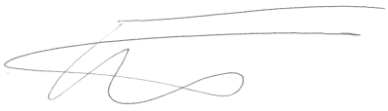
Disruption of the Secondary Metabolism Regulators LaeA and VeA in *Trichoderma hamatum* GD12

Submitted by Odette Wills to the University of Exeter as a dissertation for the degree of Master by Research in Biological Sciences, September 2014



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Abstract

Modern agriculture currently depends upon the application of environmentally-degrading chemical pesticides and the use of unsustainable fossil fuels. Low-impact methods of pest control and plant growth promotion (PGP) are required to enable sustainable agronomic intensification. *Trichoderma hamatum* GD12 simultaneously possess both PGP and biocontrol abilities, making it a suitable alternative for polluting agrochemicals. Co-culturing of GD12 with the plurivorous pathogen *Sclerotinia sclerotiorum* has shown enhancement of PGP activities. It is hypothesized that during pathogenic interactions GD12 secondary metabolite gene clusters - which are standardly quiescent – are activated allowing the production of novel PGP compounds. The global regulator methyltransferease, LaeA, forms a trimeric complex with velvet proteins VeA and VelB. Together this complex determines the production of secondary metabolites as well as asexual and sexual development. The aim of this study was to delete velvet complex elements LaeA and VeA to determine effect on fungal morphology and biocontrol capabilities. *ΔThLaeA::hph* deletion was deemed unsuccessful by southern blot. *ΔThVeA::hph* mutants showed a reduction in hyphal growth, condia production, and alterations in over-growth capabilities when confronted with *Scerotinia* pathogens. *Scerotinia* pathogens in confrontation with mutants also produced an increase number and weight of over-wintering sclerotia. This investigation demonstrates that, within GD12, *VeA* is required for normal morphological development and biological disease control.

Key words

Trichoderma hamatum GD12, LaeA, VeA, biocontrol and plant growth promotion.

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Abbreviations

%	Percent
°C	Degree Celcius
µg	Microgram
µL	Microliter
µm	Micrometre
µM	Micromolar
bp	Base pair
cm	Centimetre
DIG	Digoxigenin-dUTP
EDTA	Ethylenediaminetetraaceticacid
g	Gram
hph	Gene conferring HYG resistance
hr(s)	Hour(s)
HY/YG	Split marker HYG
HYG	Hygromycin
Kb	Kilobase
KOH	Potassium hydroxide
L	Litre
LaeA	Global regulator protein
LF	Left flank
M	Molar
Mb	Megabase
mg	Milligram min(s) Minute(s)
mL	Millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PEG	Polyethylene glycol
PGP	Plant growth promotion
pH	Power of hydrogen' measure of acidity/basicity
pM	Picomolar
RF	Right flank
SM(S)	Secondary Metabolite(s)
UV	Ultraviolet
VeA	Velvet protein A
VelB	Velvet-like protein B
vol	Volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

1. Introduction

It is predicted that by 2050 the global population will reach around 9 billion resulting in increased pressure on food production (Godfray *et al*., 2010). Presently up to 16% of crops are lost to pests and pathogens before harvest, with decreases from fungi and oomycetes making up enough to feed 8.5% of the world's current population (Bebber *et al.*, 2013). Climate change - causing variation in weather and CO_2 levels - will enable pathogen migration to new, currently unsuitable, regions further burdening future food production (Bebber *et al.*, 2013). Broad spectrum fungicides are often used to tackle pathogenic threats (El-Hassan *et al.*, 2013). However, these agrochemicals, along with artificial fertilizer, can detrimentally pollute the environment, adversely impact human health and negatively affect beneficial soil fungi through the disruption of normal fungal ecosystems (El-Hassan *et al*., 2013; Hermosa *et al*., 2012). Environmentally sound replacement methods need to be obtained, which control pathogens and raise plant growth facilitating sustainable agronomic intensification.

Trichoderma species are prevalent saprotrophs located across a variety of environments, including soil, root ecosystems (inside the plant rhizophere) and foliar settings (Harman *et al*., 2004; Ryder *et al.*, 2012). Beneficial strains of *Trichoderma* can promote plant growth by; enhancing nitrogen use efficiency, maintaining soil quality and solubilising nutrients which would otherwise be unattainable (Doni, *et al.,* 2014; Harman *et al*., 2004). Some particular *Trichoderma* strains can concurrently promote plant growth and biologically control pathogenic microbes within the soil (Harman *et al.*, 2004). Members of the *Trichoderma* genus with biocontrol ability can prevent disease caused by bacteria, fungi and oomycetes, such as damping off (i.e. *Rhizoctonia solani* and *Pythium*), bacterial spot (i.e. *Xanthomonas*) and blight (i.e. *Alternaria* species) (Fontenelle *et al.*, 2011; Verma *et al.,* 2007). Implementation of biocontrol comes through: out-competition for nutrients resources, physical competition (mycoparastism), and production of anti-microbial compounds which benefit biocontrol agents (Harman *et al*., 2004; Hermosa *et al*., 2012).

The strain *T.hamatum* GD12 unusually possesses both biocontrol and PGP properties. Pre- and post-emergence soil pathogens are controlled by GD12 and induced systemic resistance has been shown against foliar pathogens (Studholme *et*

al., 2013). GD12 shows biocontrol protection against dicot (brassicas and lettuce) and monocot (rice) pathogens while simultaneously amplifying PGP via the creation of water-soluble secondary metabolites (Studholme *et al.*, 2013). Even addition of small quantities of autoclaved bran extracts from GD12, i.e. 50 µl, have been shown to raise lettuce plant growth (Studholme *et al.*, 2013). The plurivorous necrotrophic pathogen *Sclerotinia sclerotiorum* has caused serious plant damage, with a host range of over 400 plant species, resulting in large annual economic loss (Williams, *et al.*, 2011). Necrosis is achieved by this pathogen through the production of lytic enzymes, toxins and other key substances such as oxalic acid (Williams, *et al.*, 2011). However, by co-culturing disease causing organisms, such *S. sclerotiorum*, with GD12 plant pathogens are controlled and additional stimulation of PGP activity is seen.

Secondary metabolites (SMs) are small bioactive low-molecular-weight compounds - not needed for normal growth - produced by copious fungal species, bacteria and plants (Bayram, 2011). These products are thought to be created for communication purposes and to chemically protect fungi against competitors - such as other microorganisms - enabling niche specialization (Jain & Keller, 2013; Bayram *et al*, 2010). SMs are also often involved with sexual and asexual sporulation, as well as other fungal morphological developments (Roberts, 2013; Jain & Keller, 2013). High levels of SMs are frequently seen in *Tricoderma* species (Mukherjee *et al*, 2012). Key examples of biocontrol SMs found within these species include; gliotoxins and the antibiotic gliovrin, which aid the control of antagonistic fungal pathogens i.e. *Rhizoctonia solani* and *Pythium ultimum* (Vinale *et al*, 2014). Other *Trichoderma* SMs assist in the development and growth of plants i.e. Koninginins and harzianolide (Vinale *et al*, 2014).

