Characterization of the *Bc01G03900-Bc01G03910* gene cluster and its bi-directional promoter, and identification of the Cys_6-like transcription factor as a candidate element binding the GAE1 motif present in the *Bcgar2-Bclga1* gene cluster of the galacturonic acid catabolic pathway

Pablo R. Vargas R.

M.Sc. Thesis

PHP-80436

Characterization of the *Bc01G03900-Bc01G03910* gene cluster and its bi-directional promoter, and identification of the Cys_6-like transcription factor as a candidate element binding the GAE1 motif present in the *Bcgar2-Bclga1* gene cluster of the galacturonic acid catabolic pathway

M.Sc. thesis

Submitted to the Laboratory of Phytopathology of Wageningen University as a requirement for the degree of

MASTER OF SCIENCE in PLANT SCIENCES

With the specialisation

PLANT PATHOLOGY AND PLANT BREEDING

By

Pablo Roberto Vargas Ribera

November 2014

Title

Characterization of the *Bc01G03900-Bc01G03910* gene cluster and its bi-directional promoter, and identification of the Cys_6-like transcription factor as a candidate element binding the GAE1 motif present in the *Bcgar2-Bclga1* gene cluster of the galacturonic acid catabolic pathway

Keywords

protoplast transformation, deletion construct analysis, yeast-one hybrid

Institute

Wageningen University and Research Centre (WUR)

Author

Pablo Roberto Vargas Ribera

pablo.vargasribera@wur.nl

Reg. Nr. 890905852070

Subject code

PHP-80436

Instructor

Dr. J.A.L. van Kan

Examiners

Dr. J.A.L. van Kan

Index

Declaration

The work presented in this thesis has been carried out in the Laboratory of Phytopathology, Wageningen University between April 2014 and September 2014, under the supervision of J.A.L. van Kan. The work was done by the author except when indicated otherwise in the text. This thesis has not been submitted for any other degree at any other university.

Pablo R. Vargas R.

November 2014

Acknowledgement

I want to thank dr. J.A.L. van Kan for providing me the opportunity of conducting this thesis work in the Laboratory of Phytopathology in Wageningen University. I want to thank him as well for the constant supervision, attention, regular recommendation on my work, and especially for pushing me to the limit of my knowledge.

I want to thank to all the people working in the Laboratory of Phytopathology for helping and supporting me whenever necessary with advice and guidance.

Summary

Characterization of Bc01G03900-Bc01G03910 *gene cluster*. Effector proteins encoded by pathogens attacking plants are believed to modulate and facilitate host infection. Effectors share common characteristics that distinguish them from other proteins: small size (<400 amino acids in length), presence of signal peptide (tag for secretion), and the presence of cysteine residues. By making use of genome annotations and RNA-sequencing, the identification of effector proteins has become more straightforward. In this research, the *Bc01G03900-Bc01G03910* gene cluster has been identified as a candidate effector protein. The characterization of the *Bc01G03900-Bc01G03910* gene cluster was assessed by creating knock-out mutants. Furthermore, the bi-directional promoter shared by the *Bc01G03900-Bc01G03910* gene cluster was analysed by creating deletion constructs fused to a *gfp* reporter gene.

Identification of transcription factor binding GAE1 motif. Transcription factors (TFs) orchestrate the complete set of genes in a cell's nucleus. They are responsible of what, where, and when genes are expressed. TF bind to specific sequences in the DNA dubbed transcription factor-binding sites (TFBSs). TFs and their respective TFBSs may act simply as switches activated by distinct signals like changes in external temperature or presence/absence of a certain nutrient. The gray mould fungus, *Botrytis cinerea*, contains a set of genes (*Bcgar1*, *Bcgar2*, *Bclgd1*, and *Bclga1*) that are highly expressed in presence of D-galacturonic acid, one of the main constituents of pectin present in the plant's cell wall. The *Bcgar2* and *Bclga1* genes are located in a gene cluster sharing a bi-directional promoter containing the conserved motif GAE1. In this research, a candidate Cys_6-like TF binding to the GAE1 motif present in said bi-directional promoter was found via the yeast-one hybrid (Y1H) approach.

1. Introduction

1.1. Plant immune system

Plants are capable of converting radiant energy (sunglight) into chemical energy (carbohydrates) via photosynthesis, becoming rich sources of nutrients and water. As a result, plants are constantly under attack from microbes including bacteria, fungi, and oomycetes. Since plants are nonmobile organisms, unlike animals, they rely on two layers of defense to protect themselves from microbial pathogens: preformed defenses and innate immune system. Preformed defenses include the cell wall and wax cuticle, which act as physical barriers to microbial colonization. On the other hand, the plant innate immune system is a set of concerted actions that can be represented as a "zig-zag model" comprising four phases: PAMP-triggered immunity (PTI), effector-triggered susceptibility (ETS), effector-triggered immunity, and diversification via natural selection (Jones and Dangl, 2006). In the first phase, molecules characteristic of many pathogens known as pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) in the plant, producing PAMP-triggered immunity (PTI) and arresting subsequent colonization (Jones and Dangle, 2006; Boller and Felix, 2009; Newman *et al*., 2013). In the second phase, more adapted pathogens utilize special molecules known as effectors that contribute to pathogen virulence. Effectors are capable of interfering with PTI, producing effector-triggered susceptibility (ETS), and thus allowing the pathogen to infect the plant. In the third phase, a given effector is recognized by nucleotide binding-leucine rich repeat proteins (NB-LRR) encoded by plant resistance (*R*) genes. This recognition produces effector-triggered immunity (ETI). ETI results in disease resistance and is mostly accompanied by a hypersensitive cell death response (HR) at the infection site (Katagiri and Tsuda, 2010; Newman *et al*., 2013). The fourth phase implies natural selection directing pathogens to avoid ETI either by changing the recognized effector gene or by obtaining new effectors in order to hamper ETI. Natural selection as well drives the plant to produce new R (resistance) proteins in order to keep ETI active (Jones and Dangl, 2006).

1.2. Botrytis effectors

The ascomycete *Botrytis cinerea* Pers. Fr. (teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of the gray mould disease affecting more than 200 plant species, worldwide. *B. cinerea* is an airborne plant pathogen with a necrotrophic lifestyle that causes mainly soft rotting of all aerial plant parts of vegetables and fruits. The pathogenicity of this fungus is based on the production of a range of cell wall-degrading enzymes (including pectin methyl-esterases, endo-polygalacturonases, and endo-β-1,4-xylanases), secretion of toxins (e.g.: oxalic acid, botrydial), and the initiation of a host-induced oxidative burst and programmed cell death (Choquer *et al*., 2007; Williamson *et al*., 2007). A number of necrotrophic fungi, including *B. cinerea*, secrete effector proteins that interact with the host in a gene-for-gene relationship to trigger disease, although

in an inverse manner compared to biotrophs (Oliver and Solomon, 2010). The inverse gene-for-gene relationship in necrotrophs refers to the fact that cell death promotes virulence rather than resistance, which is the case with (hemi)biotrophs when infecting plants (Coll *et al*., 2011; Govrin and Levine, 2000).

The infection process of *B. cinerea* is usually described in four stages: penetration of the host surface, killing of host tissue and primary lesion formation, lesion expansion and tissue maceration, and sporulation. *B. cinerea* is equipped with several cell wall-degrading enzymes (CWDEs) that allow plant tissue colonization and the subsequent release of carbohydrates, such as pectins, for consumption (Choquer *et al*., 2007). Pectin is a complex of polysaccharides present abundantly in the plant cell wall and is defined by the presence of galacturonic acid. Pectic polysaccharides include galacturonans (homogalacturonan, substituted galacturonans, and rhamnogalacturonan-II) and rhamnogalacturonan-I. Homogalacturonan is a polymer of D-galacturonic acid that accounts for more than 60% of pectins in the plant cell wall and is the final product released from pectin degradation by *B. cinerea* (Caffall and Mohnen, 2009). The D-galacturonic acid catabolic pathway has been genetically characterized in *B. cinerea* implicating four genes: *Bcgar1*, *Bcgar2*, *Bclgd1*, and *Bclga1*. Co-expression of these genes is important for D-galacturonic acid utilization by *B. cinerea*, suggesting the presence of a central regulatory mechanism (Zhang *et al*., 2011). The conserved motif GAE1 was found in the promoter region of the bi-directional gene cluster *Bcgar2-Bclga1*. This motif is likewise present in the promoter of all eight genes specifically induced by D-galacturonic acid and in several pectinolytic genes in *B. cinerea* (Zhang 2013). Moreover, the same motif is present in the promoters of several co-expressed pectinolytic genes of *Aspergillus niger*, posting GAE1 as a candidate conserved element implicated in pectin degradation and D-galacturonic acid utilization (Martens-Uzunova and Schaap, 2008).

1.3. Botrytis transformation

Functional analysis of candidate genes involved in pathogenicity can be assessed with relatively high efficiency through the creation of knock-out mutants by targeted gene inactivation. *B. cinerea* knock-out mutants can be generated using one or more of the selection systems available including resistance cassettes for phleomycin, hygromycin, nourseothricin and glufosinate. Using linear transformation cassettes yields high homologous recombination rates (70-100%). As a result, the use of linear transformation cassettes is a standard procedure for gene replacement using long, 500-1000 base pairs (bp) flanking sequences homologous to the intended integration site. Linear transformation cassettes are commonly obtained by Polymerase Chain Reaction (PCR). The strain B05.10 is frequently used as the recipient strain when creating knock-out mutants because it is highly virulent on several host plants and is genetically stable. Moreover, said strain has been used for genome sequencing,

allowing to retrieve information about gene sequence, structure, location, and surrounding (Williamson *et al*., 2007).

Additionally, gene expression profiles can be assessed using GFP reporter gene constructs especially tailored for Botrytis transformation (Schumacher, 2012). For integration of reporter gene constructs, the nitrate reductase system targeting the non-essential *niaD* locus, has been used successfully. Homologous integration is monitored by PCR or by antibiotic resistance of the transformants (Schumacher 2012).

1.4. Transcription factors (TFs) and transcription factor-binding sites (TFBSs)

Regulatory regions of DNA, located relatively near the transcription start site (TSS), control the transcription of each gene in a cell. These regulatory regions may act simply as switches activated by single signals like changes in external temperature or depletion of a certain nutrient (Alberts *et al*., 2008; Weake and Workman 2010). These genetic "switches" are composed of two elements: short stretches of DNA composed of 6-12 bp long degenerate sequences known as transcription factor-binding sites (TFBSs) and gene regulatory proteins that recognize and bind to them known as transcription factors (TFs; Alberts *et al*., 2008).

Regions upstream of genes contain two types of regulatory elements: sequences found in most genes involved in the basic process of transcription (constitutive promoters) and sequences only found in genes transcribed in a particular tissue or in response to a specific signal (inducible promoters). A sequence involved in the basic process of transcription is the TATA box, which is an AT rich sequence (consensus TATAA/TAA/T) found about 30 bp upstream the TSS in most genes. The region comprised between the TATA box and the TSS has been defined as the gene promoter or core promoter. Moreover, the CCAAT box and the Sp1 box (GC rich sequence) are typically found upstream of the TATA box and are known as upstream promoter elements (Latchman 2008). Conversely, among the sequences only found in genes transcribed in response to a specific signal are the promoter of the HSP70 gene in *Drosophila melanogaster*, the thyroid hormone receptor, and the glucocorticoid receptor. The promoter of the hsp70 gene in *D. melanogaster* is a heat-inducible promoter binding, among others, the heat-shock transcription factor (HSF) and allows cell development at 37 °C. The HSF is considered an activator TF since it turns "on" a specific gene (Wang and Lindquist, 1998). The thyroid hormone response element binding the thyroid hormone receptor represses and inhibits transcription of the thyroid hormone in humans. The thyroid hormone response element is considered a repressor TF since it turns "off" a specific gene (Latchman 2008).

