# Functional analysis and expression of genes involved in D-galacturonic acid utilization and pectin degradation by *Botrytis cinerea*

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**MSc Thesis Report**

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## Abstract:

Plant cell wall is a very complex structure and acts as the first line of defence against pathogens. The main components of plant cell wall polysaccharides are cellulose, hemicelluloses and pectin. Pectin is structurally the most complex polysaccharide in nature. The necrotrophic fungal pathogen, *Botrytis cinerea* produces cell wall degrading enzymes to hydrolyse cell wall polysaccharides and consumes the monosaccharides released. The full consumption of the available carbon source requires the concerted action of depolymerising enzymes, monosaccharide transporters and catabolic enzymes. The expression of the genes encoding these catabolic enzymes is co-upregulated by D-galacturonic acid. The arabinan mutants and double knockout mutants of endo polygalacturonases were also tested on tomato and *N. benthamiana* plants. The transcript levels of hexose transporter genes *Bchxt*13 and *Bchxt*15 and the putative D-galacturonic acid regulator gene *Bcgalar*1 were induced in medium containing D-galacturonic acid as the carbon source. The knockout mutants of these genes were tested on solid media with various carbon sources and on tomato and *Nicotiana benthamiana* plants but no significant difference were observed. An RNA sequence analysis was conducted which resulted in the induction in transcript level of 31 genes in the presence of pectate. Only 6 out of these 31 genes were specifically induced in the presence of D-galacturonic acid when a qRT-PCR analysis was done.

## Acknowledgement:

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## List of figures:

Figure 1: Structure of pectic substances (Perez et al.2000). ..... 1
Figure 2: Colony diameter of the growth of the knockout mutants of Δ*Bcgalar*1. ..... 8
Figure 3: Lesion diameter of the knock out mutants of Δ*Bcgalar*1 on *Nicotiana benthamiana* and tomato leaves..... 8
Figure 4: Colony diameter of the growth of the knockout mutants of Δ*Bchxt*13 and Δ*Bchxt*15.. 10
Figure 5: Lesion diameter of the growth of the knockout mutants of Δ*Bchxt*15 and Δ*Bchxt*13 .. 11
Figure 6: Lesion diameter of the growth of the knockout mutants of Δ*Bcara*1 on *Nicotiana benthamiana* and tomato leaves. ..... 12
Figure 7: Lesion diameter of the growth of the knockout mutants of Δ*Bcpg*1, Δ*Bcpg*2 and double mutant Δ*Bcpg*1/*Bcpg*2 on *Nicotiana benthamiana* and tomato leaves. ..... 13
Figure 8: The results of the qRT-PCR analysis with various carbon sources..... 16
Figure 9: The results of qRT-PCR analysis done to confirm the 6 specifically induced genes.... 17
Figure 10: Colony diameter of the growth of the knock out mutants Δ*Bchxt15* ..... 22
Figure 11: Colony diameter of the growth of the knock out mutants Δ*Bchxt13* and Δ*Bchxt15* in the presence of different concentration of D-galacturonic acid of 1 mM and 0.1 mM ..... 23

# Table of Contents


1. Introduction ..... 1
1.1. Plant cell wall composition ..... 1
1.2. *Botrytis cinerea*, a necrotrophic fungus ..... 2
1.3. Endo polygalacturonases ..... 2
1.4. Arabinanases ..... 2
1.5. D-galacturonic acid catabolism ..... 3
2. Materials and methods: ..... 4
2.1. Fungal strains and growth conditions ..... 4
2.2. Growth assay: ..... 5
2.3. Measurement of biomass: ..... 5
2.4. Virulence assay: ..... 5
2.5. Quantitative RT-PCR ..... 6
3. Results: ..... 7
3.1. Functional analysis of a putative D-galacturonic acid regulator gene *Bcgalar*1: ..... 7
3.1.1. Growth Assay: ..... 7
3.1.2. Virulence assay: ..... 7
3.2. Functional analysis of a putative D-galacturonic acid transporter genes *Bchxt*13 and *Bchxt*15: ..... 9
3.2.1. Growth assay: ..... 9
3.2.2. Virulence assay: ..... 11
3.3. Virulence assay of Δ*Bcara*1 knockout mutants: ..... 12
3.4. Virulence assay of Δ*Bcpg* knockout mutants: ..... 12
3.5. QRT-PCR analysis ..... 13
4. Discussion: ..... 18
5. References: ..... 21
Appendix 1: ..... 22
Appendix 2: ..... 23
Appendix 3: ..... 24

# 1. Introduction

## 1.1. Plant cell wall composition

The plant cell wall is a very complex structure, provides strength and structure to the plant tissue and acts as the first line of defence for plants against different pathogens. A pathogen attacks the plant cell wall mainly by cellulose, hemicellulose and pectin degradation. These polysaccharides form a 3D network along with lignin and proteins. The polysaccharide fraction mainly consists of cellulose, hemicellulose and pectin. Cellulose contributes 20-30% of the dry mass of cell walls**.** Cellulose have β-1,4-linked D-glucose residues that form un-branched polymeric chains (Nishiyama et al.2002), hemicellulose have β-1,4-linked backbones including xyloglucans, xylans, mannans and glucomannans, and β-(1,3;1,4)-glucans. Pectins are series of polymers of D-galacturonic acid found in the middle lamella of primary cell wall of dicotyledonous and non graminaceous monocotyledonous plants and constitute about 70% of the host cell wall. Pectin includes homogalacturonan (HG), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), and xylogalacturonan (XGA). (Perez et al.2000) (the structures of which are shown in Fig 1). Along with the hemicelluloses, pectin contributes to the integrity and coherence of plant tissues as well as texture of vegetables and fruits. The composition of pectin differs between plants and also depends on the age and maturity of the plant parts.

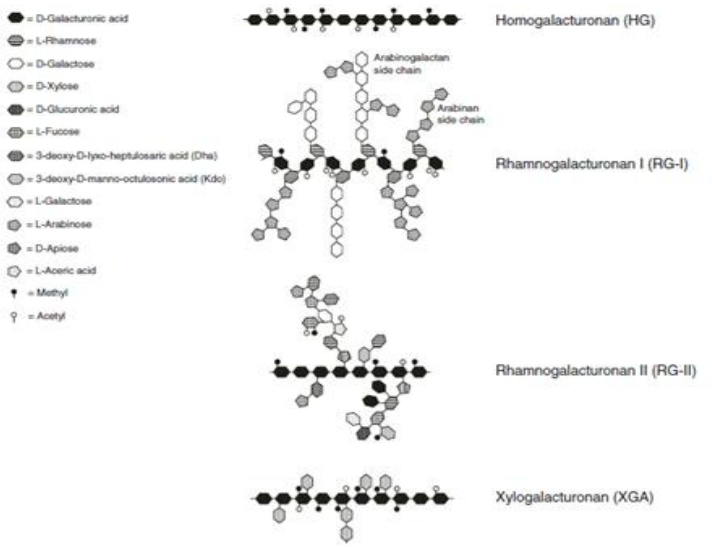


**Figure 1: Structure of pectic substances (Perez et al.2000).**

## 1.2. *Botrytis cinerea*, a necrotrophic fungus

*Botrytis cinerea* is a necrotrophic fungal pathogen which produces different enzymes to degrade the plant cell wall. It can attack over 200 plant species. The attack of this fungus is characterised by formation of necrotic lesions with extensive fungal growth, which appears to be like grey mould. The hyphae penetrate the cell wall causing the cell wall to swell. This is due to the pectin hydrolysis. The host cells surrounding these swollen cells collapse and die (Kars et al. 2005). The pectin hydrolysis thus weakens the host cell wall for colonization by the pathogen and provides carbon sources to the pathogen for its growth. During the infection process, *B.cinerea* secretes cell wall degrading enzymes (pectinases), which break down the cell wall polymers and contribute in penetration through the surface. *B.cinerea* changes the host cell wall to fungal biomass and takes up carbon and nutrients for its growth.

## 1.3. Endo polygalacturonases

Among the various cell wall degrading enzymes of *B. cinerea*, the first one to be secreted is the endo polygalacturonase (ten Have et al. 1998). These endo polygalacturonases breaks down pectin polymers by depolymerising the homogalacturonan (Kristen et al. 1998). The fungus contains 6 endo poly-galacturonases genes (Wubben et al.2000), The mutant of Δ*Bcpg*1 showed reduced virulence on tomato, apple (ten have et al.1998), broad bean, *N. benthamiana, A. thaliana*. (Unpublished data). The Δ*Bcpg*2 mutant showed reduced virulence only on tomato and broad bean (Kars et al. 2005). The mutants of *Bcpg* 3 to *Bcpg*6 showed the same level of virulence as the wild type strain.

## 1.4. Arabinanases

Arabinan, a neutral pectin polysaccharide binds to the HG chain promoting the wall flexibility and binding to the cellulose surface (Jones et al. 2003). Arabinan is highly flexible and interacts with galactan to form a temporary entangled matrix. It plays a crucial role in the response of guard cells to turgor pressure. Arabinanase can hydrolyse these polysaccharides. The knockout mutants Δ*Bcara*1 showed reduction of virulence on Arabidopsis leaves (Zhang and van Kan, 2013), but no information is available on the virulence of this mutant on other host plants.