LaeA, VeA and VelB all interact forming a trimeric complex (VelA-VeB-LaeA) in the nucleus (Figure 1.) (Bayram *et al*., 2008; Bayram and Braus, 2012; Ahmed *et al*., 2013; Palmer and Keller, 2010). Often *Aspergillus nidulans* is used to study this trimeric complex, the results below discuss the finding within this species. Through activation from external stimuli the LaeA-VeA-VelB association can modify heterochromatin (DNA inactive state) to eurochromatin (DNA active state), both directly and indirectly, allowing the production of novel secondary metabolites (Figure 1.) (Palmer & Keller, 2010; Bayram *et al*., 2008). Novel standardly silent, secondary metabolite clusters can be activated in physical exchanges between a fungus and a

pathogen, as shown in *Aspergillus* – bacterial interactions (Schroeckh *et al*, 2009). Some SMs are only found during co-cultures of pathogens, for example trichosetin (an antimicrobial metabolite) was produced only during a *T. harzianum* and calli of *Catharathus roseus* interation, and not in single cultures (Vinale *et al*, 2014).

Generally the roles of VeA and VelB are however antagonistic to LaeA, and permit the rigorous regulation of fungal morphology and asexual/sexual development (Jain and Keller, 2013; Bayram and Braus, 2012). Light conditions enable LaeA to decrease VeA and VelB levels, allowing asexual development (Bayram and Braus, 2012; Jain & Keller, 2013; López-Berges, *et al*., 2014). Whereas under dark conditions, in the absence of LaeA, velvet proteins are not repressed enabling sexual development (Calvo, 2008; Crespo-Sempere, *et al*., 2013; Jain & Keller, 2013).

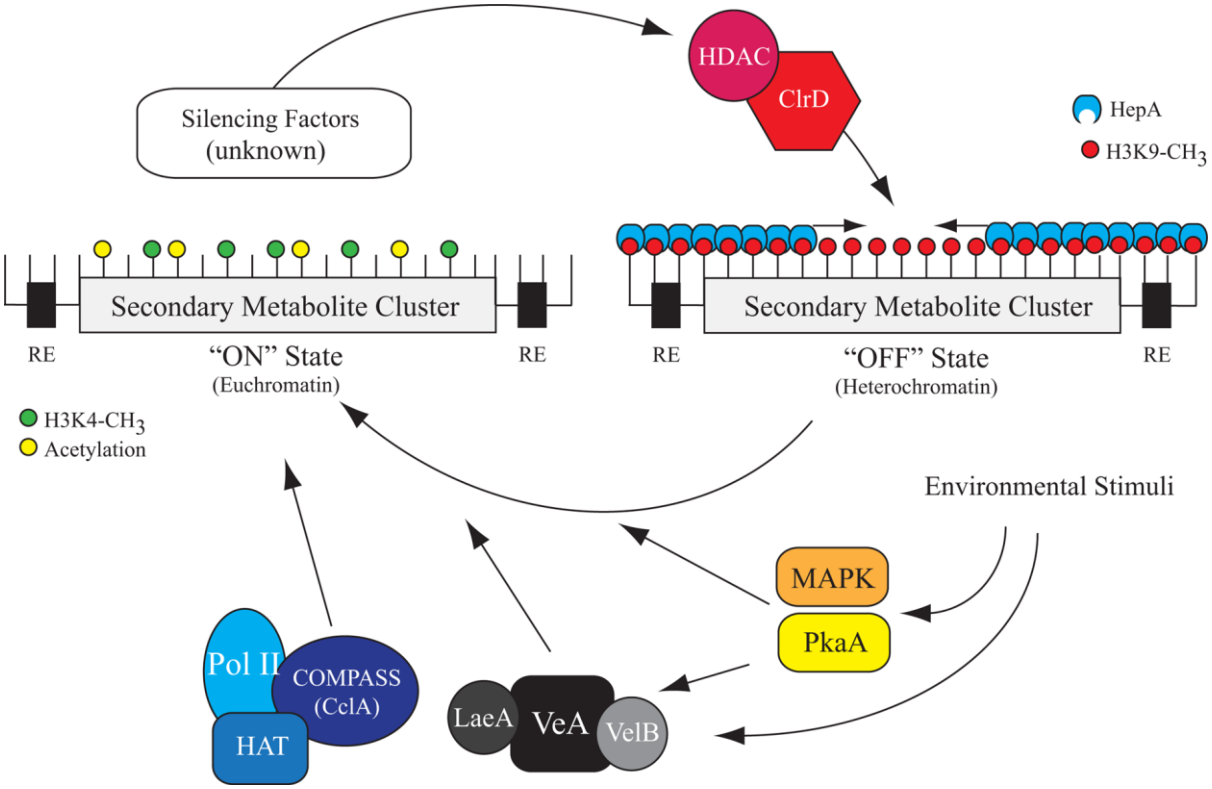


Figure 1. Association of the trimeric complex (VelA-VeB-LaeA). Figure taken from Palmer & Keller, 2010, showing the association of the trimeric complex (VelA-VeB-LaeA) and the requirement of external stimuli to enable the modification of heterochromatin to euchromatin allowing secondary metabolite production.

VeA was the first velvet protein discovered in *Aspergillus nidulans* in the 1960's, VeA point mutants showed an increase in conidia and a reduction in fruiting bodies

(Käfer, 1965; Bayram and Braus, 2012). In later studies deletion mutants of *Aspergillus* confirmed that VeA was linked to development and secondary metabolism, due to an inability to produce sexual fruiting bodies and mycotoxins. A lack of mycotoxin synthesis was determined to be due to an absence of transcripts for the corresponding biosynthetic enzymes, establishing a link between fungal secondary metabolism, development and biocontrol (Kato *et al*, 2003; Bayram and Braus, 2012). *T. hamatum* GD12 contains novel genomic regions in comparison to other *Trichoderma* genomes, these areas may encode for unique bioactive metabolites (Studholme *et al.*, 2013). Many gene clusters are seen within the *T. hamatum* genome which are key in the synthesis of SMs, i.e. polyketide synthases (Studholme *et al.*, 2013).

Secondary metabolism pathways are often affected in deletion mutants. Within *Aspergillus, Penicillium* and *Fusarium* species mycotoxin (i.e. aflatoxins, fumonsins, fusarin) and conidial production pathways have been shown to be altered as a result of VeA loss (Bayram and Braus, 2012). In *Trichoderma virens* deletion of Vel1 (VeA homologue) resulted in disruption to regulation of secondary metabolism encoding genes, including those for; glitoxin production, *O*-methyl transferase and cytochrome P450 (Mukherjee & Kenerley, 2010). Alterations were seen in condial production, mycoparastism and biocontrol ability (Mukherjee & Kenerley, 2010). Demonstrating that the secondary metabolism pathways affected in different fungi result in similar alteration in morphological and biocontrol characteristics.

We hypothese that interactions between *S. sclerotiorum* (or other soil dwelling pathogens) and GD12 results in chromatin re-modelling, activating cryptic gene clusters which were formerly dormant, producing unique SM PGP compounds. Previously work was conducted with the aim of deleting LaeA from the *Trichoderma* GD12 genome. Putative ∆*ThlaeA::hph* mutants were generated using a split marker method, which aims to replace the Lae gene ORF with a hygromycin resistance gene. Multiple round PCR reactions were performed to generate split marker cassettes. This project set out to use an alternative gene deletion cassette production method, yeast plasmid recombination, to enable VeA gene deletion and replacement with a hygromycin resistance gene. True mutants of LaeA and VeA, confirmed by southern blotting, will undergo morphological characterization to determine effect of gene loss on developmental function and biocontrol activities.