Transcription factors promote transcription in eukaryotes by establishing and stabilizing the multiprotein preinitiation complex (PIC), consisting of RNA polymerase II and the general transcription factors (GTFs) TFIII, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Warren 2002). Prior to the establishment of the PIC, TFs

commonly induce structural changes in target DNA, such as chromatin remodelling, to expose specific DNA sequences so that these can be targeted by transcription factors and let the PIC be established (Latchman 2008; Weake and Workman 2010). Chromatin remodelling also prevents inhibitory effect of histones and nucleosomes on transcription initiation (Latchman 2008, Spitz and Furlong 2012).

Although there is a vast diversity of TFs recognizing specific DNA sequences, many of them contain either one or another of a small set of DNA-binding structural motifs. In order to bind to complementary DNA sequences, these motifs generally use either α-helices or β-sheets and the most commonly known are: Helix-Turn-Helix, Zinc fingers, β-sheets, and Leucine zippers. The Helix-Turn-Helix (HTH) motif is one of the simplest and most common DNA-binding motifs; it consists of two alpha helices connected by a short extended chain of amino acids, which constitutes the turn. HTH motifs bind as symmetric dimers, are solely composed of amino acids, and use the α-helix to recognize the respective DNA binding sequence. Zinc finger motifs include one or more zinc atoms as structural components. There are two main types of Zinc finger TFs: in the first type the zinc atom holds an α-helix and a β-sheet together and it is mostly found in tandem clusters; in the second type two α-helices are packed together with zinc atoms and it is mostly found as dimers. Zinc finger motifs as well use the α-helix to recognize the respective DNA binding sequence. The β-sheet motifs are composed of two-stranded β-sheets with protruding amino acid side chains, and use β-helix to recognize the respective DNA binding sequence. Leucine zipper motifs are composed of two α-helices joined together forming a short coiled-coil and use α-helix to recognize the matching binding sequence (Warren 2002; Alberts *et al*, 2008;).

Transcription factors are responsible for the coordinated transcription of specific genes in response to particular signals, including those involved in nutrient perception. Depending on the availability of nutrients, organisms transcribe a set of genes that allow them to develop under certain environmental efficiently. A thoroughly studied case is the regulation of *GAL* genes by Gal4p in *S. cerevisiae* in order to catalyse the conversion of galactose into glucose. When yeast cells are grown in the absence of galactose, the *GAL* genes are largely not transcribed. However, if galactose is the only available carbon source, then the *GAL* genes are activated. Conversely, the presence of glucose will trigger the repression *GAL* genes and its transcriptional regulator, the Zn_2Cys_6 transcription factor Gal4p (Campbell *et al*., 2008; Weake and Workman, 2010).

1.5. TFs in fungi

Researchers have encountered taxon-specific TFs that are found only in bacteria, plants and animals (Yoshiaki *et al*., 2005; Yamasaki *et al*., 2013; Wang and Zhang, 2009), however little is known about TFs specific for fungi and their relatedness to TFs in other eukaryotes. The number of experimentally proven TFs in fungi is lower than in other eukaryotes. This can be due to two possible

explanations: either fungal TFs are less abundant since fungi are less complex organisms or they have not yet been identified (Amoutzias et al., 2007; Shelest 2008). A set of 12 domain superfamilies, retrieved from the DBD-Superfamily database, has been predicted to occur in fungi. Similarly a set of 37 PFAM domains, retrieved from the DBD-PFAM database, have been predicted to occur in fungal species. The main difference between these two database is that the latter is hand-curated. Three domain superfamilies and three PFAM families of TFs are fungal-specific, meaning that they are present only in fungi and are not found in another kingdom (Shelest, 2008).

The three domain superfamilies of fungal-specific TFs that were found in the DBD-Superfamily database are: Zn_2/Cys_6 (Zn cluster), MBP1-like DNA-binding domain, and Zinc domain conserved in yeast copper-regulated TFs. The largest class of fungal-specific superfamily domain is the Zn_2/Cys_6 (Zn cluster) superfamily. Its DNA-binding domain consists of six cysteine residues that bind two zinc atoms. This type of TFs can interact with DNA as monomers or as homo- or heterodimers. The zinc binuclear cluster is capable of regulating several cellular processes: sugar and amino acid metabolism, respiration, vitamin synthesis, cell cycle, chromatin remodelling, nitrogen utilization, and drug resistance. The two other fungal-specific domain superfamilies present less functions: DNA-binding domain of Mlu1-box-binding protein MBP1 (MBP1-like) being involved in cell cycle and Zinc domain conserved in yeast copper-regulated TFs being involved in copper utilization and stress response (Shelest 2008; Todd *et al*., 2014).

The three domain families of fungal-specific TFs that were found using the DBD-PFAM database contain the following domains: fungal-specific transcription factor domain, APSES, and MATα1. The largest class of fungal-specific domains in this group is the fungal-specific transcription factor domain. Experimentally, this domain has always been found located downstream of the zinc-cluster domain and it regulates several cellular functions: sugar and amino acid metabolism, respiration, fatty acid catabolism, and nitrate assimilation. The two other fungal-specific domain families present less functions: APSES being involved in developmental complexity, yeast-hyphal transitions, and cell cycle; and MATα1 being involved in the activation of genes specific to mating type a (Shelest 2008; Todd *et al*., 2014).

1.6. TFs in *B. cinerea*

The complete genome of *B. cinerea* has been sequenced and subsequent gene annotation has identified 392 and 410 TF-encoding genes for strains T4 and B05.10, respectively. Overall, 96 TFs are *B. cinerea*-specific and have no counterpart in *Sclerotinia sclerotiorum* nor orthologs in other fungal genomes (Amselem *et al*., 2011). *Simon et al*. (2013) have found, via Y1H library screening, a Cys_2His_2 Zn finger TF, dubbed BcYOH1, which is involved in regulating the expression of the botrydial biosynthesis gene cluster (*BcBOT1-BcBOT5*). Schumacher *et al*. (2008) have proven, by heterologous expression in

yeast (*Saccharomyces cerevisae*), the activity of BcCRZ1 as a TF acting downstream of *BCG1* and calcineurin in *B. cinerea*. Schumacher *et al*. (2014) identified, via random mutagenesis, a virulence-related gene encoding a GATA transcription factor, dubbed BcLTF1 for light-responsive TF1. Schumacher *et al*. (2014) confirmed the predicted role of the BcLTF1 TF in virulence by deletion and over-expression analysis; moreover, they discovered its functions in regulation of light-dependent differentiation, the equilibrium between production and scavenging of reactive oxygen species (ROS), and secondary metabolism.

1.7. Aim of this research

The *Bc01G03900-BcG03910* gene cluster is located in chromosome 1 of the *B. cinerea* genome and comprises roughly 2000 bp encoding for three proteins: Bc01G03900.1 with 87 amino acid residues, Bc01G03910.1 with 66 amino acid residues, and Bc01G03910.2 with 116 amino acid residues (Fig. 1). Each of the three encoded proteins contains a signal peptide according to SingalP 4.0 (Petersen *et al*., 2011). According to RNA-sequencing data (van Kan, unpublished) this cluster is highly expressed *in planta* whereas *in vitro* shows negligible expression. The *Bc01G03900-Bc01G03910* gene cluster is considered a candidate effector based on its small size (<400 amino acids in length), presence of signal peptide (tag for secretion), and the presence of cysteine residues. The promoter region of this gene cluster acts in both directions, thus considered a bidirectional promoter, and is composed of 646 bp. Bidirectional promoters are defined as those regulating adjacent genes that are organized in a divergent fashion (head to head orientation) and separated by <1 kb. Most of the time these co-expressed genes function in a common metabolic pathway (Dhadi *et al*., 2013).

The aim of this research was to assess the importance of the gene cluster of interest by creating knock-out mutants using hygromycin B as selectable marker. Additionally, various promoter deletion constructs fused to a *GFP* reporter gene were created to identify putative TFBS present in the promoter shared by the gene cluster of interest. Reporter activity was monitored in *N. benthamiana* plants.

Concurrently 44 positive colonies retrieved from a library of *B. cinerea* TFs library (INRA, France) was surveyed by PCR and subsequent sequencing to find candidate TFs regulating the galacturonic acid catabolic pathway. The positive colonies were retrieved from a *B. cinerea* TFs library containing >60000 yeast transformants screened against GAE1 element by yeast-one hybrid (Y1H) assay.

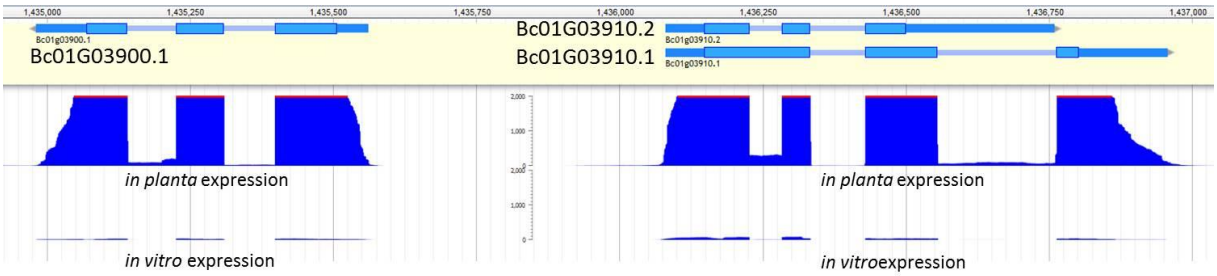


Figure 1. *Bc01G03900-Bc01G03910* gene cluster. Depiction of the *Bc01G03900-Bc01G03910* gene cluster showing exons, introns, direction of transcription, and relative expression *in planta* and *in vitro*.

2. MATERIALS AND METHODS

2.1. Fungal strain and growth conditions

Botrytis cinerea strain B05.10 (van Kan *et al*., 1997), *B. cinerea* strain PoliC::GFP (Schumacher, 2012) and mutant strains were grown and maintained on malt extract agar (Oxoid, Basingstoke, UK) at 20 °C. For liquid cultures conidia were harvested from 10-day old plates and used to inoculate 1% ME liquid culture (Wubben *et al*., 2000).

2.2. Gene replacement strategy to obtain Knock-out mutants

Gene replacement strategy. The amplification of two-target gene fragments was performed with primers (5.1 and 5.3 or 3.1 and 3.3; Table 4, Appendix 1) chosen just outside the sequence of interest with an extension complementary to 22 or 24 base pairs (bp) of the selection marker cassette, respectively. The amplification was carried out in a reaction volume of 25 µL using 1 U Taq DNA polymerase (Promega, Fitchburg, WI, USA) with appropriate PCR buffer, 0.4 µM of each primer, 0.4 mM of dNTP mix and ~32 ng DNA. The PCR conditions were as follows: heating at 95 °C for 3 min; 30 cycles of 94 °C for 1 min, 54 °C for 30 sec and 72 °C for 2 min; followed by a final extension at 72 °C for 5 min.