## 1.5. D-galacturonic acid catabolism

The action of endo polygalacturonases and exo polygalacturonases releases D-galacturonic acid (D-galacturonic acid) monosaccharides, which are taken up by the *B.cinerea* with the help of hexose transporters. D-galacturonic acid catabolic pathway has been characterised in *B. cinerea*, which consists of three catalytic steps converting D-galacturonic acid to pyruvate and L-glyceraldehyde. The pathway involves two non-homologous galacturonate reductase genes (*Bcgar*1 and *Bcgar*2), a galactonate dehydratase gene (*Bclgd*1) and a 2-keto-3-deoxy-L-galactonate aldolase gene (*Bclga*1) (Zhang et al., 2011). Knockout mutants in each step of the pathway (*ΔBcgar*1/*ΔBcgar*2, *ΔBclgd*1, and *ΔBclga*1) were affected in growth on D-galacturonic acid, pectate, or pectin as the sole carbon source (Zhang et al., 2011) and in virulence on *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves (Zhang and van Kan, 2013). The transcript levels of two hexose transporter genes *Bchxt*13 and *Bchxt*15 were induced in medium containing D-galacturonic acid as the carbon source. However the function of these genes in D-galacturonic acid uptake remains unclear.

Previous studies showed that several genes involved in pectin decomposition and D-galacturonic acid catabolism are induced *in vitro* by D-galacturonic acid (Wubben et al., 2000; Zhang et al., 2011). The co-expression pattern of these genes suggests that a central regulatory mechanism is present in *B. cinerea*. The transcript levels of a transcription factor gene *Bcgalar*1 was induced in medium containing D-galacturonic acid as the carbon source. However the function of this genes in D-galacturonic acid utilization remains unclear.

The aims of this study are:

1. To analyse the role of putative D-galacturonic acid transporters and a transcriptional regulator in virulence of *B. cinerea*.

2. To analyse the role of knockout mutants Δ*Bcara*1 and double-knockout mutants Δ*Bcpg*1/Δ*Bcpg*2 in virulence.

3. To study the expression of pectate-induced genes in presence of different monosaccharides by qRT-PCR.

## 2. Materials and methods:

### 2.1. Fungal strains and growth conditions

The *Botrytis cinerea* strains used in this study are listed in Table 1. Strains were grown in Malt extract agar (50g/l) in the dark at 20$^{o}$C for 2 to 3 days.

**Table 1: Different *Botrytis cinerea* strains used in this study.**

| *Botrytis cinerea* strains | |
|---|---|
| Wild type | B05.10 |
| Individual mutant of putative D-galacturonic acid regulator | Δ*Bcgalar*1-1 |
| Individual mutant of putative D-galacturonic acid regulator | Δ*Bcgalar*1-4 |
| Individual mutant of putative D-galacturonic acid regulator | Δ*Bcgalar*1-8 |
| Individual mutant of putative D-galacturonic acid transporter 13 | Δ*Bchxt*13-3 |
| Individual mutant of putative D-galacturonic acid transporter 13 | Δ*Bchxt*13-8 |
| Individual mutant of putative D-galacturonic acid transporter 13 | Δ*Bchxt*13-9 |
| Individual mutant of putative D-galacturonic acid transporter 15 | Δ*Bchxt*15-2 |
| Individual mutant of putative D-galacturonic acid transporter 15 | Δ*Bchxt*15-4 |
| Individual mutant of putative D-galacturonic acid transporter 15 | Δ*Bchxt*15-10 |
| Putative endo-polygalacturonase mutant 1 | Δ*Bcpg*1 |
| Individual mutant of putative endo-polygalacturonase mutant 2 | Δ*Bcpg*2-7A |
| Individual mutant of putative endo-polygalacturonase mutant 2 | Δ*Bcpg*2-9B |
| Individual mutant of putative endo-polygalacturonase mutant 2 | Δ*Bcpg*2-14B |
| Double knockout mutant of putative endo-polygalacturonase 1 and 2 | Δ*Bcpg*1/Δ*Bcpg*2-3 |
| Double knockout mutant of putative endo-polygalacturonase 1and 2 | Δ*Bcpg*1/Δ*Bcpg*2-6 |
| Double knockout mutant of putative endo-polygalacturonase 1 and 2 | Δ*Bcpg*1/Δ*Bcpg*2-7 |
| Individual mutants of putative arabinan mutants from B05.10 strain | B11 |
| Individual mutants of putative arabinan mutants from B05.10 strain | B13 |
| Individual mutants of putative arabinan mutants from IK2018 strain | IK10 |
| Individual mutants of putative arabinan mutants from IK2018 strain | IK12 |
| Individual mutants of putative arabinan mutants from IK2018 strain | IK18 |

## 2.2. Growth assay:

For the growth assays, spores (2ul of $10^6$ conidia/ml) of the strains were inoculated on Gamborg's B5 agarose media supplemented with 10mM $(NH_4)H_2PO_4$ and as carbon source either glucose (50 mM), D-Galacturonic acid (50 mM, 1 mM and 0.1 mM), citrus fruit pectin (1% w/v), sodium pectate (1% w/v), galactose (50 mM), xylose (50 mM), arabinose (50 mM), rhamnose (50 mM). The cultures were grown at 20$^o$C.

For pH buffered assay, two different phosphate buffers (10 mM) were prepared and were set at pH4 and pH5. The spores ($10^7$/ml, 2ul) of the strains were inoculated on Gamborg's B5 agarose media supplemented with these pH optimised medium containing as carbon source 50 mM glucose, 50 mM D-galacturonic acid, citrus fruit pectin (1% w/v), or sodium pectate (1% w/v). The cultures were grown at 20$^o$C.

## 2.3. Measurement of biomass:

The fungal biomass in liquid culture was determined by using a Quickstix$^{TM}$ kit for *B. cinerea* (Enviro-Logix, Portland, Maine; dewey et al, 2008). Liquid cultures with Gamborg's B5 with 10mM $(NH_4)H_2PO_4$ and carbon sources as glucose (50 mM), D-galacturonic acid (50 mM), galactose (50 mM), xylose (50 mM) were set up in three replicates starting with $10^5$ conidia in 20 ml of each medium. The culture filtrate was sampled for the measurement of biomass from 1 to 4 days post culturing. Signal intensity for mutant strains was determined with optical reader and then compared to the wild type strain.

## 2.4. Virulence assay:

The virulence of mutants was evaluated on tomato and *Nicotiana benthamiana* leaves. Droplets of a suspension of conidia of the wild type and mutant strains (2 ul of $10^6$ conidia/ml of 1.2% PDB) were inoculate on opposite sides on the central vein of the leaf (2 droplets for *N. benthamiana* and 3-4 droplets for tomato leaf half). The comparison of the wild type and mutants, on the tomato leaves were performed on four leaflets of one tomato leaf, on two leaves per plant, and two plants per experiment. The lesion size was measured with a calliper on 3 days

post inoculation (dpi). Each mutant was tested in at least two independent experiments. The lesions which did not expand were eliminated from the measurements.

## 2.5. Quantitative RT-PCR

Gene expression was quantified by Quantitative RT-PCR analysis on different carbon sources. The conidia of the wild type strain B05.10 were incubated in Gamborg's B5 liquid culture supplemented with 10mM $(NH_4)H_2PO_4$ and 30mM glucose at 20$^{o}$C, 150 rpm (Zhang et al, 2011). After 16 hours of growth, the mycelium was harvested and transferred to fresh Gamborg's B5 medium supplemented with 10 mM $(NH_4)H_2PO_4$ and 50 mM glucose, 50 mM D-galacturonic acid, 50 mM arabinose, 50 mM rhamnose, 50 mM xylose, 50 mM galactose as carbon source. Mycelium was harvested at 3 hours post transfer and RNA was isolated. cDNA synthesis was then done with Superscript III Reverse Transcriptase (Invitrogen). QRT-PCR was performed using an ABI7300 PCR machine in combination with the qPCR Sensimix kit with gene specific primers which are listed in the table (appendix). The QRT-PCR was run for 40 cycles and under the following conditions: 95$^{o}$C initial denaturation step for 10 minutes followed by 95$^{o}$C denaturation step for 15 seconds and then annealing at 60$^{o}$C for 45 seconds. The data was analysed on the 7300 system SDS software. A constitutively expressed *Bcrpl*5 gene was used to normalize the gene expression of the other genes.

# 3. Results:

## 3.1. Functional analysis of a putative D-galacturonic acid regulator gene *Bcgalar*1:

In RNA sequence analysis, the putative D-galacturonic acid regulator gene showed an induction in pectate and repression in glucose. A qRT-PCR was done to confirm this result and Bcgalar1 was found to give maximum induction in the presence of D-galacturonic acid. Knockout mutants were created and three individual mutants were made. These were then tested on plates (growth assay) and plants (virulence assay). It was thought that the mutants shall give reduced growth of the colony or lesion for the mutants compared to wild type on plates and plants respectively.