2. Materials & Methods

2.1. LaeA

2.1.1. Development of the LaeA construct

Please see the appendix for work previously undertaken to produce the LaeA construct. This work has been conducted by myself and presented in: Wills, O. (2013), Disruption of the Global Secondary Metabolism Regulator LaeA in *Trichoderma hamatum* GD12 (SWDTP lab rotation project 2). This work details how the knockout construct was created for the attempted deletion of the *LaeA*. This information is included for reference only.

Additional transformations

Two rounds of transformations (labelled as -1810 and -3110) were also performed to give 25 putative Δ*ThlaeA*::*hph* mutants, in addition to the 11 created before (labelled -0407). The transformations were undertaken using the methods as before (see appendix for 'Transformation of *Trichoderma hamatum* GD12').

2.1.2. Single spore isolation

All putative LaeA knockouts were twice single spore isolated (SSI) to ensure pure and stable colonies. To achieve this, fungal cultures were grown on V8 media (200ml V8 juice; 800ml dH_2O; 10g glucose; 20g agar) for 7-14 d under a 16h fluorescent light regime at 26^{o}C, to encourage a high level of growth and sporulation. Plates were agitated using sterile MilliQ water and the spore suspension was filtered through sterile Miracloth before being centrifudged at 148,000 rpm for 5 mins. The supernatant was decanted and spores re-suspended in 1 ml of sterile MilliQ water. A serial dilution was performed using a dilution factor of 1 in 10 (with vortexing between each dilution to ensure an even distribution) to create a step wise procedure starting from the stock spore suspension and resulting 1:1,000,000 spore dilution. 250 µl of dilutions 5 (1:100,000 of stock solution) and 6 (1:1,000,000 of stock solution) were plated out on PDA and hygromycin plates (383 mg/ml). Plates were allowed to dry in a laminar flow hood and grown at 26 ^{o}C under a 16h fluorescent light regime. Growth of plates was monitored to ensure that the individual colonies isolated originated from a single point of growth, and hence a single spore. Selected colonies were plated back onto V8

media, to repeat the SSI process, or onto PDA and hygromycin if colonies had already undergone two rounds of SSI.

2.1.3. DNA extraction

Please see appendix (under *Trichoderma hamatum* GD12 DNA extraction) for methods used to grow up liquid cultures of putative Δ*ThlaeA*::*hph* mutants and subsequent DNA extraction.

2.1.4. PCR reactions undertaken

Unless stated otherwise PCR reactions are undertaken by the following: GoTaq® Green Master Mix on a Pelitier thermal cycle 200. 25 µl samples were consisted of: 50 ng of DNA template, 12.5 µl GoTaq® Green Master Mix, 1 µl (0.2 pmol) each primer (forward and reverseand double processed tissue culture water (Sigma-Aldrich) to volume.

PCR products were separated via gel electrophoresis using 0.8% (w/v) agarose gels for 1.5 hours at 100 v, molecular weights were determined using a 1 kb ladder (Invitrogen)

2.1.5. PCR screening of putative Δ*ThlaeA*::*hph*

Each putative Δ*ThlaeA*::*hph* strain underwent four PCR reactions, as an initial screen, to determine whether these mutants contained the HYG ORF and lacked the laeA ORF (Table 1.). Those deemed successful by this method were taken forward for southern blotting.

PCR cycles were performed as follows: Initial denaturing 3 mins at 94^0C, denaturing 30 s at 94^OC, annealing (LF+ in ORF 30 s at 54^OC; LF + in HYG 30 s at 56^OC; RF + in ORF 30 s at 54^OC; RF + in HYG 30 s at 56^OC), extension (LF+ in ORF 70 s at 72^OC; LF + in HYG 90 s at 72^OC; RF + in ORF 60 s at 72^OC; RF + in HYG 90 s at 72^OC), final extension of 10 mins at 72^OC and hold at 15^OC.

	Name of reaction	Primers used		PCR gel band size (kb)
Successful Δ*ThlaeA*::*hph* mutants	LF + in HYG	KOC_LaeA_preLF	KOC_inHY	1.6
	RF + in HYG	KOC_LaeA_preRF	KOC_inYG	1.4
Unsuccessful Δ*ThlaeA*::*hph* mutants	LF + in ORF	KOC_LaeA_preLF	KOC_LaeA_inORF_LF	1.2
	RF + in ORF	KOC_LaeA_preRF	KOC_LaeA_inORF_RF	0.8

Table 1. Determination of successful Δ*ThlaeA*::*hph* mutants through PCR reactions (primer sequences: Figure A.4. and Table A.2. (appendix))

2.1.6. Digoxigenin-dUTP (DIG) southern blot analysis

2.1.6.1. PCR of the LaeA open reading frame and *hph* gene

PCR reactions were undertaken to construct the open reading frames of LaeA and Hygromycin (primer sequences: Figure A.5. and Table A.3. (appendix)) The LaeA open reading frame (ORF), using SKOC_LaeA_ORF_F and SKOC_LaeA_ORF_R (Table A3. (appendix)), and the hygromycin ORF, using SKOC_LaeA_HYG_F and SKOC_LaeA_HYG_R (Table A.3. (appendix)), were amplified by PCR from *T.hamatum* GD12 and Phyg plasmid respectively. PCR cycles were as follows; 94^{O}C for 3 mins, followed by 35 cycles of: 94^{O}C for 30 s, 56^{O}C for 30 s, 72^{O}C for 70 s (LaeA ORF) or 80 s (Hygromycin ORF), then one cycle at 72^{O}C for 10 mins. The samples were then held at 4^{O}C until use. Products were then purified using the gene clean method (see appendix 'Gene clean of PCR products' for details)

2.1.6.2. Amplification of the DIG labelling probe

The DIG labelled probe was created by PCR; 10µl HF buffer 5X (Promega), 5 µl DIG labelled nucleotides (Roche), 1 µl forward primer (LaeA ORF: SKOC_LaeA_ORF_F and Hygromycin ORF: SKOC_LaeA_HYG_F), 1 µl reverse primer (LaeA ORF:

SKOC_LaeA_ORF_R and Hygromycin ORF: SKOC_LaeA_HYG_R), 0.5 µl of Phusion® Taq DNA Polymerase (New England BioLabs, PROMEGA), and 75ng of gel purified DNA, and water to make the reaction to 50 µl. The cycle was run; 98^{O}C

for 30 s, followed by 35 cycles of: 98^{O}C for 10 s, 56^{O}C for 30 s, 72^{O}C for 35 s (LaeA ORF) or 40 s (Hygromycin ORF), then one cycle at 72^{O}C for 10 mins. The samples were then held at 4^{O}C until use. Products were then gel purified (see appendix 'Gene clean of PCR products' for details).