One type of selection marker cassette was used in the gene replacement procedure: a cassette carrying the *hph* cassette (2.7 kb) for resistance to hygromycin B. The resistance gene was flanked by the *Aspergillus nidulans* oliC promoter and the *Botrytis cinerea* β-tubuline terminator. The *hph* cassette was carried by the plasmid pLOB7 (Zhang 2011). The selection marker cassette was amplified using primers 20 and 21 (Table 4; Appendix 1). The amplification was carried out in a reaction volume of 25 µL using 1 U of Taq polymerase (Promega, Fitchburg, WI, USA) with appropriate PCR buffer, 0.4 µM of each primer, 0.4 mM of dNTP mix and 50-100 ng plasmid DNA. The PCR conditions were: heating at 95 °C for 2 min; ten cycles of 95 °C for 15 s, 50 °C for 45 s and 72 °C for 3 min; 20 cycles of 95 °C for 15 s, 50 °C for 45 s and 72 °C for 3 min increasing at 5 s per cycle; followed by a final extension at 72° for 5 min.

Overlap extension PCR was performed to fuse the two target-gene fragments to the selection marker cassette in a single PCR amplification step using primers 5.2 and 3.2 (0.4 µM each; Table 4, Appendix 1). The amplification was carried in a reaction volume of 25 µL with 0.4 mM of DNTP mixture, 2.25 U Expand High Fidelity Polymerase (Roche, Basel, Switzerland) and the appropriate PCR buffer. For gene fusion to the hygromycin cassette, equal amounts (~20 ng each) of the three templates were used. The PCR conditions for the amplification of the *hph* construct were: an initial cycle of 94 °C for 5 min, 58 °C for 2 min and 72 °C for 8 min; 30 cycles of 92 °C for 30 s, an increase of 1.2 °C to 58 °C, 58 °C for 2 min, an increase of 0.1 °C/s to 72 °C, 72 °C for 4 min, and an increase of 0.2 °C/s to 92 °C; followed by a final extension at 72 °C for 8 min. Due to low yielding of the desired construct, further ligation in a pGEMT Easy-vector (Promega, Fitchburg, WI, USA) was performed to augment the yield. The ligation

was carried in a reaction volume of 20 µL with 50 ng of pGEMT Easy-vector, ~55 ng of construct, 3 U of T4 DNA ligase, and the appropriate ligation buffer. For optimal ligation, the construct was treated before with at 72 °C for 5 min in a volume of 10 µL with 1 mM of dATP, ~30 ng of construct, 1 U of Taq DNA Polymerase (Promega, Fitchburg, WI, USA), with appropriate buffer. The ligation product was then transformed into *Escherichia coli* TOP10 competent cells (Life Technologies – Thermo Fisher Scientific, Waltham, MA, USA). Colony PCR was performed in selected colonies to check for successful ligation of the construct of interest. Plasmids were isolated from *E. coli* using the QIAprep Spin miniprep kit (QIAGEN, Hilden, Germany) and subsequently checked with primers 5.2-5.3 and 3.2-3.3. The construct of interest was afterwards amplified by PCR in a reaction volume of 25 µL using 1 U Taq DNA polymerase (Promega, Fitchburg, WI, USA) with appropriate PCR buffer, 0.4 µM of each primer (5.2 and 3.2), 0.4 mM of dNTP mix and ~50 ng DNA. The PCR conditions were as follows: heating at 95 °C for 3 min; 30 cycles of 94 °C for 1 min, 54 °C for 30 sec and 72 °C for 2 min; followed by a final extension at 72 °C for 5 min. DNA was precipitated and resuspended in KC buffer for subsequent transformation procedure.

The amplified constructs were purified either from PCR mixture or gel using Nucleospin Gel and PCR clean-up kit (Macherey-Nigel, Düren, Germany) according to the manufacturer's conditions.

2.3. Promoter deletion mutants

For the study of the regulation of the bidirectional promoter of the putative effector *Bc01G03900-Bc03G03910* gene cluster in *B. cinerea*, a yeast recombination-based cloning vector pNDH-GFP was used (Schumacher, 2012; Fig. 4, Appendix 2). The full-length promoter region of *Bc01G03900-Bc03G03910* gene cluster and six promoter deletions were amplified from genomic DNA using primers listed in Table 4 (Appendix 1). The full-length promoter region was inserted in forward orientation upstream the *GFP* reporter gene (FLF) and in reverse orientation upstream the *GFP* reporter gene (FLR). Three deletions were done with the promoter in forward orientation upstream the *GFP* reporter gene (viz. Del1F, Del2F, Del3F) and three deletions were made with the promoter in reverse orientation upstream the *GFP* reporter gene (viz. Del1R, Del2R, Del3R; Fig. 2). The amplified promoter regions, along with pNDH-GFP linearized with NcoI, were co-transformed into the uracil-auxotrophic yeast strain FY834 (Winston *et al*., 1995), as described by Schumacher (2012), and plated in synthetic defined (SD) –ura media (Clontech, Mountain View, CA, USA). The transformants were confirmed by PCR using primers LZ182/183 and plasmids were rescued with Zymoprep yeast plasmid miniprep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Plasmids were subsequently transformed into electro-competent *Escherichia coli* DH5α cells, further isolated using QIAprep Spin miniprep kit (QIAGEN, Hilden, Germany) and subsequently checked with primers LZ182/183. Positive constructs were double digested with enzymes SacII and ApaI. Digestion was checked visually in a 1%

agarose gel. DNA was precipitated and resuspended in KC buffer for subsequent transformation procedure.

The amplified constructs were purified either from PCR mixture or gel using Nucleospin Gel and PCR clean-up kit (Macherey-Nigel, Düren, Germany) according to the manufacturer's conditions.

2.4. Botrytis cinerea transformation.

The preparation of protoplasts and its subsequent transformation were carried out according to Hamada *et al*. (1994) and ten Have *et al*. (1998) with slight modifications.

Preparation of protoplasts. *Botrytis cinerea* strain B05.10 was used for transformation experiments. B05.10 was grown on potato dextrose agar (Oxoid, Basingstoke, UK) at 20 °C. Overnight treatment of the fungus with near-UV light induced conidiation. Mature conidia were harvested with water using a glass-wool filter, 1 ml of 1 x 10^8 conidia was inoculated in 0.5 L of 1% ME liquid culture (1% malt extract, Difco Laboratories, Detroit, MI, USA) and incubated for 2 h at RT. From this incubation, four 125 ml aliquots were poured in round-bottom flasks and incubated for 16 hours at 20 °C and 180 rpm shaking. Mycelium was harvested by filtration using 22.4 µm filter and washed twice with 1 V of KC buffer (0.6 M KCl, 50 mM CaCl2). After filtration of mycelium, conidia were suspended in 20 ml of KC buffer and 20 ml of Trichoderma lysing enzyme solution (200 mg of enzyme in 20 ml of KC). The suspension was incubated for 2 h at RT with shaking at 80 rpm. The digested suspension was filtered first over 20 µm filter. The filtrate suspension was collected in 100 ml Erlenmeyer flasks. Protoplasts were pelleted by centrifugation at 1200 rpm for 10 min and subsequently re-suspended in 5 ml KC buffer first and then 35 ml KC were added to get a final volume of 40 ml protoplast solution. Protoplasts were counted and re-suspended at 10^7 protoplasts per 100 µl and kept on ice.

Protoplast transformation. A suspension of 100 µl containing 10^7 protoplasts was kept on ice for 5 min. Ten micrograms of linear DNA in 95 µl of KC buffer mixed with 5 µl of 50 mM spermidine and 100 µl of 25% PEG 3540 solution (with 50 mM Cacl2 and 10 mM Tris-HCl, pH 7.5) were gently added to the protoplasts. The mixture was incubated 20 min at RT. Five-hundred microliters 25% PEG was added additionally and incubated 10 min at RT. Finally the volume was adjusted to 1 ml by adding 200 µl KC buffer. The whole (1 ml) transformation solution was poured into 100 ml flask of SH agar [0.6 M sucrose, 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2ethanesulfonic acid), 1 mM $(NH_4)H_2PO_4$ and 1.2% agar (w/v)], plated in 10 ml aliquots and incubated for 15 h at 20 °C to allow mycelium regeneration. An overlay of 10 ml SH agar containing 100 µg/ml of hygromycin B (Duchefa, Haarlem, the Netherlands) was applied on regenerating protoplasts in order to select transformants. Hyphal tips of hygromycin B resistant colonies were transferred to MEA (Oxoid) plates containing 100 µg/ml

of hygromycin B. Germlings from single conidia were isolated and transferred to MEA plates.

Genomic DNA was isolated from knock-out mutants for PCR screening using DNeasy kit (QIAGEN, Hilden, Germany). Primers 22, 23, 5.1, and 3.1 were used in different combinations in order to assess ectopic or targeted transformation.

2.5. Plant infection assay and GFP screening

Detached leaves of 5-6 week-old tomato (*Solanum lycopersicum*) and attached leaves of 5 week-old *Nicotiana benthamiana* plants were inoculated with *B. cinerea*. Droplets of a suspension of conidia of 10 day-old wild-type (WT) and promoter deletion mutants (Del1F, Del3F, Del1R, Del3R; 2 ul, 10^6 conidia/ml in potato dextrose broth) were inoculated on opposite sides of the central vein (for *S. lycopersicum* 3-4 droplets per leaf half, for *N. benthamiana* 1-3 droplets per leaf). Each comparison of WT and mutant was performed on 4 leaflets of one composite tomato leaf; or 3-4 leaves per *N. benthamiana* plant, and 2 plants per mutant. Samples were visually scored 2 days post-inoculation using a Blak-Ray B-100AP high intensity UV lamp (365 nm; UltraViolet Prodcuts, Upland, CA, USA).

Three leaves of 5 week-old *Nicotiana benthamiana* plants were inoculated with 10-day-old *B. cinerea* WT and knock out mutant cultures. Droplets of WT (2 µl, 10^6 conidia/ml in potato dextrose broth) and agar plugs of knock-out mutant were inoculated on opposite sides of the central vein. Each comparison of WT and knock-out mutant was performed on 3 leaves per *N. benthamiana* plant, on 2 plants.

2.6. Total protein extraction and Western Blotting

Selected samples from *N. benthamiana* and tomato leaves(FL, Del1F, B05.10, PoliC::GFP) were frozen in liquid nitrogen, freeze-dried, and ground to get a fine powder. Extraction buffer (250-500 µl) and proteinase inhibitor (3 µl) were added to each sample. Samples were spun at 5000 RPM for 20 min. Supernatant was transferred to clean 1.5 ml Eppendorf tubes and stored at -20°C until use.

Samples were run in a 12% SDS gel at 110 V for 2.5 hours and a Precision Plus Protein was used as a marker (Bio-Rad, Veenendaal, Netherlands). 50 µl of each sample were loaded with appropriate loading buffer (1:1). Gel was blotted onto a PVDF membrane and run for at 20 V overnight. Blotting was blocked by adding PBS-tween + 5% milk powder for 1 h at RT. Blot was treated with 1:5000 agfp-hrp (anti-gfp horse radish peroxide) in 5 ml PBS-Tween + 5% milk powder for 2 h at RT. Blot was developed with 150 µl femto substrate in Chemidoc.