### 3.1.1. Growth Assay:

The growth of knockout mutants Δ*Bcgalar*1 was conducted on solid media with various carbon sources: glucose, galacturonic acid, pectate, pectin, xylose, galactose. Δ*Bcgalar*1-1, Δ*Bcgalar*1-4, Δ*Bcgalar*1-8 did not show any significant difference in colony diameter as compared to that of the wild-type strain B05.10 on the carbon sources tested. The growth of all the strains on pectin was faster than on other carbon sources and hence the growth was observed till 4 days post incubation (dpi), whereas the growth of all the strains on xylose and galactose was slow and the growth was observed till 7 dpi (Figure 2).

The knockout mutants of Δ*Bcgalar*1 were grown in a liquid culture with various carbon sources. The mycelium was collected and fungal biomass was measured with the help of Quickstix$^{TM}$ kit for *B. cinerea* (Enviro-Logix, Portland, Maine; dewey et al, 2008). There was no significant difference observed in the biomass of mutants and wild type.

### 3.1.2. Virulence assay:

A virulence assay of the knockout mutants of ΔBcgalar1 was performed by inoculating the tomato and *Nicotiana benthamiana* leaves. The mutants Δ*Bcgalar*1-1, Δ*Bcgalar*1-4, Δ*Bcgalar*1-8 showed the same average lesion size as that of the wild type strain (Figure 3).

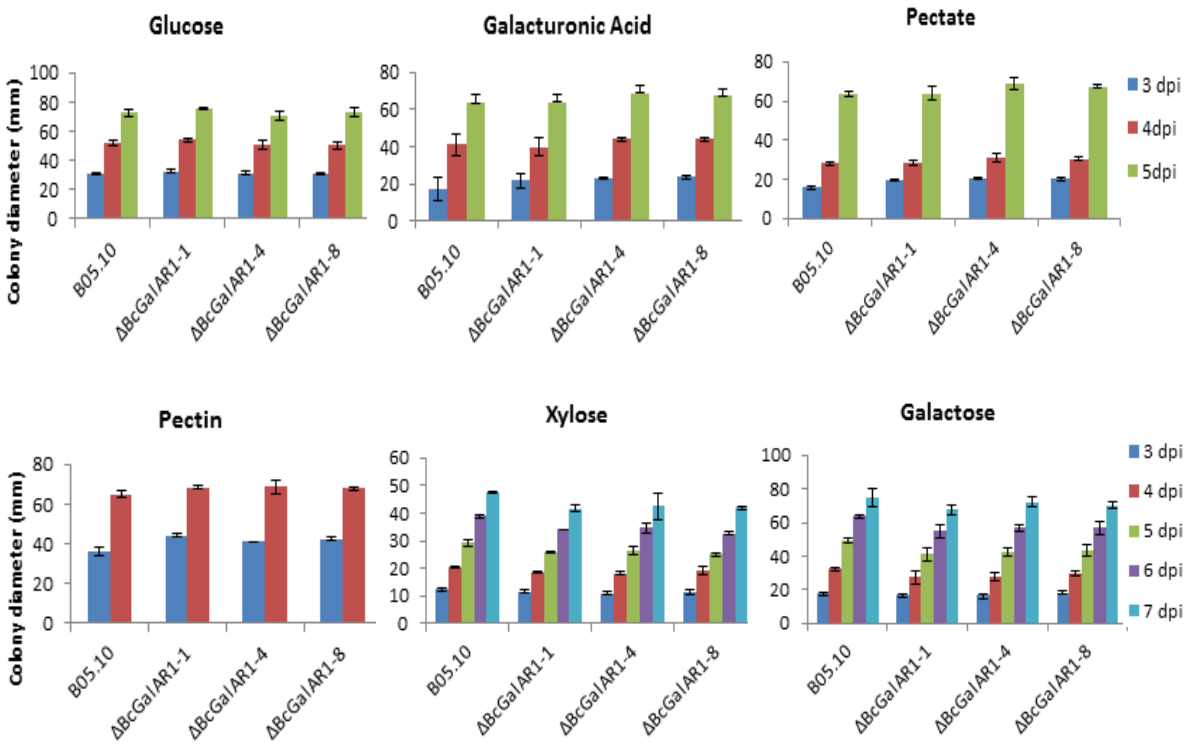


**Figure 2: Colony diameter of the growth of the knock out mutants of Δ*Bcgalar*1 compared to wild type strain B05.10 in the presence of different carbon sources: glucose (A), galcturonic acid (B), pectin (C), pectate (D), arabinose (E) and rhamnose (F). No significant difference was observed between the Δ*Bcgalar*1-1, Δ*Bcgalar*1-4, Δ*Bcgalar*1-8 when compared to the wild type strain B05.10.**

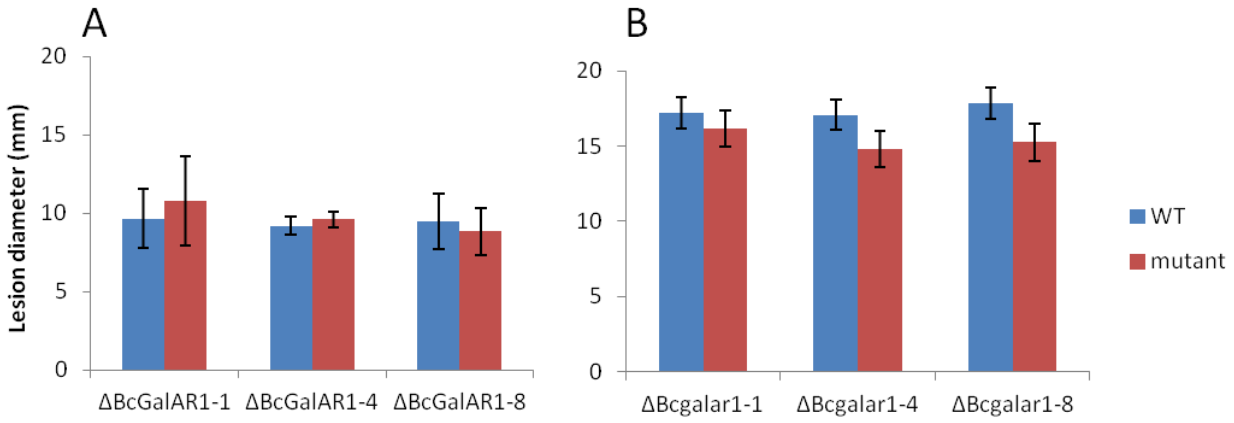


**Figure 3: Lesion diameter of the knock out mutants of Δ*Bcgalar*1 compared to wild type strain B05.10 on *Nicotiana benthamiana* (A) and tomato (B) leaves. No significant difference was observed between the Δ*Bcgalar*1-1, Δ*Bcgalar*1-4, Δ*Bcgalar*1-8 when compared to the wild type strain B05.10.**

## 3.2. Functional analysis of a putative D-galacturonic acid transporter genes *Bchxt*13 and *Bchxt*15:

The putative hexose transporter genes showed an induction in the presence of pectate and repression in glucose, in an RNA sequence analysis. A qRT-PCR was done to confirm this result and *Bchxt*15 and *Bchxt*13 was found to give maximum D-galacturonic acid induction. Knockout mutants were created and three individual mutants were made for each gene. These were then tested on plates (growth assay) and plants (virulence assay). It was thought that the mutants shall give reduced growth of the colony or lesion for the mutants compared to wild type on plates and plants respectively.

### 3.2.1. Growth assay:

The growth of knockout mutants Δ*Bchxt*13 and Δ*Bchxt*15 was conducted on solid media with various carbon sources: glucose, galacturonic acid, pectin, pectate, arabinose, rhamnose, xylose, galactose. Δ*Bchxt*15-2, Δ*Bchxt*15-4, Δ*Bchxt*15-10, Δ*Bchxt*13-3, Δ*Bchxt*13-8, Δ*Bchxt*13-9 did not show any significant difference in colony diameter as compared to the wild type strain B05.10 on D-galacturonic acid, pectin, xylose, rhamnose and arabinose. But on solid media with glucose, pectate and galactose one of the three individual mutants shows significant difference. The growth on the plates were observed from 3 dpi till 4 dpi for pectate and 7 dpi for xylose and galactose, as there was slow growth on the sources (Figure 4).

Thus the liquid assay was performed to check and quantify the fungal biomass of the knockout mutants and wild type with the Quickstix$^{TM}$ kit for *B. cinerea* (Enviro-Logix, Portland, Maine; Dewey et al, 2008). There was no significant difference observed in the biomass of mutants and wild type.

pH plays a very important role in the function of several genes. It was thus assumed that the transporter genes could also behave differently in their action of transporting galacturonic acid, in medium with different pH values. To evaluate this hypothesis, the media was prepared with two different pH (pH 4.0 and pH 5.0). The knockout mutants of *Bchxt*15 were grown on these media, but no significant difference was observed between the wild type and Δ*Bcxht*15-2, Δ*Bcxht*15-4, Δ*Bcxht*15-10 under both the pH concentrations (Appendix 1).