2.1.6.3. Digestion of genomic DNA

Genomic DNA was extracted from putative Δ*ThlaeA*::*hph* mutants as previously described (see appendix). Twenty units of enzyme – *Sal*I (Promega) for LaeA ORF and *Hind*III (Promega) for Hygromycin ORF - was added to 20 µg this genomic DNA and that of GD12 for a control. To this digestion 5 µl of buffer – D (Promega) for LaeA ORF and E (Promega) for Hygromycin ORF - was added with sterile milliQ water to a final volume of 25 µL. Restriction digests were incubated overnight at 37 °C. See appendix – Figure A.6. and Figure A.7. – for details of digestion cutting sites.

2.1.6.4. Confirmation of the Δ*ThlaeA*::*hph* mutants

Digested samples were run on a 0.8% TBE gel for 16 h at 10v. The gel was then laid with the wells downwards and depurated by adding 0.25M HCl and placing on a tilter shaker for 15 mins at 15rpm. Next the gel was neutralised by the addition of 0.4M NaOH and tilter shaken for 15 mins at 15 rpm.

A blot was constructed to enable the transfer of the digested DNA to a membrane. The large trough was filled with NaOH and a Perspex® plastic sheet placed over the middle. A long piece of Whatman paper was laid over the plastic 'bridge' to ensure that the blot remained moist with NaOH. The gel membrane was placed on top of the Whatman paper with the wells facing upwards. Capilary effect was enhanced by the addition of Seran™ wrap on each side of the gel. A section of Amersham Hybond-NX membrane (GE Healthcare), cut to the size of the gel, was placed on the surface of the gel, followed by two gel sized Whatman paper pieces. A stack of paper towels was placed on the top followed by another sheet of Perspex® plastic, and pressure applied. The blot was left overnight.

The blot was dismantled, retaining only the membrane which was added to a hybridization tube. To this tube 50 mL of southern hybridization buffer (500 mL 1M $NaPO_4$, 350 mL 20% SDS, 150 mL dH_20) was added and incubated for 30 min at 62^{O}C. The DIG labelled probe was added to 40 mL southern hybridization buffer and

boiled at 100^{O}C for 10 minutes for denaturation. This solution was added to the membrane and incubated for overnight at 62^{O}C. The probe was poured off and washed twice with Southern Wash Buffer (100 mL 1 M $NaPO_4$, 50 mL 20% SDS, 850 mL dH_2O) at 62^{O}C for 15 mins.

The membrane was removed from the hybridization tube and tilter shaken (15 rpm) at room temperature in DIG-wash buffer (0.3 % Tween 20 with DIG Buffer 1 (23.21 g maleic acid, 60 mL 5M NaCl, adjusted to pH 7 with NaOH)) for 5 mins. The solution was removed and DIG buffer 2 (1% skimmed milk powder in DIG buffer 1) added for 30 mins to enable blocking. Next the membrane was incubated with 40 mL of antibody solution (4µl anti-DIG AB (Roche) and 40 mL DIG buffer 2) for 30 mins. The membrane was then washed twice in DIG wash buffer for 15 mins, before being incubated for 5 mins in DIG buffer 3 (Tris-HCl (200 mL 1 M), NaCl (40 ml 5M) MgCl2 (100 mL 1M), dH_20 (1660 mL), adjusted to pH 9.5 with HCl). The membrane was removed from the tilter shaker and the buffer discarded. The membrane was placed in a plastic envelope and 1 mL of CDP-Star (Roche) solution was pipette over the surface and the solution left for 5 mins. The CDP-star was drained onto blue towel and sealed in a new plastic envelope onto a film cassette. The cassette was incubated at 37^{O}C for 30 mins. In a dark room the membrane was exposed to X-ray film (Fujifilm) and incubated for 5 mins (at room temp) before the film was developed.

2.2. VeA

2.2.1. VeA deletion cassette production using yeast plasmid recombination

Yeast recombination, using *Saccharomyces cerevisiae,* was used to create a deletion cassette to enable the replacement of the *VeA* open reading frame with a hygromycin resistance cassette. NODE_4214 was identified to contain the *VeA* ortholog within the fungus *Trichoderma hamatum* GD12, previously sequenced in-house (Le Cocq, 2012). Primers were developed to flank the VeA open reading frame (ORF), with the left flank encompassing 1 Kb upstream and the right flank encompassing 1.2 Kb downstream (Figure A.8.; Table A.4.). The larger size of this right flank was due to a 0.2 Kb un-sequenced region. The Phyg-YR, a *S. cerevisiae* plasmid with previously inserted hygromycin resistance marker, was used for this method.

2.2.2. Construction of the Left flank (LF) + HY and right flank (RF) + YG cassettes

PCR reactions were undertaken using Phusion® High-Fidelity PCR kit (NEB). Unless otherwise specified, samples contained: 0.25 µl Phusion® DNA polymerase, 5µl Phusion® HF, 0.5 µl dNTPs, 1.25 µl forward primer, 1.25 µl reverse primer, 50 ng DNA template, and double processed tissue culture water (Sigma-Aldrich) to make a total volume of 25 µl. PCR products were separated via gel electrophoresis by using 0.8% (w/v) agarose gels for 1.5 hours at 100v, and molecular weights were determined using a 1kb ladder.

2.2.2.1. Left flank (LF) + right flank (RF) amplification from GD12 and Hygromycin (HYG) from Phyg

The following cycling parameters were used for PCR: Initial denaturation 30 s at 98^{O}C, followed by 35 cycles of; denaturation (7.5 s at 98^{O}C); annealing (30 s at 72^{O}C for LF and RF; 30 s at 60^{O}C HYG); extension (30 s at 72^{O}C), and a final extension for 10 mins at 72^{O}C. Samples were held at 15^{O}C for immediate use or stored at -20^{O}C for later.

2.2.2.2. Gel purification of PCR products

A Wizard® SV gel and PCR clean-up system (NEB, Southampton, UK) was used to gel purify either excised gel DNA fragments post electrophoresis or PCR reactions mixes directly. Membrane binding solution was added to each gel slice as a 1:1 (v/w)

ratio. Samples were vortexted before being incubated at 65^{O}C in order to dissolve the gel fragment. For PCR amplifications an equal volume of membrane binding solution was added. For both procedures the DNA was then bound by inserting a mini-coloumn, inside a collection tube, and incubating for 1 min. Subsequently samples were centrifuged at 16,000 x *g* for 1 min; the flow-through was discarded. (see appendix 'Gene clean of PCR products' for details).

2.2.3. Digestion of Phyg-YR with *Eco*RI and *Hind*III

The pHYG-YR plasmid contains a trpC promoter and a hygromycin resistance region, these are neighboured by unique *Eco*RI and *Hind*III cut sites within the plasmid. The pHYG-YR plasmid (10 µg) was digested with the *Eco*RI (3 µl), *Hind*III (3 µl), 10 µl cut smart buffer and water to make a final volume of 100 µl. The digest was run out on a gel and the 9.5 kb band gel band of the linearized plasmid purified.