2.7. TFs library screening

Selected colonies from a *B. cinerea* transcription factors (TFs) library in yeast were received from Adéline Simon (INRA) and re-plated in SD –ura agar medium. Each colony was inoculated in 3 ml of SD –ura broth and incubated overnight at 28 °C. Subsequent plasmid isolation was performed using Zymoprep yeast plasmid miniprep kit (Zymo Research, Irvine, CA, USA) according to the

manufacturer's instructions. A PCR was performed to check for the size of insert present in each yeast construct. The amplification was carried in a reaction volume of 25 uL using 1 U Taq DNA polymerase (Promega, Fitchburg, WI, USA) with appropriate PCR buffer, 0.4 uM of each primer, 0.4 mM of dNTP mix and ~32 ng DNA. The PCR conditions were as follows: heating at 95 °C for 3 min; 10 cycles of 94 °C for 1 min, 52 °C for 30 sec and 72 °C for 2 min; 20 cycles of 94 °C for 1 min, 65 °C for 30 sec and 72 °C for 2 min; followed by a final extension at 72 °C for 5 min. Primers used are listed in Table 4. Subsequently, a restriction digestion with HaeIII was performed in order to check the sequence similarity or dissimilarity amongst the 44 yeast constructs. The digestion was carried out in a reaction volume of 20 uL using 10 U HaeIII (Promega, Madison, WI, USA) with appropriate digestion buffer and ~150 ng PCR product for 15 min at 37 °C. Samples were grouped according to the digestion pattern and one representative of each group was subsequently amplified, purified, and sent for sequencing to MACROGEN (Amsterdam, the Netherlands). Sequencing results were blasted against the *Botrytis cinerea* T4 genome using the blastx program.

The amplified constructs were purified either from PCR mixture using Nucleospin Gel and PCR clean-up kit (Macherey-Nigel, Düren, Germany) according to the manufacturer's conditions.

3. Results

3.1. Bc01G03900-910 gene cluster KO mutants.

To determine the importance of the *Bc01G03900-Bc01G03910* gene cluster in virulence in *B. cinerea*, knock-out mutants were created by replacing the coding region of the gene cluster by the hygromycin phosphotransferase resistance gene (*hph*) in B. cinerea WT strain B05.10 background. One transformant was inoculated in *N. benthamiana* plants and *S. lycopersicum* detached leaves and showed no difference in virulence compared to the WT strain (data not shown).

3.2. Bi-directional promoter deletion mutants

To determine the promoter region contributing to the high *in planta* induction of the *Bc01G03900-Bc01G03910* gene cluster, the full-length (FL) promoter region (in the sense orientation, in the direction of transcription of the *Bc01G03910* gene) was fused with a codon-optimised *GFP* gene. A second construct was prepared in which the promoter region was fused in reverse orientation (in the direction of transcription of the *Bc01G03900* gene) with the codon-optimised *GFP* gene.

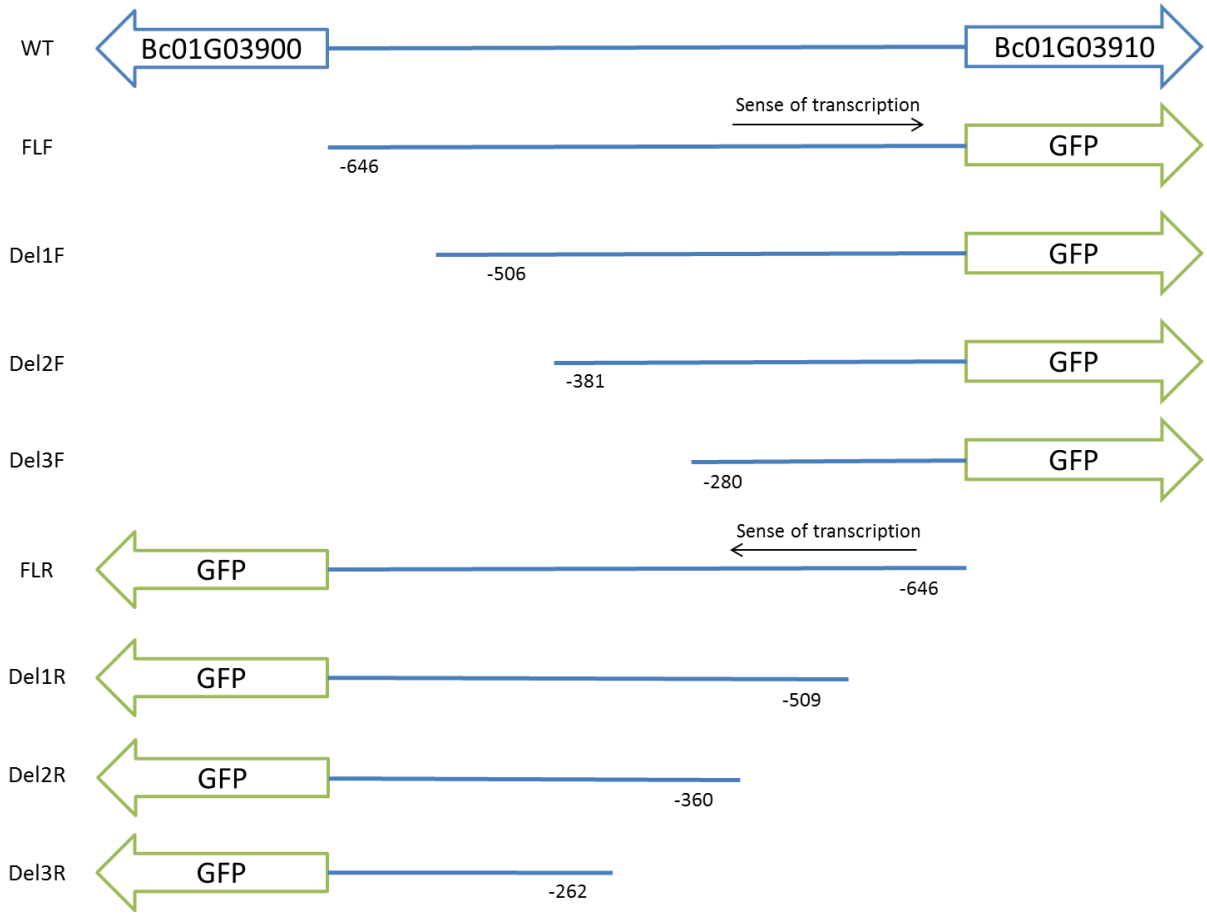


Figure 2. Promoter deletion constructs. A set of unidirectional (for Bc01G03910 expression) promoter fragments with various deletions was fused to *GFP* reporter gene and transformed into B. cinerea WT strain. WT: wild type. FLF: full length promoter in forward orientation. Del1F: deletion 1 in forward orientation. Del2F: deletion 2 in forward orientation. Del3: deletion 3 in forward

orientation. FLR: full length promoter in reverse orientation. Del1R: deletion 1 in reverse orientation. Del2R: deletion 2 in reverse orientation. Del3R: deletion 3 in reverse orientation

A set of promoter deletion constructs was generated in which different lengths of sequence were removed in the forward (Del1F-Del3F) and reverse (Del1R-Del3R) orientation (Fig. 1). The FL and deletion constructs were transformed into WT *B. cinerea* strain B05.10. The number of transformants obtained per promoter deletion construct is shown in Table 1. Plant infection assays were performed with three FLF and two Del1F transformants and surveyed for GFP expression with Blak-Ray B-100AP high intensity UV lamp. Non-GFP-expressing WT strain B05.10 was used as a negative control and the constitutive GFP-expressing strain PoliC::GFP (Schumacher, 2012) was used as a positive control. Neither the samples nor the controls showed fluorescence. The total protein extraction and subsequent Western Blotting of samples FL1, FLF3, FLF4, Del1F1, Del1F4, B05.10 WT (negative control), and PoliC::GFP (positive control) did not show valid results. The expected band of ~25 kD pertaining to the GFP was detected only from sample FLF1 for total protein extraction from *N. benthamiana* (Fig. 3A) and tomato (Fig. 3B) leaves. No band was detected from the positive control.

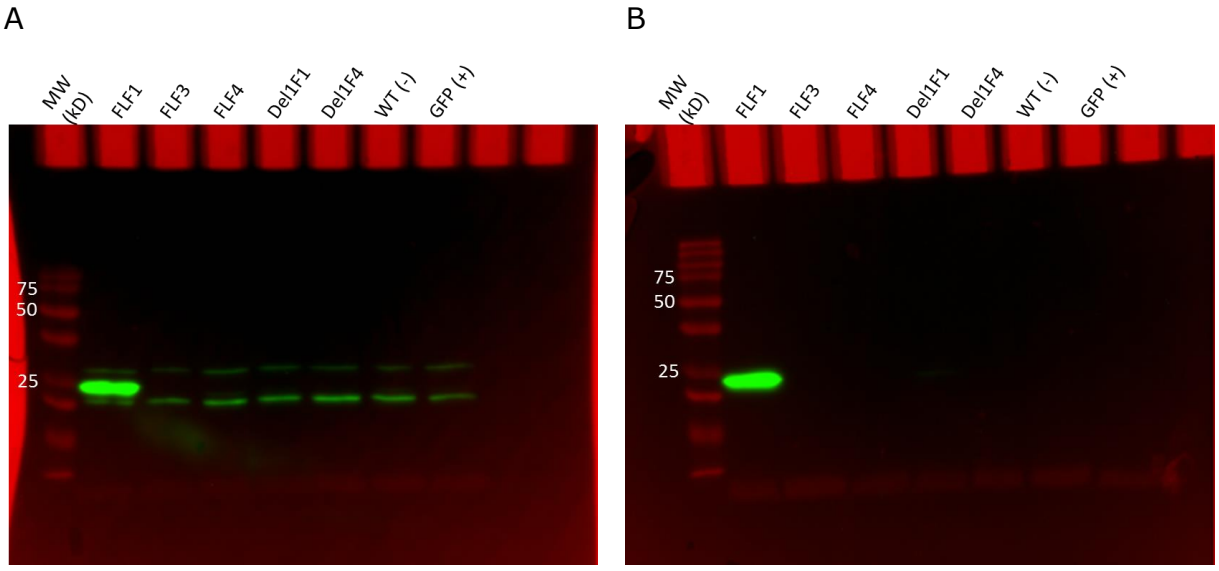


Figure 3. Western Blot analysis of promoter deletion constructs. Total protein extraction from *N. benthamiana* (A) and tomato (B) leaves of samples FLF1, FLF3, FLF4, Del1F1, Del1F4, WT (-), and GFP::PoliC (+) run in a 12% SDS gel and blotted into a PVDF membrane. After blocking, blot was treated with agfp-hrp (anti-gfp horse radish peroxide) and afterwards developed with 150 µl femto substrate in Chemidoc.

Table 1. Number of *B. cinerea* transformants obtained per construct.