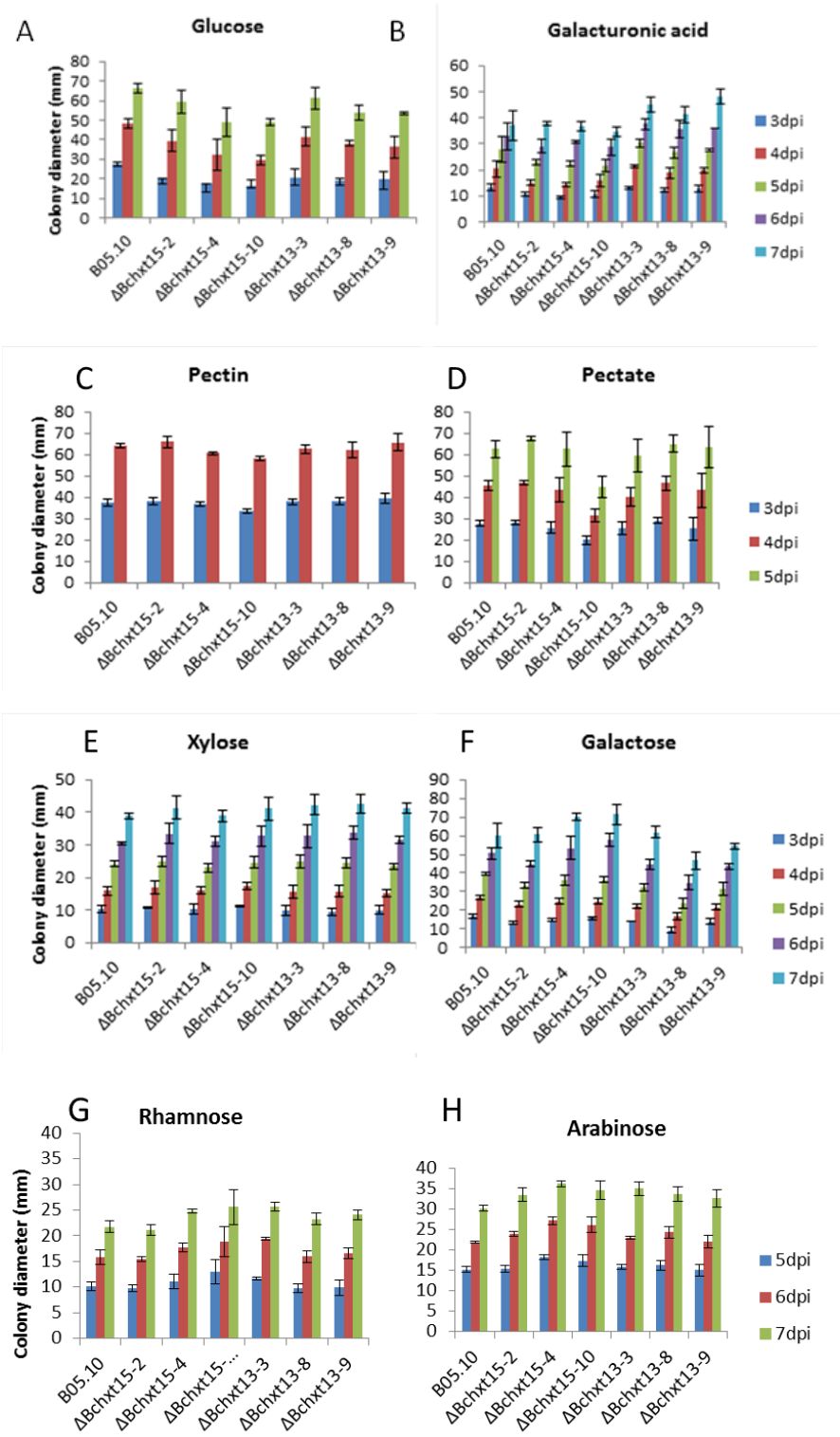


**Figure 4: Colony diameter of the growth of the knock out mutants of Δ*Bchxt*13 and Δ*Bchxt*15 compared to wild type strain B05.10 in the presence of different carbon sources: glucose (A), galacturonic acid (B), pectin (C), pectate (D), arabinose (E), rhamnose (F), xylose (G) and galactose (H). No significant difference was observed between the Δ*Bchxt*15-2, Δ*Bchxt*15-4, Δ*Bchxt*15-10, Δ*Bchxt*13-3, Δ*Bchxt*13-8, Δ*Bchxt*13-9 when compared to the wild type strain B05.10.**

It was hypothised that at high D-galacturonic acid concentration, the affinity of the hexose transporters other than D-galacturonic acid transporter can also take up D-galacturonic acid. But at a lower concentration, the other sugar transporters cannot take up the D-galacturonic acid molecules other than the BcHxt 15 and BcHxt-13 transporters (as these genes show high induction in the presence of D-galacturonic acid molecules seen in RNAseq data). To confirm this, the media was prepared with lower concentration of D-galacturonic acid (1 mM). The growth assay was done with a comparison of B05.10 and transporter mutants. But no significant difference was seen between the wildtype and mutants. The experiment was then repeated with even lower concentration of D-galacturonic acid (0.1mM). The growth of the mycelia on this medium was very poor and also no significant difference was observed (Appendix 2).

### 3.2.2. Virulence assay:

The knockout mutants of Δ*Bchxt*13 and Δ*Bchxt*15 were then tested on tomato and *N. benthamiana* leaves. The assay was done in 2 technical repeats and four biological repeats. The lesion sizes were determined at 3 days post inoculation (dpi). There was no difference observed between the mutants Δ*Bchxt*15-2, Δ*Bchxt*15-4, Δ*Bchxt*15-10, Δ*Bchxt*13-3, Δ*Bchxt*13-8, Δ*Bchxt*13-9 and the wild type.

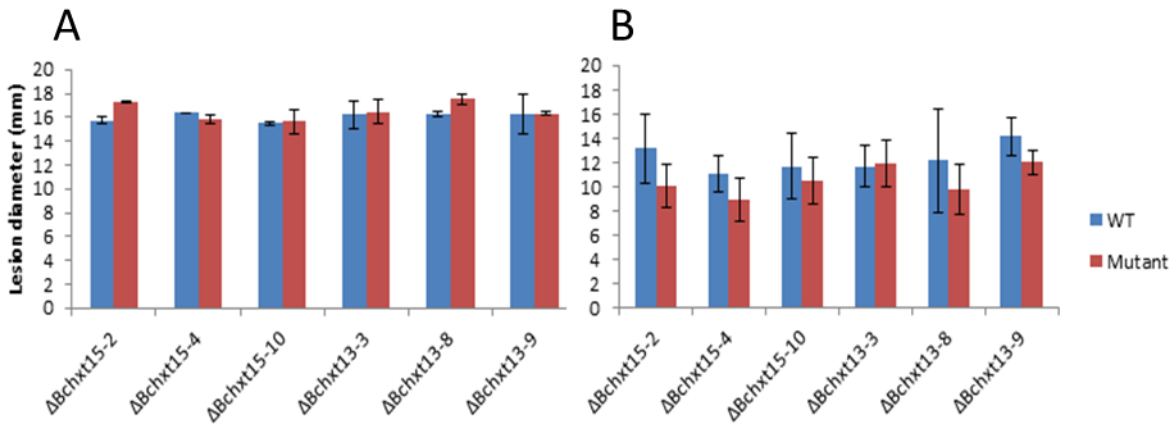


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**Figure 5: Lesion diameter of the growth of the knock out mutants of Δ*Bchxt*15 and Δ*Bchxt*13 compared to wild type strain B05.10 on and tomato (a) and *Nicotiana benthamiana* (b) leaves. No significant difference was observed between the Δ*Bchxt*15-2, Δ*Bchxt*15-4, Δ*Bchxt*15-10, Δ*Bchxt*13-3, Δ*Bchxt*13-8, Δ*Bchxt*13-9 when compared to the wild type strain B05.10.**

## 3.3. Virulence assay of *ΔBcara*1 knockout mutants:

Earlier studies of virulence assay on Arabidopsis were conducted on the arabinanase mutants made from *B. cinerea* strain IK2018 and B05.10. The mutant showed significant reduction in lesion size of the mutants as compared to the wild type strain and so was thought to show difference also in the other plant species.

The studies were conducted on tomato and *N. benthamiana* leaves also. But no significant difference was found in them. Also the two individual mutants were made from the *B. cinerea* B05.10 strain and tested on tomato and *N. benthamiana*. There were no significant differences observed except for the B11 mutant, which showed reduction in the lesion size compared to B05.10 on *N. benthamiana* leaves.