2.2.4. Plasmid construction by homologous recombination in yeast

S. cerevisiae yeast cells were grown at 30^{O}C overnight at 230 rpm in 3 ml of YPD medium (Yeast extract 5 g, Peptone 10 g, D-glucose 10 g, 500 ml dH_2O). 1 ml of this culture was added to 50 ml of YPD for 5 hours at 30^{O}C and 230 rpm. Cells were then spun down for 5 mins at 2200 rpm. The supernatant was discarded and the pellet re-suspended in 10 ml distilled water before being spun down again for 5 mins at 2200 rpm. Supernatant was removed and the pellet re-suspended in 300 µl of distilled water. Salmon sperm DNA - denatured at 100^{O}C for 10 mins prior to use – was incubated with 50 µl of *S. cerevisiae* cells; 3 µl of linearized plasmid; and 4 µl of the following (gel purified) PCR products: VeA left flank, VeA right flank and hygromycin. 32 µl 1 M Lithium acetate and 240 µl 50% PEG was added to this solution (mixed by pipetting) and tubes incubated for 30 mins at 30^{O}C. Samples were heat shocked for 15 mins at 45^{O}C. Solutions were then spun at 2200 rpm for 5 mins, supernantant removed, and the pellet re-suspended in 200 µl. 20 µl of this solution was removed and added to 180 µl dH_2O. The cells were plated out on YPD agar (500ml YPD medium and 10g agar) at 50 µl and 150 µl dilutions and incubated at 30^{O}C overnight.

2.2.5. Colony PCR

Colony PCR was performed by taking white colonies from the 50 µl and 150 µl dilution plates. A pipette tip was used to select colonies and then inserted into a reaction tube containing the left flank reverse primer (1 µl), HYsplit primer (1 µl), GoTaq® green master mix (12.5 µl) and dH_2O (10.5 µl). The subsequent cycle were undertaken: Initial denaturing 7 mins at 94^OC, followed by 35 cycles of; denaturing 30 s at 94^OC, annealing 30 s at 72^OC, extension 180 s at 72^OC, and one round of final extension; 72^OC for 10 mins. Samples were held at 15^OC until use.

2.2.6. Plasmid isolation from yeast (adapted from Singh & Weil, 2002)

Samples which indicated positive results (by colony PCR) were separated into two groups (1,4,5,+6 and 7,8+10 respectively) and added to flasks containing 10 ml Sc-ura, before being shaken at 30^OC and 230 rpm overnight.

Plasmid isolation was performed (based on the Singh and Veil protocol, 2002) using a Qiagen extraction kit. Cultures were spun down at 4000rpm to isolate cells, the supernatant was removed and cells repeated in 200 µl P1 buffer. Cells were re-suspened in 100 µl Lyticase solution (1.2M sorbitol; 0.1M $NaHP0_4$ buffer; PH 7.4, with 5 mg/ml Arthrobactor luteus lyticase) and incubated at 37^OC for 30 mins. 300 µl P2 (lysis) buffer was added to samples before being mixed by inversion and incubated at room temperature for 10 mins. Samples were mixed with 420 µl of N3 buffer and centrifuged for 10 mins at 148,000 x g. The supernatant was removed and added to QIA spin columns before being centrifuged for 1 min at 148,000 x g. Samples were washed with 500 µl PE buffer and centrifuged for 1 min at 148,000 x g. The column was then dried by spinning at 148,000 x g for 3 mins. Plasmids were eluted with EB buffer, incubated for 1 min at room temperature and spun for 2 mins at 148,000 x g.

2.2.7. Plasmid transformation into *Escherichia coli*

50 µl Ph5 alpha *Escherichia coli* competent cells were added to 5 µl eluted VeA yeast plasmids. These were incubated for 30 mins on ice before being heat shocked for 30 secs at 42^OC, before being returned to ice for 5 mis. 200 µl of LB media was then added to the reactions before being shaken at 37^OC for 90 mins. 30 µl and 220 µl

solutions were spread out onto LB and kanamycin plates (100 µg/ml) and incubated at 37^{O}C for 20 hours.

From the 30 µl diluted plates 10 colonies were picked by pipette tip and added to 5 ml LB and kanamycin (100 µg/ml) overnight at 37^{O}C 180rpm.

Four yeast lines, named VeA1, VeA2, VeA3 and VeA4, were taken forward. Overnight cultures of these samples were conducted by growing samples in 5 ml LB and kanamycin (100 µg/ml) overnight at 37^{O}C 180rpm.

2.2.8. Quiagen miniprep

Overnight cultures were spun at 8000rpm for 3 mins and the supernatant was discarded. 250 µl of P1 buffer was added and the pellet re-suspended. To this solution 250 µl of P2 buffer was added, the tube was inverted 6 times and then left at room temperature for 4 mins. Next 350 µl of N3 buffer was inserted and inverted 6 times before being centrifuged for 10 mins at 13,000rpm. Supernatant was transferred to a spin column and spun at 13,000 rpm for 1 min. The flow through was discarded and 500 µl of PB buffer was added before being spun 13,000 rpm for 1 minute. Flow through was removed and 750 µl of PE buffer was added, columns were then spun at 13,000 rpm for 1 min. Flow through was discarded and the empty tube spun at 13,000 rpm for 1 min to remove any ethanol residue. The capture column was incubated with 50 µl of water in for 1 min before being centrifuged at 13,000 rpm for 1 min. The flow through was retained.

2.2.9. Miniprep digestion (NotI and *Hind*III)

The minipreps of VeA1, VeA2, VeA3 and VeA4 were digested with NotI and *Hind*III, which gave differential band sizes for the pHYG plasmid and the pHYG plasmid with the VeA gene inserted (Figure 2.). Plasmids (5 µl) were each incubated for 3 hour at 37^{O}C with cut smart buffer (2 µl), NotI (1 µl), *Hind*III (1 µl) and water (11 µl).