Construct ID	Number of transformants
FLF	4
Del1F	4
Del2F	2
Del3F	3
FLR	9
Del1R	1
Del2R	1
Del3R	3

3.3. A Cys_6-like TF binds to the GAE1 motif present in the bi-directional promoter of Bcgar2-Bclga1 gene cluster

The *Bcgar2* and *Bclga1* genes are located next to each other, in opposite orientations, and are separated by a shared 1744 bp promoter region. The promoter region shared by this gene cluster contains a conserved GAE1 motif, eight CreA binding motifs, one PacC binding motif, and three other "unspecified" motifs (Zhang 2013). The CreA binding motif has the consensus sequence 5'-SYGGRG-3' and binds a Zinc finger TF involved in the negative regulation and repression of carbon catabolism in *Aspergillus nidulans* (Cubero and Scazzocchio, 1994). The PacC motif has the consensus sequence 5'-GCCARG-3' and binds a Zinc finger TF repressing transcription of acid-expressed genes and activating transcription of alkaline-expressed genes (Tilburn *et al*, 1995). The GAE1 motif present in the promoter region of the *Bcgar2-Bcgal1*, showing the consensus motif sequence 5'-CCNCCAA-3', is considered to be a key regulator of this gene cluster (Zhang 2013).

The GAE1 motif was amplified and inserted upstream from the *HIS3* reporter gene in pINT1-HIS3NB vector for a yeast-one hybrid (Y1H) screening. The screening was performed against a plasmid library containing 393 *B. cinerea* TFs (binding domain) inserted downstream a Gal4p activation domain. A screening of 60 000 colonies of the library resulted in the isolation of 44 colonies, expressing TFs that permitted expression of the selectable marker gene under control of the GAE1 motif (Simon *et al*., data not shown). The 44 yeast constructs containing the putative TFs binding GAE1 were further amplified by PCR and digested with HaeIII. Seven patterns were recognized and one representative sample of each pattern was sequenced. After blasting the sequences using the blastx program against the *B. cinerea* T4 genome, three gene IDs were identified and selected out of the seven samples: Bofu_T4P042280.1, BofuT4_P046390.1, and BofuT4_P100290.1. The gene IDs were selected based on their high score, low E value, and high percentage of identity (Altschul *et al*. 1997; Tables 5 and 6, Appendix 2). BofuT4_P042280.1 encodes a protein similar to transcription factor Cys6 and is considered a false positive in this research because it has appeared in other screenings unrelated to the galacturonic acid catabolic pathway. BofuT4_P046390.1, dubbed BcYOH1, encodes a protein similar to transcription factor Zinc C2H2and binds to the *BcBot1-2* promoter related to secondary

metabolite synthesis (Simon *et al*. 2013) and is considered a false positive in this research as well. BofuT4_P100290.1 encodes a protein similar to transcription factor Cys_6 composed of 842 amino acid residues, which has not been reported yet and is considered a relevant positive result in this research. BofuT4_P100290.1 contains two relevant protein domains: Zn2/Cys6 fungal-type DNA-binding domain (Interpro) or GAL4-like Zn2/Cys6 (C6 zinc) binuclear cluster DNA-binding domain (SMART) and fungal-specific transcription factor domain. Moreover, BofuT4_P100290.1 contains cysteine residues, making it a good candidate for a TF binding the GAE1 motif.

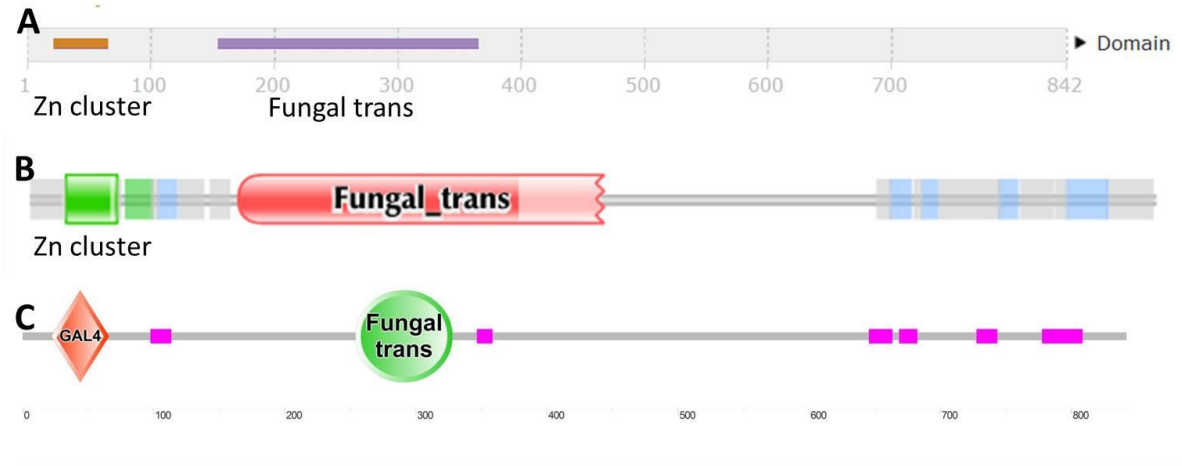


Figure 4. Relevant BofuT4_P100290.1 protein domains. Relevant protein domains in the BofuT4_P100290.1 gene according to InterPro (A), Pfam protein domain database (B), and SMART (C).

4. Discussion

Research on transcriptional networks in phytopathogenic fungi is not widely conducted currently. Trustworthy information regarding promoter consensus sequences and structure is needed in order to better interpret genome-wide expression data and matching transcription factors correctly (Basse and Farfsing, 2006). RNA-sequencing data has been proven to help identifying genes differentially expressed during infection and nutrition processes in several fungi (de Jonge *et al*., 2012; Cantu *et al*., 2013; Bruce *et al*., 2014; Mesarich *et al*., 2014). During infection process, pathogens secrete special proteins, widely known as effectors, which help them achieve a compatible interaction and successfully colonize a host. The study of RNA-sequencing data has proven to be particularly helpful to identify candidate effector proteins secreted during infection. De Jonge *et al*. (2012) identified the Ave1 effector, which interacts with tomato immune receptor Ve1 and governs resistance to race 1 strain of *Verticillium dahliae* and *Verticillium albo-atrum* by analysing RNA-sequencing data. Cantu *et al*. (2013) identified 22 candidate effector genes in *Puccinia striiformis* f. sp. *tritici* by combining RNA-sequencing data and genome annotations. Bruce *et al*. (2014) identified 15 candidate effector genes in six *Puccinia triticina* races by analysing RNA-sequencing data. In this research, we identified the *Bc01G03900-Bc01G03910* gene cluster as a candidate effector gene given the characteristics of the proteins encoded by it: small size (<400 amino acids in length), presence of signal peptide (tag for secretion), and presence of cysteine residues. Even though no radial growth measurements were made, a plant infection assay using *B. cinerea Bc01G03900-Bc01G03910* knock-out transformant showed no difference in virulence compared to WT strain B05.10. This trial was preliminary and was not measured since at least two experiment replications are needed to draw reliable inferences (Hu *et al*., 2011). Moreover, the only *Bc01G03900-Bc01G03910* knock-out transformant obtained was not molecularly characterized in order to verify if the gene replacement was site-directed, ectopic, or unsuccessful. Further molecular and pathogenic characterization of *Bc01G03900-Bc01G03910* knock-out transformant, including the creation of more independent transformants, should be performed in order to confirm these results.

To delineate the function of the regulatory region governing the expression of the *Bc01G03900-Bc01G03910* gene cluster, we constructed a series of deletion mutants in the bi-directional promoter region fused to a *GFP* reporter gene and subsequently assayed the promoter activity. Qualitative analysis of GFP expression in mutants FLF, Del1F, Del3F, Del1R, Del3R showed neither perceptible fluorescence nor difference in fluorescence when compared to both controls; i.e., none of the samples showed fluorescence. The Western Blot derived from the total protein extraction of the tested transformants did not show conclusive results. The bands detected in the Western Blot need further validation since the ~25 kD GFP band was only detected for FLF1 but not for the positive control, which is a *B. cinerea* strain constitutively expressing GFP. The

absence of fluorescence in the GFP constructs and the positive control may be ascribed to a time-dependent GFP expression, meaning that the GFP expression may be scored better using younger hyphae and/or screening earlier. Schumacher (2012) inspected fluorescence patterns in young growing hyphae as older hyphae exhibited higher autofluorescence. However, in our research no autofluorescence was perceived in older hyphae.

The GFP vector, pNDH-GFP, used for the targeted integration of the reporter gene constructs was especially tailored to be expressed in *B. cinerea*. Schumacher (2012) and Zhang (2013) have reported successful integration, expression, and detection of GFP reporter gene constructs using the previously mentioned GFP vector. Nevertheless, different factors affect the detectability of GFP such as susceptibility to photobleaching, competition with noise and background signals, and sensitivity of photodetector (Tsien 1998). The sensitivity of photodetector might be considered an issue in our research since our devise contains a 360 nm UV source, whereas Schumacher (2012) detected GFP fluorescence with a 470/40 UV source. For further research, we advise to create at least three promoter deletion mutants per construct in order to have a thorough analysis of the promoter region of interest. Additionally, check the veracity of the transformants by PCR using the appropriate set of primers, 5.1 and 23 for the 5'-end and 3.1 and 22 for the 3'-end. Finally, we advise to perform a plant infection assay using young growing hyphae and check GFP expression at different time points after inoculation.

The role of pectin degrading enzymes (endo-polygalacturonases, exo-polygalacturonases, pectin and pectate lyases) as virulence factors in *B. cinerea* has been thoroughly studied (Chilosi and Magro, 1997; ten Have *et al*., 1998; Kars *et al*., 2005); however, whether pectin degrading enzymes are involved in tissue degradation and/or releasing monosaccharide nutrients from pectic polymers is yet not clear. To clarify this, mutants with a normal set of pectinases but impaired in D-galacturonic acid catabolism might be analyzed (Zhang *et al*., 2011). Zhang *et al*. (2011) described the D-galacturonic acid catabolic pathway in *B. cinerea* as a three-step process involving four genes: *Bcgar1*, *Bcgar2*, *Bclgd1*, and *Bclga1*. Additionally, the D-galacturonic acid catabolic pathway was genetically characterized by creating mutants on each step; said mutants showed impaired growth on medium with D-galacturonic acid as the sole carbon source compared to wild-type strain B05.10. Strikingly, the same mutants did not show any difference in virulence when inoculated on tomato leaves; whereas they did show reduced virulence on *Nicotiana benthamiana* and *Arabidopsis thaliana*. The three plants tested have varying levels of D-galacturonic acid, and thus different proportions of other monosaccharides such as glucose. Tomato contains higher amounts of glucose and sucrose than *N. benthamiana* and *A. thaliana*; conversely, *N. benthamiana* and *A. thaliana* contain higher amounts of uronic acids (>95% of which is D-galacturonic acid; Zhang and van Kan, 2013). Moreover, the *ΔBclgd1* mutant impaired in a mid-step of the D-galacturonic acid catabolic pathway showed a marked reduce in virulence purportedly due to the

deleterious accumulation of the intermediate L-galactonate (Zhang and van Kan, 2013). Altogether, it is considered that the reduced virulence of the mutants impaired in D-galacturonic acid catabolism on *N. benthamiana* and *A. thaliana* is due to their inability to use an abundant carbon source as nutrient and the deleterious accumulation of catabolic intermediates (Zhang and van Kan, 2013).