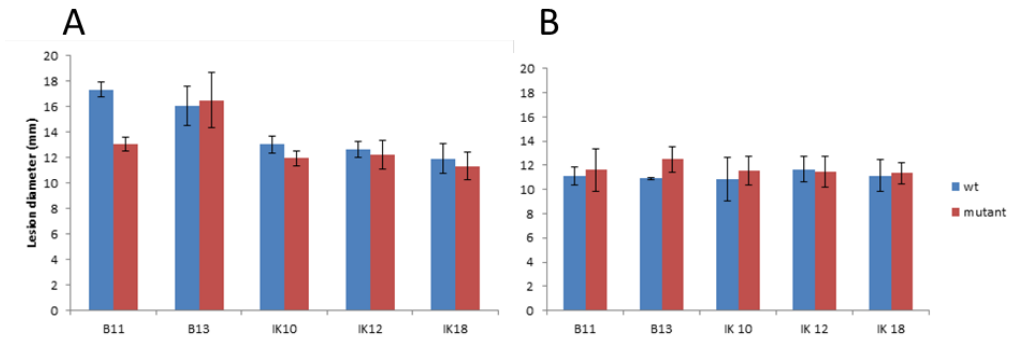



**Figure 6: Lesion diameter of the growth of the knockout mutants of Δ*Bcara*1 compared to wild type strain B05.10 on *Nicotiana benthamiana* (A) and tomato (B) leaves.**

## 3.4. Virulence assay of *ΔBcpg* knockout mutants:

There are many studies conducted on the Δ*Bcpg* mutants before including the growth and also the virulence assay. We know from these that Δ*Bcpg*1 shows reduction in the growth when compared to wild type (Te Have et al, 1998) and (Kars et al, 2005). But complete reduction of the growth on plants was not observed. Three individual mutants of Δ*Bcpg*2 *(*Δ*Bcpg*2-7A, Δ*Bcpg*2-9B, Δ*Bcpg*2-14B), double mutants *(*Δ*Bcpg*1/*Bcpg*2-3, Δ*Bcpg*1/*Bcpg*2-6, Δ*Bcpg*1/*Bcpg*2-7) were tested on the *N. benthamiana* and tomato leaves. It was thought that double mutants *(*Δ*Bcpg*1/*Bcpg*2) can help in the complete reduction of growth of *B. cinerea*. When tested, there was significant reduction in the diameter of the lesion observed but it didn't lead to the total failure of the growth of the lesion.

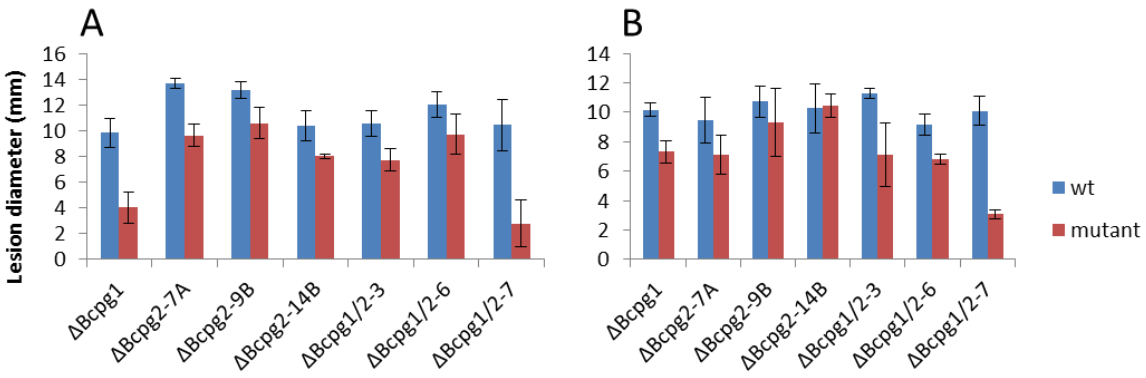



**Figure 7: Lesion diameter of the growth of the knockout mutants of Δ*Bcpg*1, Δ*Bcpg*2 and double mutant Δ*Bcpg*1/*Bcpg*2 compared to wild type strain B05.10 on *Nicotiana benthamiana* (A) and tomato (B) leaves.**

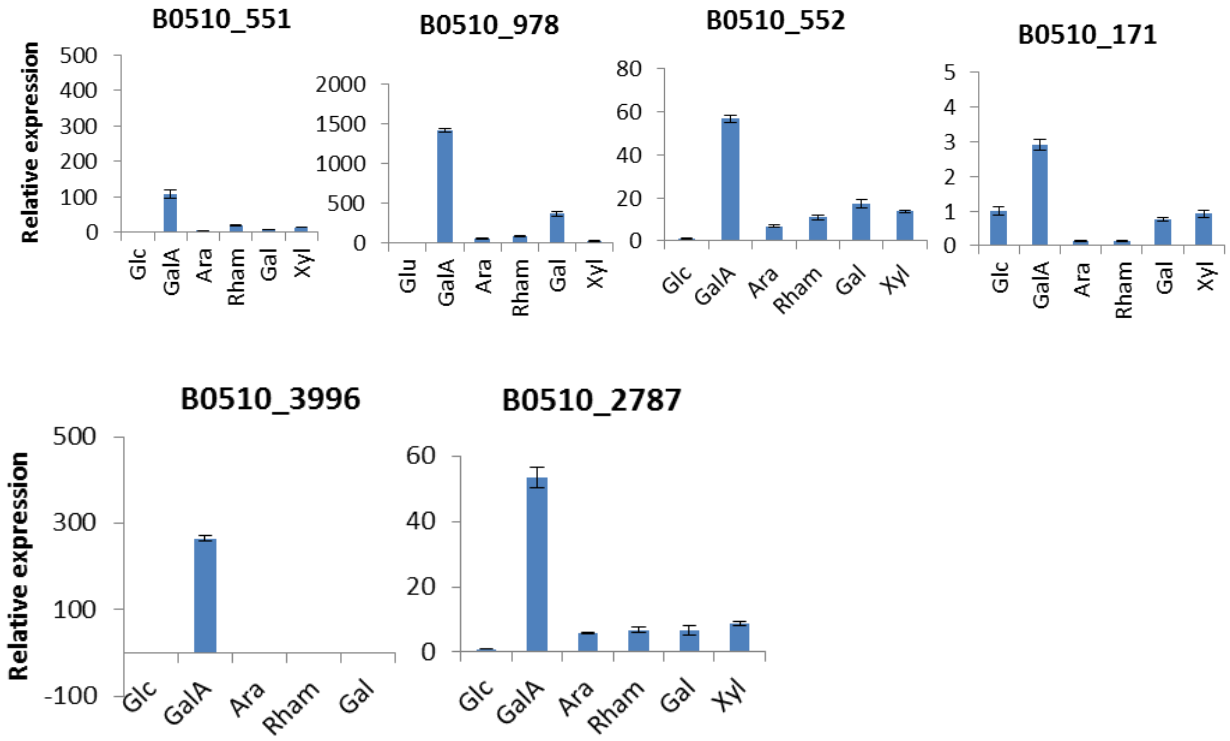
## 3.5. QRT-PCR analysis

RNA sequence analysis of the whole botrytis genome sequence was done in the presence of glucose and polygalacturonic acid (pectate). The result indicated 31 genes which are unregulated by pectate. A qRT-PCR analysis was done to confirm these results with the other two biological repeats of the RNA samples prepared for RNA sequencing with glucose (Glc1, Glc3) and galacturonic acid (PGA1,PGA2) as the template. The qRT-PCR showed almost the same log2 fold change value as that of RNA seq data (Table 2). This data is quite robust as the log2 (fold change) difference is less than 1 between two methods and samples. Only two genes showed difference more than 1, thus RNA seq data is trustworthy. This leads to the further investigation of these genes by running a QPCR in the presence of different templates of rhamnose, arabinose, xylose and galactose along with glucose and galacturonic acid of course. Out of the 31 genes, 6 genes (B0510_4887, B0510_978, B0510_551, B0510_3996, B0510_2787, B0510_171) were found to be induced by D-galacturonic acid specifically (Figure 8A), 11 genes were induced by glucose but not specifically by D-galacturonic acid (Figure 8B) and the remaining 16 genes were not at all induced by D-galacturonic acid, instead they were induced by other C sources (Figure 8C).

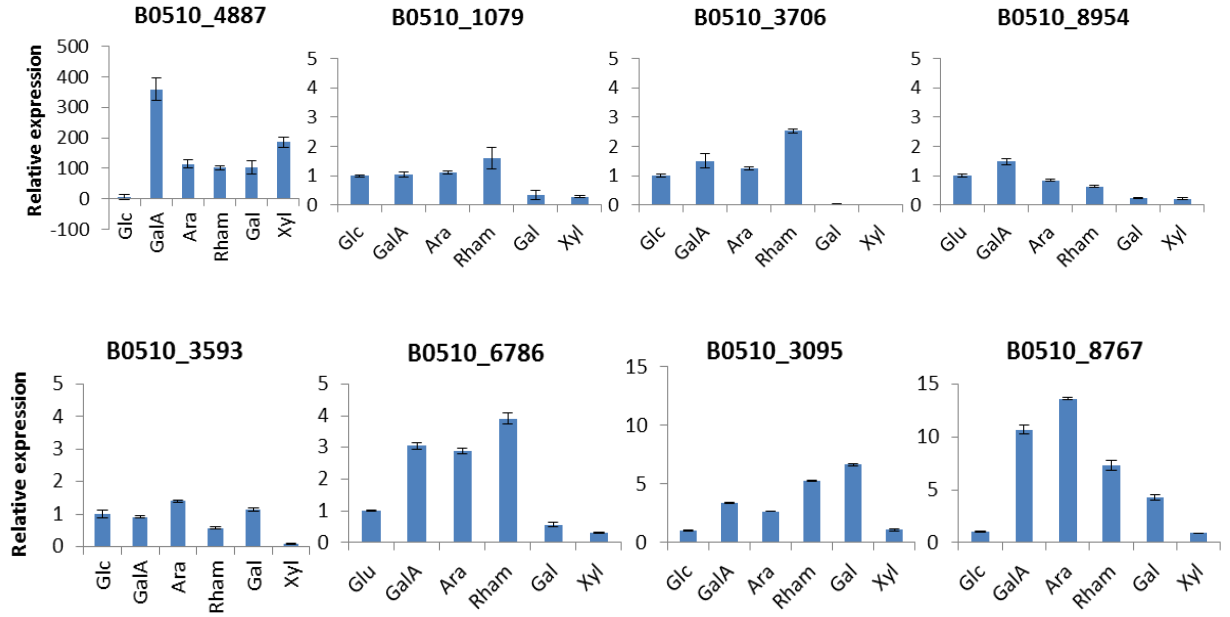
**Table 2: log2 fold change of the polygalacturonic acid and glucose according to both RNA sequence analysis data and q RT-PCR data (average of two biological repeats).**