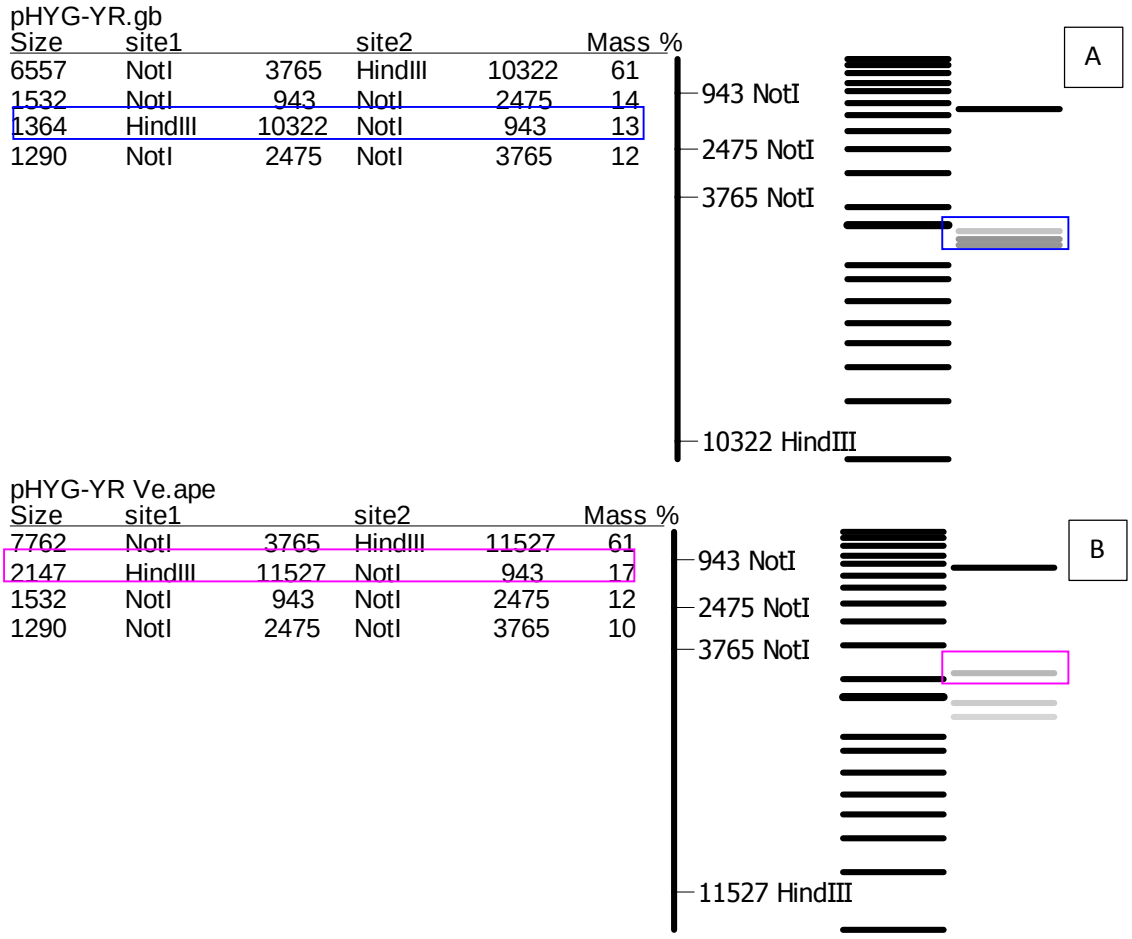


Figure 2. pHYG plasmid B. the pHYG plasmid with the VeA gene inserted. Blue and purple boxes highlight the differently band sizes obtained by digestion with NotI and *Hind*III.

2.2.10. Amplification of split-marker cassettes

PCR reactions were used to amplify out the split cassette using; GoTaq® green master mix (12.5 µl), primers left flank + HYsplit or right flank + YGsplit (1 µl of each), 25 ng VeA DNA and water (9.5 µl). Initial denaturation 5 min at 94^{O}C, followed by 35 cycles of; denaturation (30 s at 94^{O}C); annealing (30 s at 61^{O}C for LF+HY and 30 s at 60^{O}C RF+YG); extension (90 s at 72^{O}C for LF+HY and 60 s at 72^{O}C RF+YG), and a final extension for 10 mins at 72^{O}C. Samples were held at 15^{O}C for immediate use or stored at -20^{O}C for later.

Samples were run out on a 0.8% agarose TBE gel, fragments removed and gel purified (see appendix ‘Gene clean of PCR products’ for details).

2.2.11. Sequencing of recombinated *Saccharomyces cerevisiae* plasmid

The recombinated *S. cerevisiae* plasmid - containing the inserted left and right flanks in addition to the trpC promoter and hygromycin resistance cassette - was sent for sequencing. For each sample 2 µm of primer (see Table A.5. (appendix)) was added to 1600 ng of DNA and dispatched to Eurofins Genomics.

2.2.12. Transformation of *Trichoderma hamatum* GD12

Please see the appendix “Transformation of *Trichoderma hamatum* GD12” to see the method used.

2.2.13. Single hyphae isolation

As all mutants obtained were un-sporulating single hyphae isolation was performed, instead of single spore isolation, to ensure pure and stable colonies.

For each sample: a 3mm plug was taken from a PDA and hygromycin plate (383 mg/ml). This plug was plated onto water agar (8 g agar/ 1 L dH_2O) and allowed to grow for up to 7 ds. This minimal nutrient plate encouraged dispersed hyphal growth, allowing easy isolation of a single hyphal strand. This strand was removed and reinstated on to a PDA and hygromycin plate and allowed to grow. From this culture a single hyphae was again removed and placed onto a PDA and hygromycin plate. After a period of up to 7 d growth samples were placed onto PDA plates for future work.

2.2.14. DIG southern blot analysis

2.2.14.1. PCR of the VeA open reading frame and Hygromycin

PCR reactions were undertaken to construct the open reading frames of VeA and Hygromycin (see appendix for ‘PCR of the VeA open reading frame and Hygromycin’ Table A.6. and Figure A.10. for details of primer sequences). The VeA open reading frame (ORF), using SKOC_VeA_ORF_F and SKOC_VeA_ORF_R (Table A.6. (appendix)), and the hygromycin ORF, using SKOC_LaeA_HYG_F and SKOC_LaeA_HYG_R (Table A.6.(appendix)), were amplified by PCR from *T.hamatum* GD12 and Phyg plasmid respectively. PCR cycles were as follows for VeA ORF: 94^{O}C for 5 mins, followed by 35 cycles of: 94^{O}C for 30 s, 55^{O}C for 30 s, 72^{O}C for 100 s, then one cycle at 72^{O}C for 10 mins. PCR cycles were as follows for Hygromycin ORF: 98^{O}C for 30 s, followed by 35 cycles of: 98^{O}C for 30 s, 56^{O}C for 30

s, 72^{O}C for 45 s, then one cycle at 72^{O}C for 10 mins. The samples were then held at 4^{O}C until use. Products were then gel purified (see appendix 'Gene clean of PCR products' for details).

2.2.14.2. Amplification of the DIG labelling probe

The DIG labelled probe was created by PCR; 10µl HF buffer 5X (Promega), 5 µl DIG labelled nucleotides (Roche), 1 µl forward primer (VeA ORF: SKOC_VeA_ORF_F and Hygromycin ORF: SKOC_LaeA_HYG_F), 1 µl reverse primer (VeA ORF: SKOC_VeA_ORF_R and Hygromycin ORF: SKOC_LaeA_HYG_R), 0.5 µl of Phusion® Taq DNA Polymerase (New England BioLabs, Promega), and 75ng of gel purified DNA, and water to make the reaction to 50 µl. The cycle was run; 98^{O}C for 30 s, followed by 35 cycles of: 98^{O}C for 30 s, 56^{O}C for 30 s, 72^{O}C for 45 s (VeA ORF) or 40 s (Hygromycin ORF), then one cycle at 72^{O}C for 10 mins. The samples were then held at 4^{O}C until use. Products were then gel purified (see appendix 'Gene clean of PCR products' for details).

2.2.14.3. Digestion of genomic DNA

Genomic DNA was extracted from putative the Δ*ThVeA*::*hph* mutants as previously described (see appendix). 20 units of enzyme – XhoI (Promega) for VeA ORF and for Hygromycin ORF - was added to 20 µg this genomic DNA and that of GD12 for a control. To this digestion 5 µl of buffer D (Promega) was added with sterile milliQ water to a final volume of 25 µL. Restriction digests were incubated overnight at 37°C. See appendix – A.11.and Figure. A.12.– for details of digestion cutting sites.

2.2.14.4. Confirmation of the Δ*VeA*::*hph* mutants

See section 'Confirmation of the Δ*ThlaeA*::*hph* mutants' for description of DIG based southern blotting confirmation procedure.