Following the quest for identifying the role of pectin degradation in nutrition, Zhang *et al*. (2013) identified the sequence motif GAE3 present in the cis-regulatory sequence of the gene cluster *Bcgar2-Bclga1* involved in D-galacturonic acid catabolism. Regulatory sequences, widely known as promoters, contain transcription factor-binding sites that interact with transcription factor and thus function as genetic switches to either enhance or repress transcription of a specific gene or set of genes. Transcription factors play an important role in regulatory networks including the regulation of plant cell degradation in filamentous fungi (Aro *et al*., 2004). A set of 12 domain superfamilies have been predicted to occur in fungi according to the DBD-Superfamily database; similarly, 37 PFAM domains have been predicted to occur in fungi according to the DBD-PFAM database. Three domain superfamilies and three PFAM families of TFs are predicted to be fungal-specific according to the DBD-Superfamily database and DBD-PFAM database, respectively (Shelest, 2008). The Zn2/Cys6 (Zn cluster) superfamily and the fungal-specific transcription factor domain are the largest classes of fungal-specific domain superfamilies and PFAM domains retrieved from DBD-Superfamily and DBD-PFAM databases, respectively. In *B. cinerea*, 406 TFs have been predicted, harbouring 11 different DNA-binding domains (Simon *et al*., 2013). The BofuT4_P100290.1 gene found in this research, dubbed Cys6-like candidate TF, encodes a protein similar to transcription factor Cys6 belonging to the Zn cluster superfamily and containing two relevant protein domains: Zn2/C6 fungal-type DNA-binding domain (Interpro) or GAL4-like Zn2/Cys6 (C6 zinc) binuclear cluster DNA-binding domain (SMART) and fungal-specific transcription factor domain. The Zn2/C6 fungal-type DNA-binding domain contains a Cys-rich motif involved in zinc-dependent binding of DNA, where two Zn atoms are bound by six Cys residues (Pan and Coleman, 1990). The fungal-specific transcription factor domain has been found in several fungal transcription factors including transcriptional activator xlnR, yeast regulatory protein GAL4, and other transcription factors regulating a number of cellular and metabolic processes (EMBL-EBI, 2014).

The Cys6-like candidate transcription factor was found screening a Y1H library with predicted TF DNA-binding domains based on an *in silico* genome analysis of *B. cinerea* (Anselem *et al*., 2011; Simon *et al*., 2013). In the library, each clone is a putative TF DNA-binding domain in frame with the yeast GAL4 activation domain. For the screening, the DNA promoter region of *Bcgar2-Bcgal1* containing the GAE1 motif, inserted upstream from a *HIS3* reporter gene, is integrated into yeast genomic DNA. This brings the protein-DNA interaction into the natural environment of the nucleus. A drawback from this strategy is the incapability to identify unpredicted TFs.

The interaction of the GAE1 motif, present in the *Bcgar2-Bclga1* gene cluster, with the candidate Cys6-like TF was confirmed by Y1H. This candidate TF might be a global regulator in the galacturonic acid catabolic pathway. Following the observation of a physical interaction between the Cys6-like TF and the GAE1 motif, a functional analysis demonstrating the role of Cys6-like TF in expression regulation of the *Bcgar1, Bcgar2, Bclgd1, and Bclga1* genes should be performed. If the candidate Cys6-like TF turns out to be a global regulator of the D-galacturonic acid catabolic pathway, a mutant impaired on synthesizing said transcription factor will be unable to process D-galacturonic acid as a nutrient but will keep the set of pectin degrading enzymes completely functional. This scenario would support the hypothesis that pectin degrading enzymes are involved only in degrading and colonizing host tissue whereas other elements, such as those involved in D-galacturonic acid catabolism, are involved in releasing monosaccharide nutrients from pectic polymers.

5. Conclusions

The identification of candidate effector genes is crucial for a thorough understanding of economically important plant pathogens. In our research, we focused on both the identification and transcriptional regulation of candidate effector genes. We acquired one *Bc01G03900-Bc01G03910* knock-out mutant that showed no reduced virulence when compared to the WT strain. Additionally, we created promoter deletion mutants in order to assess the regulatory sequence governing the *Bc01G03900-Bc01G03910* gene cluster. Plant infection assays using three FLF and two Del1F GFP-transformants did not show fluorescence when compared with the WT and the constitutive GFP-expressing strain PoliC::GFP. We recommend for further research to obtain at least three independent transformants of *Bc01G03900-Bc01G03910* knock-out and each promoter deletion construct. Moreover, we advise to perform a molecular and pathogenic characterization of the transformants aforementioned.

The identification of TFs playing key roles in regulatory networks allows getting a better understanding of cellular processes. The Cys6-like candidate TF has been proven, via Y1H, to interact with the GAE1 motif present in the *Bcgar2-Bclga1* gene cluster that is involved in the degradation of galacturonic acid. The galacturonic acid degradation pathway includes as well two other genes, *Bcgar1* and *Bclgd1*, which might be regulated by the Cys6-like candidate TF; thus making this candidate TF a global regulator in the galacturonic acid degradation pathway. If the Cys6-like candidate TF turns out to be a global regulator of the D-galacturonic acid catabolic pathway, then a mutant impaired in synthesizing said TF would help clarifying whether pectin degrading enzymes are involved only in degrading and colonizing host tissue or additionally they have a role in releasing monosaccharide nutrients from pectin polymers. To validate this hypothesis, knock-out mutants impaired in the expression of Cys6-like TF should be created and further characterized.

6. References

Amoutzias GD, Veron AS, Weiner III J, Robinson-Rechavi M, Bornberg-Bauer E, Oliver SG, and Robertson DL. 2007. One billion years of bZIP transcription factor evolution: conservation and change in dimerization and DNA-binding site specificity. Molecular Biology and Evolution 24(3):827-835.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, and Walter P. 2008. Control of gene expression: DNA-binding motifs in gene regulatory proteins. In: Molecular Biology of the Cell (pp. 416-432). New York, NY, USA: Garland Science.

Anselem J, Cuomo CA, van Kan JAL, Viaud M, Benito EP, *et al*. 2011. Genomic analysis of the necrotrophic fungal pathogens Sclerotinia sclerotiorum and Botrytis cinerea. PLoS Genetics 7(8): e1002230. Doi: 10.1371/journal.pgen.1002230.

Aro N, Pakula T, and Penttilä M. 2004. Transcriptional regulation of plant cell wall degradation by filamentous fungi. FEMS Microbiology Reviews 29: 719-739.

Basse CW and Farfsing JW. 2006.Promoters and their regulation in Ustilago maydis and other phytopathogenic fungi. FEMS Microbiology Letters 254:208-216.

Boller T and Felix G. 2009. A renaissance of elicitors: perception of microbe-associated moleuclar patterns and danger signals by pattern-recognition receptors. Annual Review of Plant Biology 60:379-406.

Bruce M, Neugebauer KA, Joly DL, Migeon P, Cuomo CA, Wang S, Akhunov E, Bakkeren G, Kolmer JA, and Fellers JP. Using transcription of six Puccinia triticina races to identify the effective secretome during infection of wheat. Frontiers in Plant Science 4:520. doi:10.3389/fpls.2013.00520.

Caffal KH and Mohnen D. 2009. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydrate Research 344:1879-1900.

Campbell RN, Leverentz MK, Ryan LA, and Reece RJ. 2008. Metabolic control of transcription: paradigms and lessons from *Saccharomyces cerevisiae*. Biochemical Journal 414:177-187.

Cantu D, Segovia V, MacLean D, Bayles R, Chen X, Kamoun S, Dubcovsky J, Saunders DGO, and Uauy C. 2013. Genome analysis of the wheat yellow (stripe) rust pathogen Puccinia striiformis f. sp. tritici reveal polymorphic and haustorial expressed secreted proteins as candidate effectors. BMC Genomics 14:270. doi:10.1186/1471-2164-14-270.

Cao Y, Yao Z, Sarkar D, Lawrence M, Sanchez GJ, Parker MH, MacQuarrie KL, Davison J, Morgan MT, Ruzzo Wl, Gentlemen RC, and Tapscott SJ. 2010.

Genome-wide MyoD binding in skeletal muscle cells : a potential for broad cellular reprogramming. Developmental Cell 18:662-674.

Coll NS, Epple P, and Dangl JL. 2011. Programmed cell death in the plant immune system. Cell Death and Differentiation 18:1247-1256.

Chilosi G and Magro P. 1997. Pectin lyase and polygalacturonase isoenzyme production by botrytis cinerea during the early stages of infection on different host plants. Journal of Plant Pathology 78:61-69.

Choquer M, Fournier E, Kunz C, Levis C, Pradier JM, Simon A, and Viaud M. 2007. Botrytis cinerea virulence factors: new insights into a necrotrophic and polyphageous pathogen. Federation of European Microbiological Societies 277:1-10.

Cubero B and Scazzocchio C. 1994. Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of Aspergillus nidulans. EMBO Journal 13(2):407-415.

Dalmais B, Schumacher J, Moraga J, Le Pecheur P, Tudzynski B, Gonzalez Collado I, and Viaud M. 2011. The Botrytis cinerea phytotoxin botcinic acid requires two polyketide synthases for production and has a redundant role in virulence with botrydial. Molecular Plant pathology 12(6):564-579.

Dhadi SR, Aparna D, Driscoll K, and Ramakrishna W. 2013. Major cis-rgulatory elements for rice bidirectional promoter activity reside in the 5'-untranslated regions. Gene 526:400-410.

EMBL-EBI. Interpro, protein sequence analysis and classification: Transcription factor domain, fungi (IPR007219). 2014. Web. 14 Oct, 2014.

Farfsing JW, Auffarth K, and Basse CW. 2005. Identification of *cis*-active elements in *Ustilago maydis mig2* promoters conferring high-level activity during pathogenic growth in maize. Molecular Plant-Microbe Interactions 18(1):75-87.

Farnham PJ. 2009. Insights from genomic profiling of transcription factors. Nature Reviews Genetics 10:605-616.

Feng J, Bhadauria V, Liu G, Selvaraj G, Hughes GR, and Wei Y. 2011. Analysis of the promoter region of the gene *LIP1* encoding triglyceride lipase from *Fusarium graminearum*. Microbiological Research 166:618-628.

Fry CJ and Peterson CL. 2002. Unlocking the gates to gene expression. Science 295:1847-1848.

Govrin EM and Levine A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Current Biology 10(13): 751-757.

Hamada W, Reignault Ph, Bompeix G, and Boccara M. 1994. Transformation of *Botrytis cinerea* with the hygromycing B resistance gene, *hph*. Current Genetics 26:251-255.

Hu L, Bao X, and Wang Q. 2011. The repetition principle in scientific research. Journal of Chinese Integrative Medicine 9(9):937-940.

ten Have A, Mulder W, Visser J, and van Kan J.A.L. 1998. The endopolygalacturonase gene Bcpg1 is required for full virulence of Botrytis cinerea. Molecular Plant-Microbe Interactions 11(10):1009-1016.

John S, Sabo PJ, Thurman RE, Sung MH, Biddie SC, Johnson TA; Hafer GL, and Stamatoyannopoulos JA. 2011. Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. Nature Genetics 43:264-268.