| | RNA seq data | qPCR data |
|---|---|---|
| Genes | log2(fold change) | log 2 (fold change) |
| B0510_171 | 7.168 | 7.373 |
| B0510_9316 | 6.942 | 3.966 |
| B0510_1685 | 6.113 | 2.897 |
| B0510_4051 | 5.263 | 3.265 |
| B0510_8116 | 5.179 | 4.646 |
| B0510_9368 | 4.785 | 0.281 |
| B0510_4887 | 4.758 | 4.195 |
| B0510_1156 | 4.589 | 3.662 |
| B0510_7189 | 4.432 | 2.470 |
| B0510_552 | 4.421 | 3.945 |
| B0510_551 | 4.270 | 3.942 |
| B0510_3593 | 4.238 | 4.924 |
| B0510_1684 | 4.050 | -2.586 |
| B0510_8767 | 3.973 | 2.543 |
| B0510_3095 | 3.900 | 3.177 |
| B0510_5927 | 3.833 | 4.370 |
| B0510_9071 | 3.756 | 2.880 |
| B0510_8243 | 3.635 | 3.210 |
| B0510_9363 | 3.604 | 4.148 |
| B0510_3901 | 3.499 | 3.437 |
| B0510_3996 | 3.472 | 5.194 |
| B0510_2787 | 3.445 | 3.240 |
| B0510_8492 | 3.405 | 5.227 |
| B0510_7215 | 3.265 | 5.515 |
| B0510_10339 | 3.260 | 3.874 |
| B0510_978 | 3.259 | 2.758 |
| B0510_8954 | 3.205 | 2.188 |
| B0510_6060 | 3.199 | 4.002 |
| B0510_6786 | 3.019 | 3.035 |
| B0510_1079 | 2.947 | 3.554 |
| B0510_3706 | 2.868 | 3.645 |

A:


B0510_551
B0510_978
B0510_552
B0510_171
B0510_3996
B0510_2787
Relative expression
Glc
GalA
Ara
Rham
Gal
Xyl
Glu

B:


B0510_4887
B0510_1079
B0510_3706
B0510_8954
B0510_3593
B0510_6786
B0510_3095
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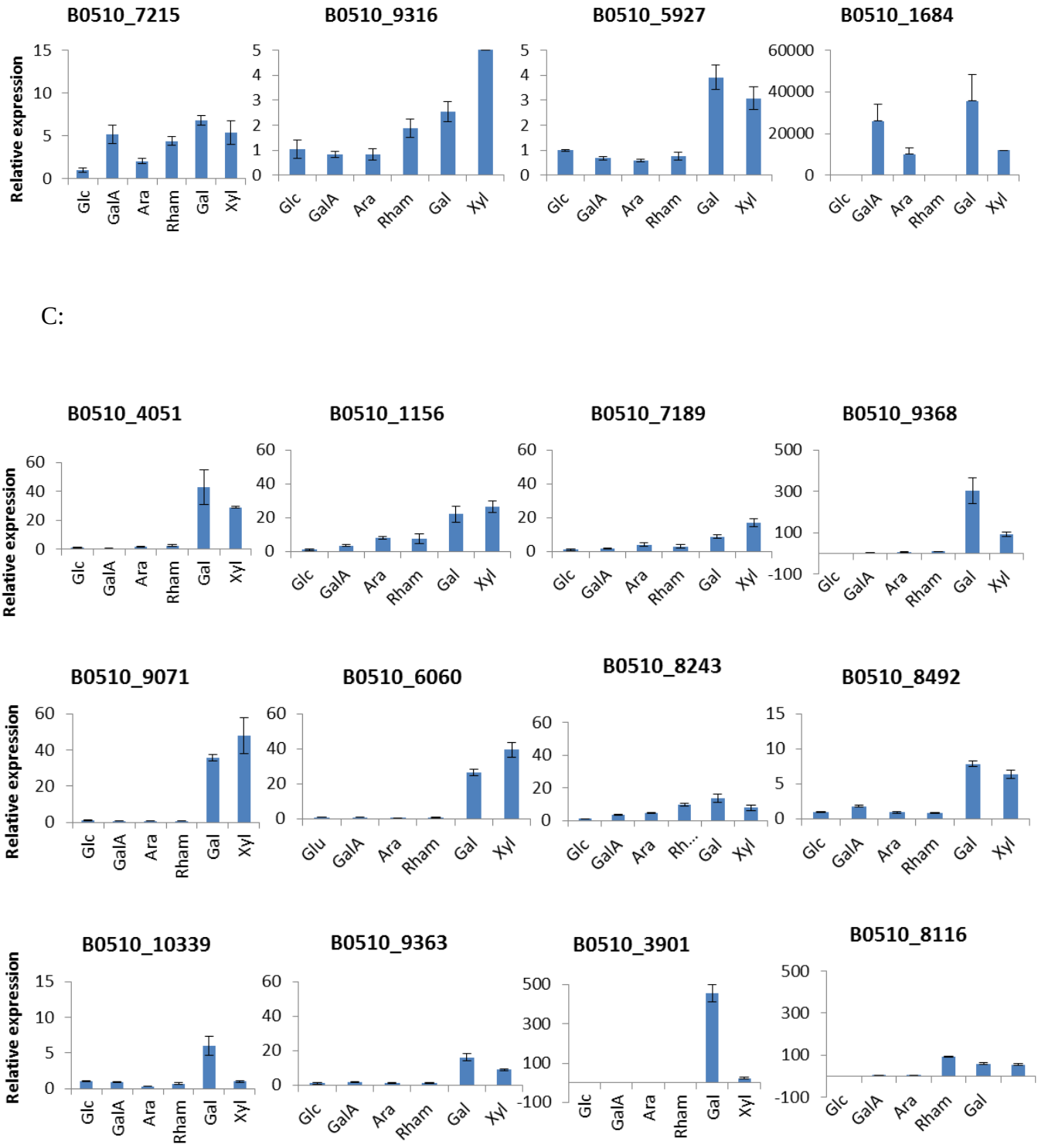
Relative expression
Glc
GalA
Ara
Rham
Gal
Xyl
Glu




C:




**Figure 8: The results of the qRT-PCR analysis with various carbon sources glucose (Glc), D-galacturonic acid (D-galacturonic acid), arabinose (Ara), rhamnose (Rham), galactose (Gal) and xylose (Xyl). (A) represents the genes which are induced specifically by galacturonic acid. (B) represents the genes induced by galacturonic acid but are not specific. (C) represents the genes which are not at all induced by galacturonic acid.**

To confirm the 6 genes specifically induced by D-galacturonic acid, a qRT-PCR was run with two other biological repeats of glucose and D-galacturonic acid as the template. It showed a result equivalent to the previous QPCR run (Figure 9).

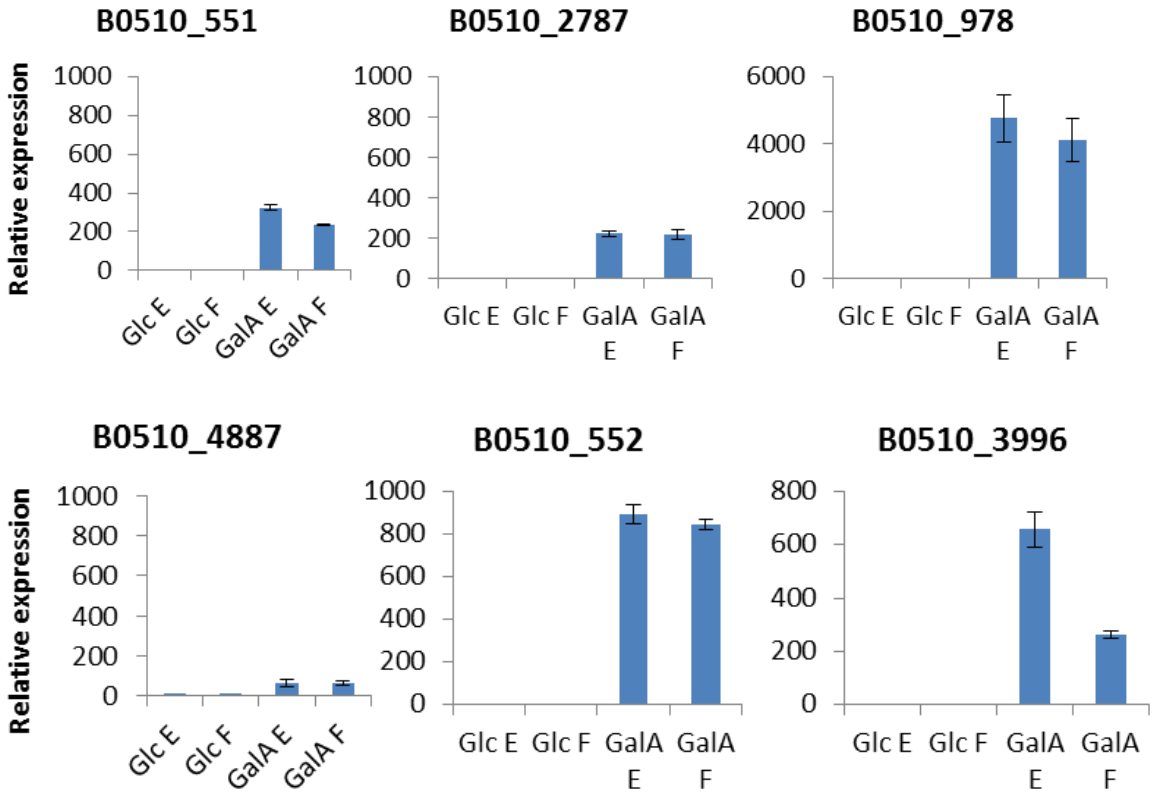



**Figure 9: The results of qRT-PCR analysis done to confirm the 6 specifically induced genes obtained from the first qRT-PCR run.**