2.2.15. Phenotypic analysis

2.2.15.1. Growth curves

T. hamatum GD12 and Δ*ThVeA*::*hph* confirmed mutants were grown on 15 cm PDA square petri dishes at 26^{O}C under a 16 hr light regime, in both dark and light conditions. An Epson Perfection V750 Pro scanner was used to scan plates daily over a 7 day period. Mycelia growth (mm^2) was measured using imageJ.

2.2.15.2. Spore count

After 7 days *T. hamatum* GD12 and Δ*ThVeA*::*hph* growth curve plates were used to perform spore counts. Plates were washed using 20 ml of sterile MilliQ water and filtered through Mira cloth. Solutions were centrifuged at 148,000 rpm for 5 mins and the spores re-suspended in 1 ml of sterile MQ water before being counted using a haemocytometer.

2.2.15.3. Confrontation assays with *Sclerotinia sclerotiorum*

To determine the effect of loss of VeA biocontrol mutants (1605-14, 1605-21, 1605-22) and GD12 (control) were confronted with various *S. sclerotiorum* strains (BF5, GFR1, GFR11, M448). Mycelia plugs (5 mm) were taking from the leading edge of 2 d old cultures and placed at opposite ends of a standard PDA petri dish (9cm). Plates were incubated at 26^{O}C under a 16 hr light regime and scanned 6 dpi to view interactions.

2.2.15.4. Sclerotia number and weight from 12 d old confrontation assays with S*clerotinia sclerotiorum*

12 d old *S. sclerotiorum* confrontation assay plates were scanned and the sclerotia counted. Sclerotia were removed and dried in 1.5ml tubes at 70^{O}C for 21 hours; the dry weight (mg) of these samples were then recorded.

2.2.16. Statistical analysis

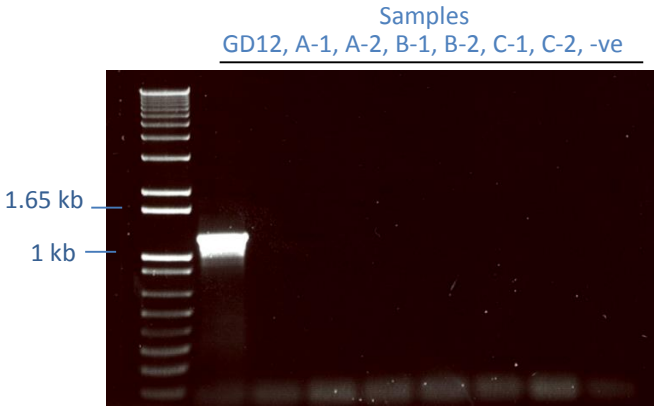
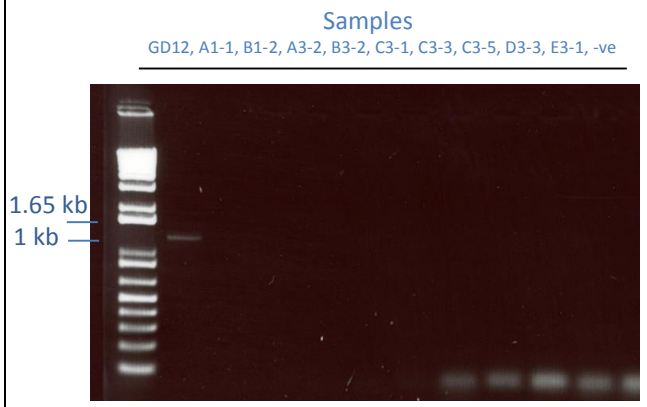
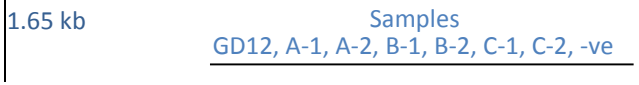
All statistical analysis was performed using R. Means of more than two groups were compared via ANOVA. Post hoc Tukey-Kramer analysis was subsequently performed to determine which groups were significantly different from one another.

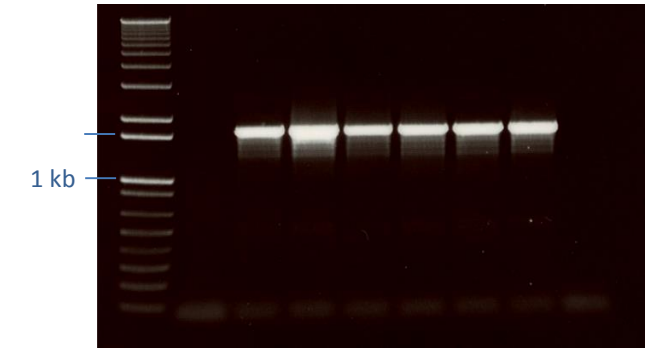
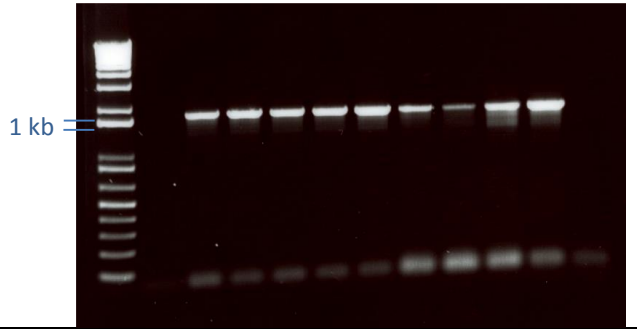
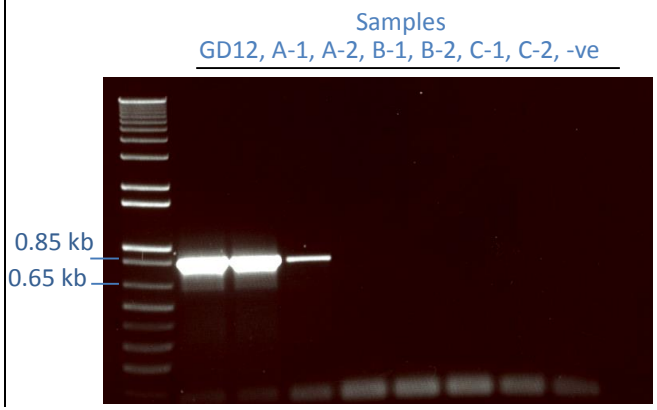
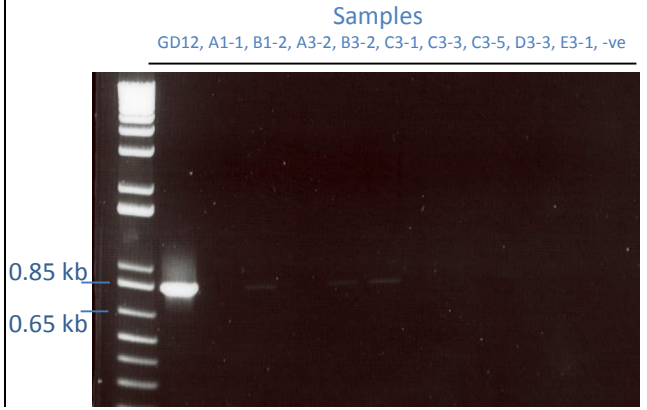
3. Results

3.1. LaeA

3.1.1. PCR screening of putative Δ*ThlaeA*::*hph*

The split marker method of homologues recombination was used to construct Δ*ThlaeA*::*hph* mutants by substituting the laeA ORF with a hygromycin resistance cassette. Samples were single spore isolated leaving 15 putative Δ*ThlaeA*::*hph* mutants remained. These samples underwent PCR screening. PCR results indicated that samples B0407-1, B0407-2, C0407-1, C0407-2, A3110-2, C3110-3, C3110-5 and E3110-1 (Table 2.) were potential Δ*ThlaeA*::*hph* mutants and were thus taken forward for southern blotting.