Jones JDG and Dangl JL. 2006. The plant immune system. Nature 444(16):323-329.

de Jonge R, van Esse HP, Maruthachalam K, Bolton MD, Santhanam P, Saber MK, Zhang Z, Usami T, Lievens B, Subbarao KV, and Thomma BP. Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. Procedures of the National Academy of Sciences USA 109(13):5110-5115.

van Kan JAL, van 't Klooster JW, Wagemakers CAM, Dees D, and van der Vlugt-Bergmans CJB. 1997. Cutinase A of Botrytis cinrea is expressed, but not essencial, during penetration of gerbera and tomato. Molecular Plant-Microbe Interaction 10:30-38.

Kars I, McCalman M, Wagemakers L, and van Kan JAL. Functional analysis of Botrytis cinerea pectin methylesterase genes by PCR-based targeted mutagenesis: Bcpme1 and Bcpme2 are dispensable for virulence of strain B05.10. Molecular Plant Pathology 6(6):641-652.

Katagiri F and Tsuda K. 2010. Understanding the plant immune system. Molecular Plant-Microbe Interactions 23(12):1531-1536.

Kaufmann K, Pajoro A, and Angenent GC. 2010. Regulation of transcription in plants: mechanisms controlling developmental switches. Nature Reviews Genetics 11:830-842.

Latchman DS. 2008. DNA sequences, transcription factors and chromatin structure. In: Latchman DS, Eukaryotic transcription factors (pp. 1-24). London, UK: ELSEVIER.

Levis C, Fortini D and Brygoo Y. 1997. Transformation of Botrytis cinerea with the nitrate reductase gene (niaD) shows a high frequency of homologous recombination. Current Genetics 32:157-162.

Levo M and Segal E. 2014. In pursuit of design principles of regulatory sequences. Nature Reviews Genetics 15:453-468.

Li XY, Thomas S, Sabo PJ, Eisen MB, Stamatoyannopoulos JA, and Biggin MD. 2011. The role of chromatin accessibility in directing the widespreasd, overlapping patterns of Drosophila transcription factor binding. Genome Biology 12:R34.

Martens-Uzunova ES, and Schaap PJ. 2008. An evolutionary conserved D-galacturonic acid metabolic pathway operates across filamentous fungi capable of pectin degradation. Fungal Genetics and Biology 45:1449-1457.

Mesarich CH, Griffiths SA, van der Burgt A, Okmen B, Beenen HG, Etalo Desalegn W, Joosten MHAJ, and de Wit PJGM. 2014. Transcriptome sequencing uncovers the Avr5 avirulence gene of the tomato leaf mold pathogen Cladosporium fulvum. Molecular Plant-Microbe Interactions 27(8):846-857.

Newman MA, Sundlein T, Nielsen JT, and Erbs G. 2013. MAMP (microbe-associated molecular pattern) triggered immunity in plants. Frontiers in Plant Science 4(139):1-14.

Oliver RP and Solomon PS. 2010. New developments in pathogenicity and virulence of necrotrophs. Current Opinion in Plant Biology 13:415-419.

Pan T and Coleman JE. 1990. GAL4 transcription factor is not a "zinc finger" but forms a Zn2Cys6 binuclear cluster. Proceedings of the National Academy of Sciences of the United States of America 87(6):2077-2081.

Rutherford J. 2011. Direct sensing of nutrient availability by fungi. Fungal Biology Reviews 25:111-119.

Petersen TN, Brunak S, von Heijne G, and Nielsen H. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods 8:785-786.

Schleif RF. 2013. Modulation of DNA binding by gene-specific transcription factors. American Chemical Society 52:6755-6765..

Schumacher J, de Larrinoa IF, and Tudzynski B. 2008. Calcineurin-responsive zinc finger transcription factor CRZ1 of Botrytis cinerea is required for growth, development, and full virulence on bean plants. Eukaryotic Cell 7(4):584-601.

Schumacher J. 2012. Tools for *Botrytis cinerea*: New expression vectors make the gray mold fungus more accessible to cell biology approaches. Fungal Genetics and Biology 49:483-497.

Schumacher J, Simon A, Cohrs KC, Viaud M, and Tudzynski P. 2014. The transcription factor BcLTF1 regulates virulence and light responses in the necrotrophic plant pathogen *Botrytis cinerea*. PLoS Genetics 10(1):e1004040.

Shelest E. 2008. Transcription factors in fungi. Federation of European Microbiological Societies 286:145-151.

Simon A, Dalmais B, Morgant G, and Viaud M. 2013. Screening of a Botrytis cinerea one-hybrid library reveals a Cys_2His_2 transcription factor involved in the regulation of secondary metabolism gene clusters. Fungal Genetics and Biology 52:9-19.

Spitz F and Furlong EEM. 2012. Transcription factors: from enhancer binding to developmental control. Nature Reviews Genetics 13:613-626.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, and Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25:3389-3402.

Tilburn J, Sarkar S, Widdick DA, Espeso EA, Orejas M, Mungroo J, Peñalva MA, and Arst HN Jr. 1995. The Aspergillus PacC zinc finger transcripti9n factor mediates regulation of both acid- and alkaline-expressed genes by ambient pH. EMBO Journal 14(4):779-790.

Todd RB, Zhou M, Ohm RA, Leeggangers HACF, and Visser Loek. 2014. Prevalence of transcription factors in ascomycete and basidiomycete fungi. BMC Genomics 15:214.

Tsien RY. 1998. The green fluorescent protein. Annual Review of Biochemistry 67:509-544.

Wang Z and Lindquist S. 1998. Developmentally regulated nuclear transport of transcription factors in Drosophila embryos enable the heat shock response. Developent 125: 4841-4850.

Wang Z and Zhang Q. 2009. Genome-wide identification and evolutionary analysis of the animal specific ETS transcription factor family.

Warren AJ. 2002. Eukaryotic transcription factors. Current Opinion in Structural Biology 12:107-114.

Weake VM and Workman JL. 2010. Inducible gene expression: diverse regulatory mechanisms. Nature Reviews Genetics 11:426-437.

Williamson B, Tudzynski B, Tudzynski P, and van Kan J.A.L. 2007. *Botrytis cinerea*: the cause of grey mould disease. Molecular Plant Pathology 8(5):561-580.

Wubben JP, ten Have A, van Kan JAL, and Visser J. 2000. Regulation of endopolygalacturonase gene expression in Botrytis cinerea by galacturonic acid, ambient pH and carbon catabolite repression. Current Genetics 37:152-157.

Yamasaki K, Kigawa T, Seki M, Shinozaki K, and Yokoyama S. 2013. DNA-binding domains of plant-specific transcription factors: structure, function, and evolution. Trends in Plant Science 8(5):267-276.

Yoshiaki M, Homma K, and Nishikawa K. 2005. Genome-wide survey of transcription factors in prokaryotes reveals many bacteria-specific families not found in Archaea. DNA Research 12(5):269-280.

Zaret KS and Caroll JS. 2011. Pioneer transcription factors: establishing competence for gene expression. Genes and Development 25:2227-2241.

Zhang L, Thiewes H, and van Kan JAL. 2011. The D-galacturonic acid catabolic pathway in *Botrytis cinerea*. Fungal Genetics and Biology 48:990-997.

Zhang L. 2013. Pectate-induced gene expression and cis-regulatory elements. In: Zhang L, Stasen J, Chatterjee S, Cornelissen M, and van Kan JAL; Pectin degradation by Botrytis cinerea: recognition of endo-polygalacturonases by an Arabidopsis receptor and utilization of D-Galacturonic acid (pp. 129-160). Wageningen, the Netherlands: Wageningen University

Zhang L and van Kan JAL. 2013. *Botrytis cinerea* mutants deficient in D-galacturonic acid catabolism have a perturbed virulence on *Nicotiana benthamiana* and Arabidopsis, but not on tomato. Molecular Plant Pathology 14:19-29.

Appendix 1

Table 2. Media used for culture of microorganisms.

ID	Medium	Components for 1000 ml
1	LB medium	10 g bacteriological tryptone 5 g yeast extract 10 g NaCl for plates add 15 g technical agar before autoclaving
2	YPD medium	10 g yeast extract 20 g bacteriological peptone 20 g glucose For plates add 15 g technical agar before autoclaving
3	SH agar	203.38 g sucrose 5 mM HEPES pH 6.5 1 mM (NH4)H2PO4 12 g technical agar
4	KC buffer	0.6 M KCl 50 mM CaCl2

Table 3. Solutions and buffers used for protein work.

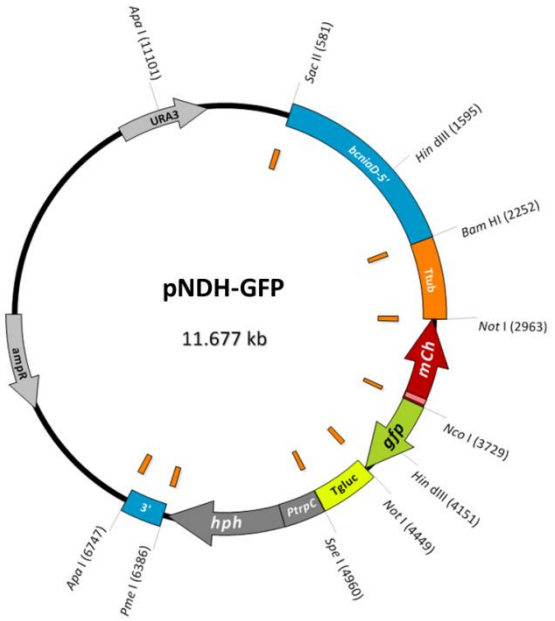
ID	Medium	Components
1	Running buffer 10x	For 1000 ml: 144 g glycine 30 g Tris 10 g SDS
2	Transfer buffer (blotting buffer 10x)	For 900 ml: 30.5 g Tris 138 g glycine
3	Transfer buffer / blotting buffer 1x	For 1000 ml: 3.05 Tris 13.8 g glycine 100 ml EtOH
4	TBS 10x	For 1000 ml: 12.1 g Tris 14.6 g NaCl Set pH to 7.5 and add 1 ml tween
5	Running gel*	For 1 gel: 1.7 ml dH2O 2.0 ml acrylamide/bis solution (30%) 1.3 ml 1.5 M Tris (pH 8.8) 50 ul SDS (10%; w/v) 50 ul APS (NH4)2S2O8 (10%; w/v) 2 ul Tetramethylethylendiamin TEMED (Invitrogen)
6	Stacking gel*	For 1 gel: 0.68 ml dH2O 0.17 ml acrylamide/bis solution (30%)

		0.13 ml 1.0 M Tris (pH 6.8) 10 ul SDS (10%; w/v) 10 ul APS (NH4)2S2O8 (10%; w/v) 1 ul Tetramethylethylendiamin TEMED (Invitrogen)
7	Blotting buffer	For 1000 ml: 1.5 g Tris → 12 mM 7.2 g glycine → 95 mM

Table 4. Primers used for cloning.