## 4. Discussion:

*Botrytis cinerea* can infect more than 200 plant species and different tissue types- leaves, stems, flower, fruit. *B. cinerea* secretes various cell wall degrading enzymes (CWDE) to decompose the cell wall polysaccharides and take up the monomers which are released (Kars et al. 2005). The plant cell wall component, pectin is degraded either by depolymerisation or altering its structure. It includes homogalacturonan (HG), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII), and xylogalacturonan (XGA) (Perez et al.2000). Pectins are degraded by endo-polygalacturonass secreted by *B.cinerea*. Several studies have been conducted before on *B.cinerea* mutants in various *Bcpg* genes. The *Bcpg*1 gene was shown to play an important role in the breakdown of the polygalacturonates. It was seen that knocking out this gene doesn't stop the lesion growth completely. The *Bcpg*2 was also knocked out and was checked for a reduced virulence. When Δ*Bcpg*1 and Δ*Bcpg*2 mutants were tested on the tomato plants, the former showed a reduction in the lesion growth while the three individual mutants of the latter didn't show any difference. When the double mutants were tested, all the three showed reduced growth. This was due to the knockout of the *Bcpg*1 gene as the reduction was the same when compared to the individual *Bcpg*1 knockout.

When the individual mutants and the double mutants were tested on the *N. benthamiana* plants, the Δ*Bcpg*1 showed a 60% decrease in the lesion size. There was significant difference in the individual mutants in the *Bcpg*2 gene too. The double mutants on *N. benthamiana* also showed significant difference in the fungal growth on plants but the reduction in them should be more than 60% as the *Bcpg*1 gene is knocked out along with *Bcpg*2. The mutants Δ*Bcpg*1/*Bcpg*2-3 showed around 30%, Δ*Bcpg*1/*Bcpg*2-6 showed 20% and Δ*Bcpg*1/*Bcpg*2-7 showed 70% reduction.

Arabinan is present as the side chain of the main homogalacturonan polymer, forming a temporary entangled matrix which gives stability to the structure. Studies have been performed previously on the arabinanase mutants made from *B.cinerea* IK2018 strains, on Arabidopsis plants. Significant differences in virulence were observed between the wild type and mutants (IK10, IK12, IK18). These mutants were studied on tomato and *N. benthamiana* plants in this study, but there was no difference observed. Two individual arabinanase mutants were also made from the B05.10 strain and were checked on tomato and *N. benthamiana* plants, but no difference was observed.

After the polygalacturonases breakdown the pectins into monomers of D-galacturonic acid, they are being taken up by the fungus with the help of transporters. Thus the putative hexose transporter genes were tested in vitro on the media plates and also on plants. There was no difference observed in the colony diameter on media plates with D-galacturonic acid, as only carbon source. It was then checked if theses mutants showed reduced colony diameter when the concentration of D-galacturonic acid in the media was lowered to 1mM and 0.1 mM due to the fact that in high concentration of D-galacturonic acid, other sugar transporter can also take up these monomers. But at lower concentration of D-galacturonic acid, only the specific transporter takes up the monomers. The virulence assay was then done and no significant difference was observed. It is hence logical that the leaves which have many other carbon sources along with D-galacturonic acid don't show any significant difference in the lesion diameter as well. The growth and virulence assay of the transporter gene mutants Δ*Bchxt*15 and Δ*Bchxt*13 suggested that these are not the only genes responsible for the transport of the D-galacturonic acid into the fungal hyphae. There may be some other gene(s) responsible which compensate for the loss of action of the *Bchxt*15 and *Bchxt*13 genes.

When RNA sequencing was done on *Bchxt*15 was present in the list of genes induced by D-galacturonic acid along with another putative transporter gene. It may be possible that new putative transporter also contributes to the function. Further research has to be done by creating knockout mutants of this new putative transporter mutant and testing them on plates and plant. Also a double mutant can be made by knocking out *Bchxt*15 and the new gene, followed by further functional assays.

D-galacturonic acid is potentially an important carbon source for fungus living on plant material. Once the D-galacturonic acid monomers enter the fungal cells, they undergo the enzymatic catabolic mechanism. In *B. cinerea*, the BcGAR1 and BcGAR2 contribute to the catabolic pathway (Zhang et al. 2011). For the full consumption of the D-galacturonic acid, continuous action of the appropriate depolymerising enzymes, monomer transporters and catabolic enzymes are required. The genes coding for these enzymes are supposed to be co regulated. The expression of these genes is high during the plant tissue decomposition (lesion expansion). Thus study has been conducted by performing RNA sequencing on botrytis genome and 31 genes were found to be upregulated by polygalacturonic acid. Necessary primers were designed for these genes. The expression profiles of these genes were then validated by determining their mRNA level by qRT-PCR in two cultures containing glucose (Glc1, Glc3) and polygalacturonic acid (PGA). The qRT-PCR showed for most genes (29 out of 31) the same result as that of the

RNA sequencing data of the 31 genes. A qRT-PCR was run but in different cultures containing glucose, galacturonic acid, arabinose, rhamnose, xylose and galactose to observe the expression of the genes in the presence of these carbon sources. Out of the 31 genes, only 6 genes showed to be specifically induced by galacturonic acid, 11 were induced by D-galacturonic acid but not specifically and the remaining 16 were not at all induced by D-galacturonic acid. This implies that all the genes which are induced in the presence of pectate aren't necessarily induced in galacturonic acid. The possible reasons behind this can be the difference in the incubation time of the mycelia in polygalacturonic acid (6 hours) and D-galacturonic acid (3 hours), the pH of the medium with PGA and D-galacturonic acid as carbon sources also differ and can lead to induction of genes in PGA but not in D-galacturonic acid. Further studies have to be carried on by performing a RNA sequence analysis in the presence of D-galacturonic acid. And also by making knockout mutants of these 6 genes and checking the expression by inoculation on plants and solid media plates with D-galacturonic aicd.

It is found that a conserved sequence motif is present in the promoter of several pectinolytic genes of *B.cinerea*. Characterisation of the regulatory elements of D-galacturonic acid was thus carried out. This includes the functional analysis of a putative galacturonic acid regulator. The results from the growth assay of the regulator genes very clearly shows that the regulator mutants don't show any significant difference in the colony diameter, on the plates with D-Galacturonic acid as the C- source between wildtype and mutants. Also in virulence assay no difference was seen on the tomato and *N.benthamiana* leaves. One reason behind it can be that that this gene gets activated only during the breakdown of the pectate and thus shows induction in the presence of it. And it is inactivated while D-galacturonic acid is taken up or is inside the fungal hyphae.

