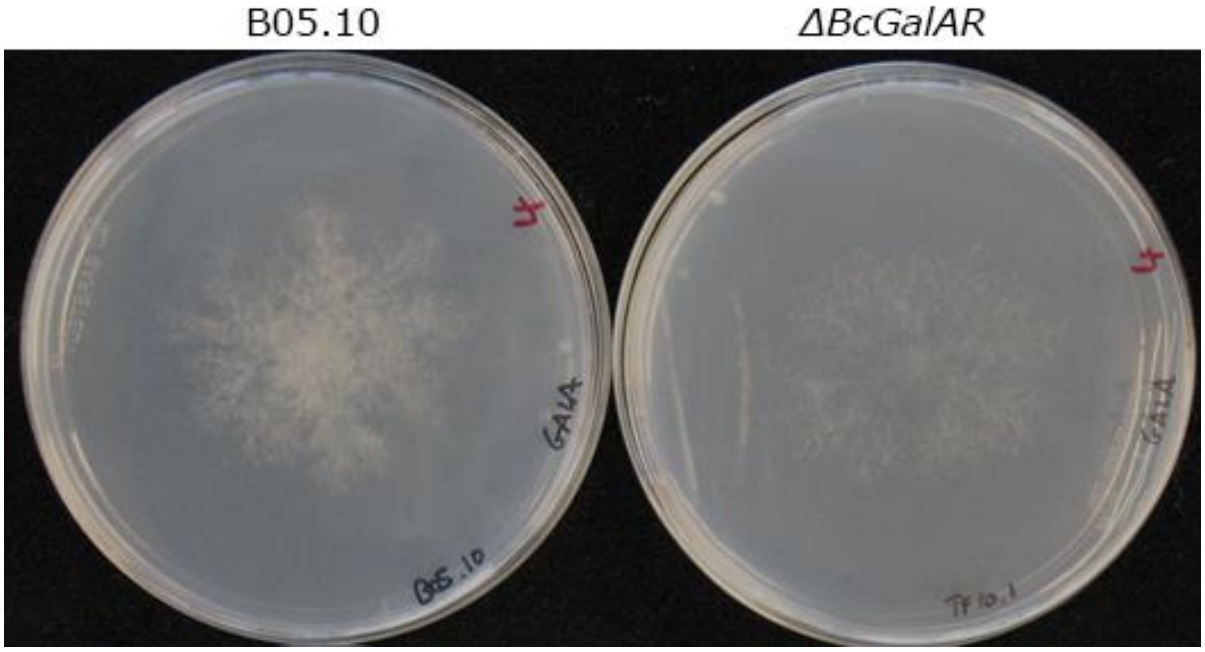
Supplemental figure 3. Growth of B05.10 *ΔBcGalAR* mutants in glucose and D-galacturonic acid. Colony diameters were measured at 2-4 days and 5-6 days. On glucose, all transformants except TF18 were able to reach the end of the plates within 6 days. On D-galacturonic acid, four transformants were not able to reach the end of the plate within 6 days.



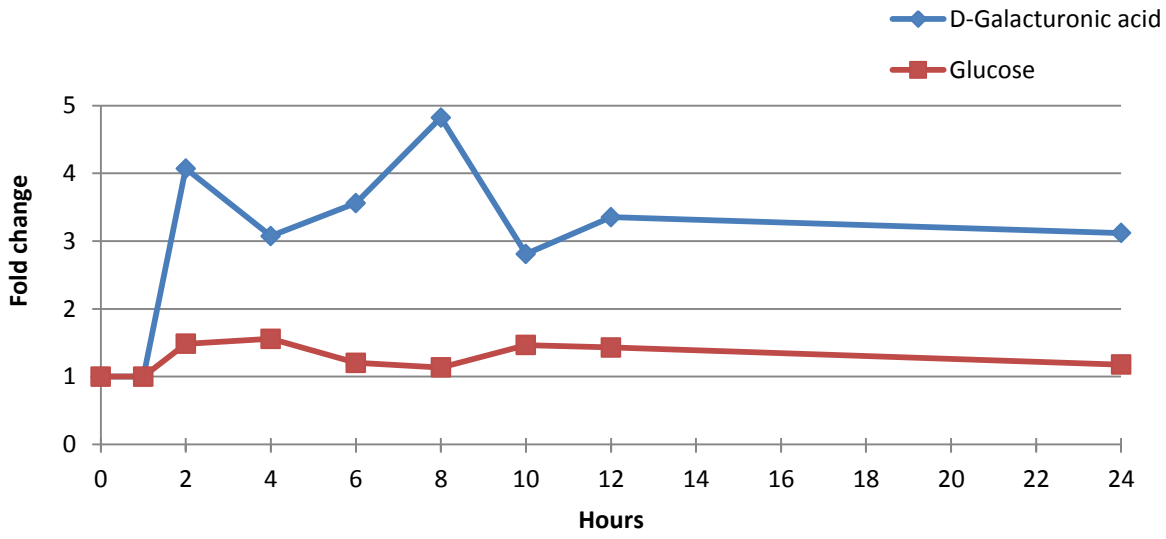
Supplemental figure 4. Screening for homo- and heterokaryotic *ΔBcGalAR* transformants. A. Screening for the Hygromycin + 3' flanking region. The expected size for hygromycin + 3' flanking region was 480bp and was detected in all transformants. WT B05.10 does not contain hygromycin so no amplification is expected. **B.** The expected size of *BcGalAR* + 3' flanking region was 759bp and was amplified in TF4, TF9, TF10.2, TF10.3 and the WT B05.10. **C.** Amplification of *BcGalAR* was done with the *BcGalAR* qRT-PCR primers and amplifies a product of 224bp. The amplified product was detected in all samples, except TF10.1, TF14 and TF16. X is the mastermix without DNA. The numbers next to the marker represent the size of the fragment in bp.



Supplemental figure 5: Screening of homokaryotic *ΔBcGalAR* transformants TF14 and TF16 single spore isolates. Screening for the Hygromycin + 3' flanking region. The expected size for hygromycin + 3' flanking region was 480bp and was detected in all transformants. WT B05.10 does not contain hygromycin so no amplification is expected. **B.** The expected size of *BcGalAR* + 3' flanking region was 759bp and was amplified only in WT B05.10. **C.** Amplification of *BcGalAR* was done with the *BcGalAR* qRT-PCR primers and amplifies a product of 224bp. The amplified product was detected only WT B05.10. X is the mastermix without DNA. The numbers next to the marker represent the size of the fragment in bp.



Supplemental figure 6. Radial growth of B05.10 and *ΔBcGalAR* on D-galacturonic acid. *ΔBcGalAR* mutants are smaller on D-galacturonic acid compared with B05.10. The mycelia of *ΔBcGalAR* mutant appeared to be thinner and were less dense compared to B05.10.



Supplemental figure 7. Expression of *BcGalAR* in B05.10 in glucose and D-galacturonic acid. Expression levels were normalized against *BcRPL5* according to $2^{-\Delta\Delta CT}$ method. Data of timepoint 2h, 4h and 8h are represented as average from 2 biological repeat with three technical replicates per repeat. For timepoint 2h and 4h, a second independent repeat was performed. Data from timepoint 1h, 6h, 10h, 12h and 24h are represented as average for three technical repeats and do not contain biological repeat.