ID	Primer sequence (5'-3')	Remarks
FF5'	F(5.1):GATACGATTGATGCCACGGG R(5.3):GGGTACCGAGCTCGAATTCAGGCGATTCTTGCAACTGTGAC	Knock-out
FF3'	F(3.3):CTCGGCGCGCCGAAGCTTGGTAAGTTCTTCTCTTATCAAGTC R(3.1):GAATATGAATTACAGAAGAGACG	Knock-out
hph	F(20):GAATTCGAGCTCGGTACCC R(21):AAGCTTGATATCTGTTAGTA	Selection marker
hph	F(22):GGGTACCGAGCTCGAATTC R(23):GATTACTAACAGATATCAAGCTT	Screenning
OE	F(5.2):GATGCCACGGGTTCAGTATAG R(3.2):CTAGCTGTAGTACAATGTCACCC	Overlap-extension PCR
FLF	F:GATTACTTACCTCGCCCTTGCTTACCATTATTGCAAGTCGGATGGTTGAT R:TGAAAAGCTCTTCACCTTTGGAAACCATATTGAAGACTCAGAGTGTTTCAG	Full length forward orientation
Del1F	F:GATTACTTACCTCGCCCTTGCTTACCATCATATCCGATTGTTAGAATAGGG R:TGAAAAGCTCTTCACCTTTGGAAACCATATTGAAGACTCAGAGTGTTTCAG	Deletion 1 forward orientation
Del2F	F:GATTACTTACCTCGCCCTTGCTTACCATCCCGTATATGCAATCCACATAGGTG R:TGAAAAGCTCTTCACCTTTGGAAACCATATTGAAGACTCAGAGTGTTTCAG	Deletion 2 forward orientation
Del3F	F:GATTACTTACCTCGCCCTTGCTTACCATCGACTGCTGTGCCACTTTCCC R:TGAAAAGCTCTTCACCTTTGGAAACCATATTGAAGACTCAGAGTGTTTCAG	Deletion 3 forward orientation
FLR	F:GATTACTTACCTCGCCCTTGCTTACCATATTGAAGACTCAGAGTGTTTCAG R:TGAAAAGCTCTTCACCTTTGGAAACCATTATTGCAAGTCGGATGGTTGAT	Full length reverse orientation
Del1R	F: GATTACTTACCTCGCCCTTGCTTACCATCCTCTTGTCAGGATTCGCCC R:TGAAAAGCTCTTCACCTTTGGAAACCATTATTGCAAGTCGGATGGTTGAT	Deletion 1 reverse orientation
Del2R	F:GATTACTTACCTCGCCCTTGCTTACCATAGAACGCACATATATGGAGTGC R:TGAAAAGCTCTTCACCTTTGGAAACCATTATTGCAAGTCGGATGGTTGAT	Deletion 2 reverse orientation
Del3R	F:GATTACTTACCTCGCCCTTGCTTACCATCAGTTGCATTAAGAACCTCC R:TGAAAAGCTCTTCACCTTTGGAAACCATTATTGCAAGTCGGATGGTTGAT	Deletion 3 reverse orientation
LZ	F(182):GTTTTCCCAGTCACGACCCTTAAATCTCATGAACTCCTTG R(183):CAGGAAACAGCTATGACCCTCTCCGCTGACTGAGAAC	To check inserts in *E. coli* transformation
AS	F(41):GCGTTTGGAATCACTACAGG R(42):CACGATGCACAGTTGAAGTG	TFs library screening

Appendix 2



bcniaD-5' flank	583..2250
T*tub*	2251..2956
mCherry	2957..3727
gfp	3730..4458
T*gluc*	4459..4958
PtrpC::hph	4965..6347
bcniaD-3' flank	6390..6741
vector backbone	pRS426ΔNcoI

Figure 4. Plasmid map of pNDH-GFP followed by sequences.

Table 5. Alignments from the seven samples sent for sequencing after running the blastx program (retrieved from NCBI).

ID	Alignment
PV7	Query 156 MAARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVPKKRGPKTDVLE 335 + ARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVPKKRGPKTDVLE Sbjct 13 LPARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVPKKRGPKTDVLE 72 Query 336 ALLKRVDGLERKLRDEKKSNSPNNDgsasgsgsgggeaesPHDDTKPKRPHLGIRI 503 ALLKRVDGLERKLRDEKKSNSPNNDGSASGSGSGGGEAESPHDDTKPKRPHL I Sbjct 73 ALLKRVDGLERKLRDEKKSNSPNNDGSASGSGSGGGEAESPHDDTKPKRPHLETTI 128
PV8	Query 117 VPDYASLGGHMAMVETLHAVAMEPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQR 296 VP SL + H +EPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQR Sbjct 378 VPHTHSLSPLTPLSSISHPSPLEPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQR 437 Query 297 EKSGVASKSSCPNCGAEFTRTTARNGHMAHEKCKQRRN 410 EKSGVASKSSCPNCGAEFTRTTARNGHMAHEKCKQRRN Sbjct 438 EKSGVASKSSCPNCGAEFTRTTARNGHMAHEKCKQRRN 475
PV11	Query 155 MAARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVPKKRGPKTDVLE 334 + ARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVPKKRGPKTDVLE Sbjct 13 LPARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVPKKRGPKTDVLE 72 Query 335 ALLKRVDGLERKLRDEKKSNSPNNDgsasgsgsgggeaesPHDDTKPKRPHLGIRI 502 ALLKRVDGLERKLRDEKKSNSPNNDGSASGSGSGGGEAESPHDDTKPKRPHL I Sbjct 73 ALLKRVDGLERKLRDEKKSNSPNNDGSASGSGSGGGEAESPHDDTKPKRPHLETTI 128
PV15	Query 161 DESTLVIATSSVMFADYQNQQAIAIPPNSTYPSASSISPTVPPSNPDDVRRPSTSAPTAV 340 DESTLVIATSSVMFADYQNQQAIAIPPNSTYPSASSISPTVPPSNPDDVRRPSTSAPTAV Sbjct 10 DESTLVIATSSVMFADYQNQQAIAIPPNSTYPSASSISPTVPPSNPDDVRRPSTSAPTAV 69 Query 341 PTGLNArscvtcrrrkvkcdkkvpcSNCTKAQSPCVFpapgraprrpraggKPISD 508 PTGLNARSCVTCRRRKVKCDKKVPCSNCTKAQSPCVFPAPGRAPRRPRAGGKPISD Sbjct 70 PTGLNARSCVTCRRRKVKCDKKVPCSNCTKAQSPCVFPAPGRAPRRPRAGGKPISD 125

PV16

```
Query  162  DESTLVIATSSVMFADYQNQQAIAIPPNSTYPSASSISPTVPPSNPDDVRRPSTSAPTAV  341
            DESTLVIATSSVMFADYQNQQAIAIPPNSTYPSASSISPTVPPSNPDDVRRPSTSAPTAV
Sbjct  10   DESTLVIATSSVMFADYQNQQAIAIPPNSTYPSASSISPTVPPSNPDDVRRPSTSAPTAV  69

Query  342  PTGLNArscvtcrrrkvkcdkkvpcSNCTKAQSPCVFpapgraprrpraggKPISD  509
            PTGLNARSCVTCRRRKVKCDKKVPCSNCTKAQSPCVFPAPGRAPRRPRAGGKPISD
Sbjct  70   PTGLNARSCVTCRRRKVKCDKKVPCSNCTKAQSPCVFPAPGRAPRRPRAGGKPISD  125
```

PV32

```
Query  119  VPDYASLGGHMAMVETLHAVAMEPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQR  298
            VP   SL     +    H   +EPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQR
Sbjct  378  VPHTHSLSPLTPLSSISHPSPLEPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQR  437

Query  299  EKSGVASKSSCPNCGAEFTRTTARNGHMAHEKCKQRRN  412
            EKSGVASKSSCPNCGAEFTRTTARNGHMAHEKCKQRRN
Sbjct  438  EKSGVASKSSCPNCGAEFTRTTARNGHMAHEKCKQRRN  475
```

PV43

```
Query  131  ASLGGHMAMEPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQREKSGVASKSSCPN  310
            +S+     +EPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQREKSGVASKSSCPN
Sbjct  391  SSISHPSPLEPRDHVRVVQSRPKPQCWDHGCNGRQFSTFSNLLRHQREKSGVASKSSCPN  450

Query  311  CGAEFTRTTARNGHMAHEKCKQRRN  385
            CGAEFTRTTARNGHMAHEKCKQRRN
Sbjct  451  CGAEFTRTTARNGHMAHEKCKQRRN  475
```

Table 6. Alignment parameters from the seven samples sent for sequencing after running the blastx program (retrieved from NCBI).

ID	Description	Max score	Total score	Query cover (%)	E value	Identity (%)	Accession
PV 7	Similar to transcription factor Cys6 [Botryiotinia fuckleiana T4]	204	204	55	$6.00E^{-61}$	96	CCD34625.1
PV 8	Similar to transcription factor Zn, C2H2 [Botryotinia fuckeliana T4]	176	176	53	$1.00E^{-52}$	83	CCD45621.1
PV 11	Similar to transcription factor Cys6 [Botryiotinia fuckleiana T4]	204	204	51	$1.00E^{-60}$	96	CCD34625.1
PV 15	Similar to transcription factor Cys6 [Botryiotinia fuckleiana T4]	151	151	48	$1.00E^{-41}$	100	CCD46617.1
PV 16	Similar to transcription factor Cys6 [Botryiotinia fuckleiana T4]	151	151	48	$1.00E^{-41}$	100	CCD46617.1
PV 32	Similar to transcription factor Zn, C2H2 [Botryotinia fuckeliana T4]	175	175	53	$5.00E^{-52}$	83	CCD45621.1
PV 43	Similar to transcription factor Zn, C2H2 [Botryotinia fuckeliana T4]	170	170	18	$4.00E^{-47}$	91	CCD45621.1

Appendix 3

Protein sequence of BofuT4_P100290.1 according to Prosite:

MSGRPAQVGIERLPARRMSNEPRESMNCKSCRKRKIKCNRLRPTCEACQVFQCPCIYDAVP
KKRGPKTDVLEALLKRVDGLERKLRDEKKSNSPNNDGSASGSGSGGGEAESPHDDTKPK
RPHLETTISNIADESAVYSPTPISEPSPSVQQDVLLDTYFVRCHGKSYHILDETSIRQRIQSN
QIPTYLLYAIYAVSARYTSHPNGYFAAVRLSEDYAHRARAEVDIDEPSIDNLQALLLLGISYT
ASGRGKKAYMMLANAVGMAVALELHRELDQNLRISPVERELRRRLFWTCYLIDRFTACGSK
RPSLIADKSIVLRLPSWSPNPAALPVEGEFFQSGSNLHFGSGKKSQGSSGMLIDIVRILGIT
NRYLAAGGVKGRFCNNHVCSSIVHCTNTDYHATKTGDSHFPWHSLSNLSKIRQDLDIWA
GGTQDVFTSVDTLFGQPDSTTLVLSKLIYHLIHCLIYRPFLPIDLAELAGTGQHQSWQIEAT
NLCFLHANAIAELVELGKQSASIEWPAFVGYCICTAGTVHVHGTHYKGGREGEVFSASADF
LSREMQQLSELRYAWSSVQHQRETLQTIYGCHSDLVKSLGSNPMRFSPVFHLEDFFDRYS
DLGQYFDGAHISFADVVTPSPEAYQGHDLYAPTQSNRNGSMNGGTGSSGSNDLTTVTAK
RKSSSSARKRALTNSLKDRPLMSPSGRSVEQLPLPQNGLLGHDPASYSNQGSSMTTLPQIP
FSPPSQNQSQSYTFSPSLSLNHGQESDFDPMFGMSHLGSGGNFSFAGMGGTSGGMMGE
EGMTPGGRSNNGSTGTSGEEKDPFLSLLEQLAENEQQRGGPSELDFFLGGGQG

Figure Index

Table Index