## 5. References: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## Appendix 1:

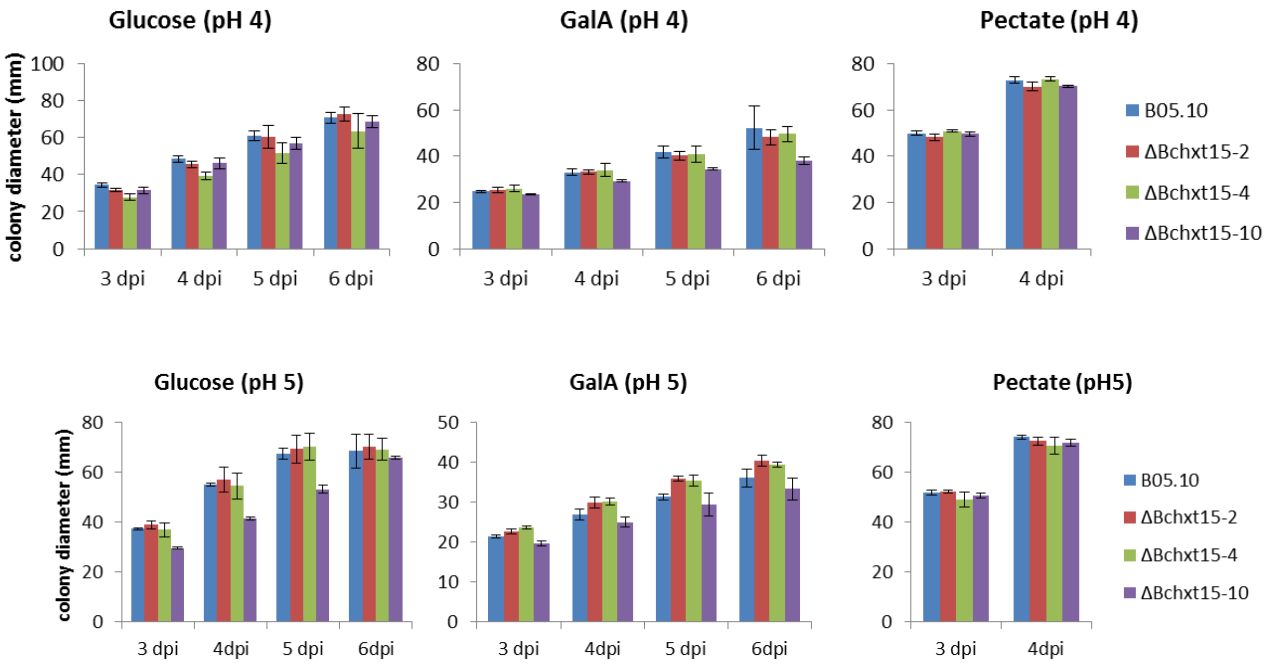


**Figure 10: Colony diameter of the growth of the knock out mutants Δ*Bchxt15* compared to wild type strain B05.10 in the presence of different carbon sources: glucose galcturonic acid, pectate. No significant difference was observed between the Δ*Bchxt*15-2, Δ*Bchxt*15-4, Δ*Bchxt*15-10 when compared to the wild type strain B05.10.**

## Appendix 2:

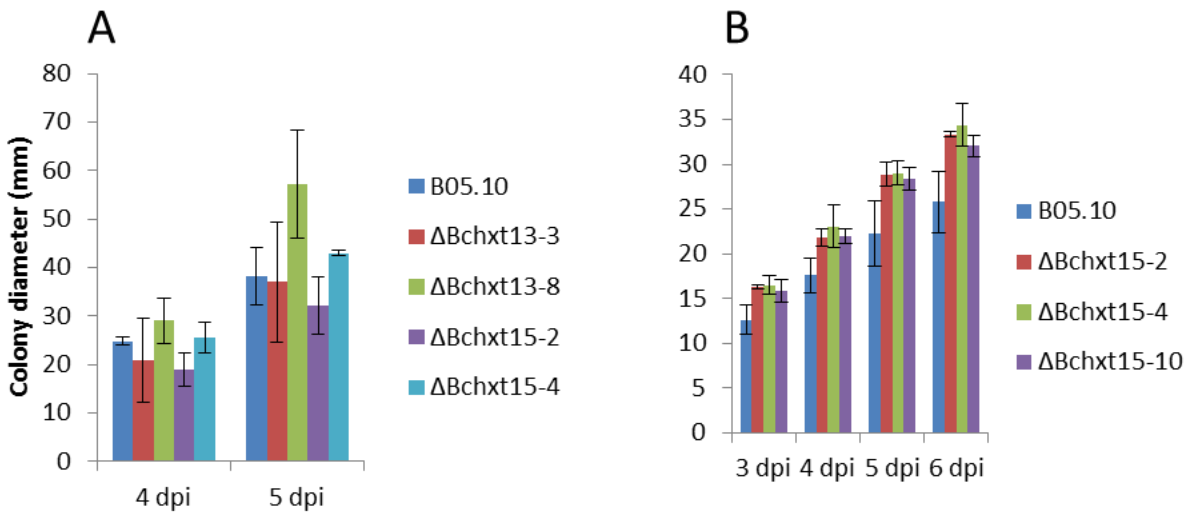


**Figure 11: Colony diameter of the growth of the knock out mutants Δ*Bchxt13* and Δ*Bchxt15* compared to wild type strain B05.10 in the presence of different concentration of D-galacturonic acid of 1 mM (A) and 0.1 mM (B). No significant difference was observed.**

## Appendix 3:

### The primers used for the quantitative RT-PCR.

| Primer name | Sequence |
|---|---|
| QB05.10_1685F | AATCCGGATCACCATAAGTTGG |
| QB05.10_1685R | CACGTTCCAACGAAACGATTGG |
| QB05.10_4051F | GAGATTATGGGAGATTGATCTTC |
| QB05.10_4051R | ACATGAAGATCCCAATTCCAAG |
| QB05.10_9368F | ATGGCTCATCTAGGTGGGATC |
| QB05.10_9368R | ACTTCTTTCAATTTGTCGCCTTG |
| QB05.10_4887F | CAGCGTATAGTCCTTTGGTCAG |
| QB05.10_4887R | TAGCATGGGTTCTCGAAATAGC |
| QB05.10_1156F | GGTCAACTATTCGCCTCCATC |
| QB05.10_1156R | CCATCACAGCAGAAGCCACAC |
| QB05.10_7189F | CTTGCGGTAGTAAACGTCTTTG |
| QB05.10_7189R | CGACAAGGTAGACATTCGAGAG |
| QB05.10_551F | CGACACATCCTTCGATTCCTTC |
| QB05.10_551R | TTACGTTCAATAGCGTAGAGAG |
| QB05.10_3593F | TTCAACTCACGTATCTCCAAGC |
| QB05.10_3593R | AACCAGGTGGTGAAGTAATCGG |
| QB05.10_1684F | TGAAGGATGTCGATATGTGGATG |
| QB05.10_1684R | GTTCCGCAGGGATGGAATCC |
| QB05.10_1684(1)F | TCGCCTCGGGAAATCCATCG |
| QB05.10_1684(1)R | ATCAGCACCCCTTTCAGCAAC |
| QB05.10_8767F | TTCTAAGTTCCTGGATCGGTTC |
| QB05.10_8767R | ACAACGGCAGCTGGGGTTGAC |
| QB05.10_3095F | GTTACGGTGGCCCTCTCATC |
| QB05.10_3095R | GTAGTACTCCTCTGCTGGAAC |
| QB05.10_5927F | TTCAGAAAGAGACTACACTTGCG |
| QB05.10_5927R | TCACCAGTCTTGCCGGTCTTG |
| QB05.10_9071F | GAGTATGGTAAGGTGATTCTTGC |
| QB05.10_9071R | CATATACCCACGGAAAGAAGTC |

| | |
|---|---|
| QB05.10_8243F | CCAGTTGAACCTCTCATCTACC |
| QB05.10_8243R | TCAGTCTCCTTCTCCACCTTG |
| QB05.10_9363F | CATGCACTTCTAGGAGGCATAG |
| QB05.10_9363R | CTCATTCCACTGATACCATGTC |
| QB05.10_3901F | TACCACAACCTACAACAGCAAC |
| QB05.10_3901R | CCATCCATATCAATCATACTTTCC |
| QB05.10_3996F | CGCCTCTACTCAATGGTTGTG |
| QB05.10_3996R | CTTCCAAAGTCTTGCCCTTCG |
| QB05.10_2787F | GCTCATTGCGAGGACTTTCAC |
| QB05.10_2787R | TAATCGTTCCATTCACACAAGTG |
| QB05.10_8492F | CATTGGTGGAAACACCGACTC |
| QB05.10_8492R | AGCTCGAGGTGTTATCATAGTC |
| QB05.10_7215F | TTATGGTGACAAGGCACTCGAC |
| QB05.10_7215R | TCTGCTTCTCATACAATCCTCTG |
| QB05.10_10339F | TGCCACTGGAGATGATTCAAGG |
| QB05.10_10339R | AAAGCGGTGGAGCTAGCGTAG |
| QB05.10_978F | GGCCAAGTGCAGTTTCTACAC |
| QB05.10_978R | AACATGGTTTACGCCTCCGAAG |
| QB05.10_8954F | TCATCTTCTATTGTGGGATCATC |
| QB05.10_8954R | ATCCATAAGCATCCCTTCGAAC |
| QB05.10_6060F | CTGCGGATTATGAACATCACAAG |
| QB05.10_6060R | TTCGTGGCGATCTGCTGCTAC |
| QB05.10_6786F | TACTTTCTGGAACTAGCGCATC |
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| QB05.10_1079F | CAAGGGTCTGGGAGTGTAGAG |
| QB05.10_1079R | GGAGGGGAATAACAACATTGTTC |
| QB05.10_3706F | ATCCTCAAAATCCTAAGCATCAC |
| QB05.10_3706R | TCTCATAACATCATTAACCAGATC |
| QB05.10_171F | TGTTGGTAGCGCAACTGTCAG |
| QB05.10_171R | CATAGTCGGTGTAACATTGCTC |
| QB05.10_9316F | CGAGGATGCTGTATGTTTTGAC |
| QB05.10_9316R | CTCACTCTTCCTCACTCCAAC |
| QB05.10_8116F | GTCTGGGGCTACAACGAATCG |

| | |
|---|---|
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| QBcRPL5 F | GATGAGACCGTCAAATGGTTC |
| QBcRPL5 R | CAGAAGCCCACGTTACGACA |
| QB05.10_551F | CAAGGTTTGGGAATTGTACAGAG |
| QB05.10_551R | GTATCCTCCATATCCATAGTAGC |