	Δ*ThlaeA*::*hph* mutants -0407	**Δ*ThlaeA*::*hph* mutants -1810 and -3110**	**Result**
Pre LF + in ORF			Unsuccessful Δ*ThlaeA*::*hph* mutants (band at 1.2 Kb)
Pre LF + in HY			Successful Δ*ThlaeA*::*hph*

			mutants (band at 1.6 Kb)
Pre RF + in ORF			Unsuccessful Δ*ThlaeA*::*hph* mutants (band at 0.8 Kb)

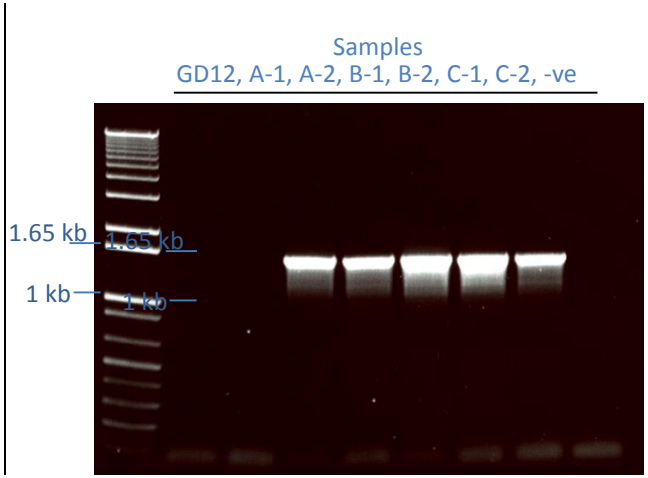
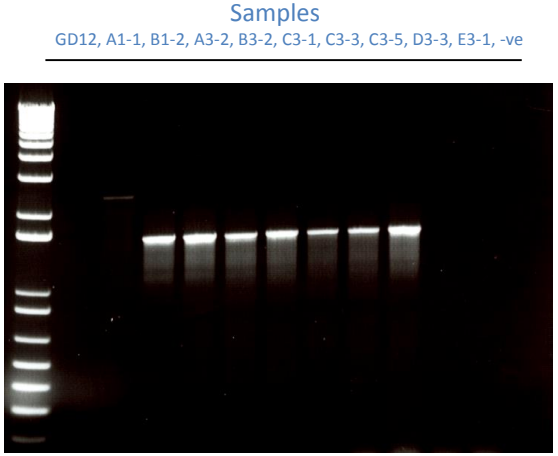
Pre RF + in YG			Successful Δ*ThlaeA*::*hph* mutants (band at 1.2 Kb)

Table 2. Electrophoresis gel images of putative Δ*ThlaeA*::*hph* mutants
Δ*ThlaeA*::*hph* mutant -0407 samples: GD12, A0407-1, A0407-2, B0407-1, B0407-2, C0407-1, C0407-2, -ve and Δ*ThlaeA*::*hph* mutant -1810 and -3110 samples: GD12, A1810-1, B1810-2, A3110-2, B3110-2, C3110-1, C3110-3, C3110-5, D3100-3, E3110-1, -ve. Lane 1 of all images contains a standard ladder to determine molecular weight (see appendix for ladder guides).

3.1.2. DIG southern blotting

3.1.2.1. Amplification of DIG-labelled probe

Southern blot probes were created and gel purified probes were checked to check for purity (Table 3.).

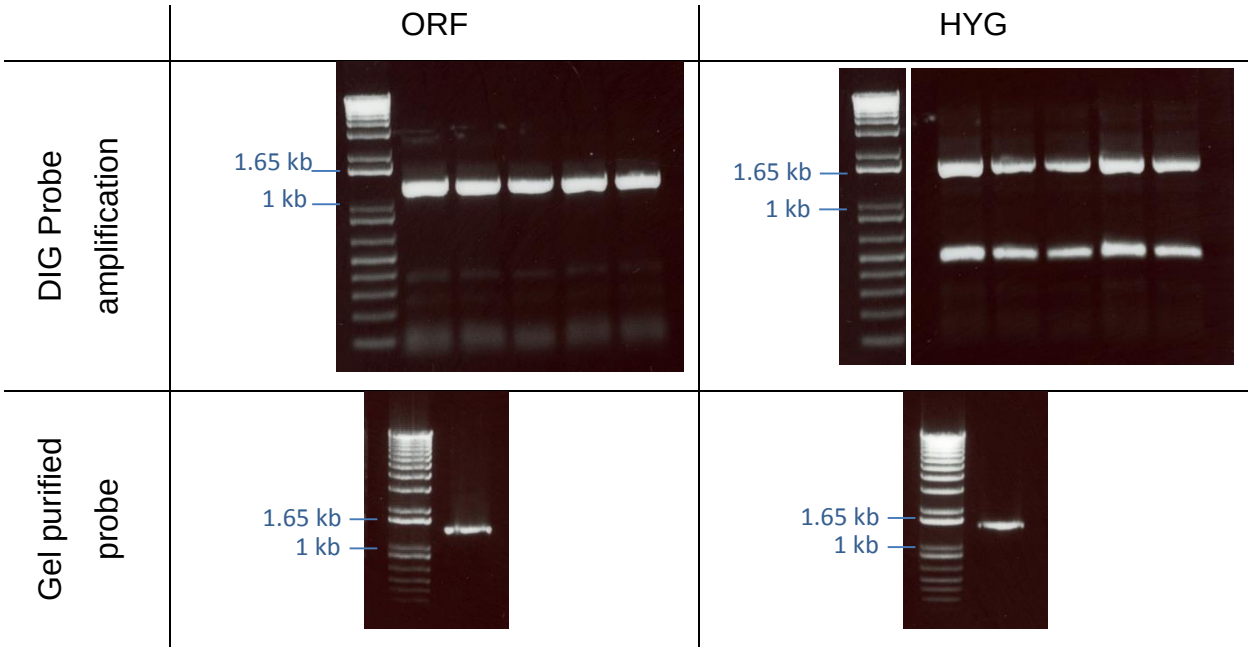


Table 3. Gel electrophoresis of DIG southern blot probes.
Lane 1 of all images contains standard ladders to determine molecular weight (see appendix for ladder guides).

3.1.2.2. DIG- Southern blot

Southern blot analysis was performed using a DIG labelled probe of either the LeaA ORF or Hygromycin ORF. Within the LaeA ORF a band size of 3.5 Kb indicates a wild-type strain (Table 4.). For the Hygromycin ORF a band of 3.14 Kb size indicates a knockout strain (Table 4.). A band of 3.14 kb on the Hygromycin ORF blot demonstrates the presence of Hygromycin. An absence of a band at 3.5 kb in the LaeA ORF blot suggests a knockout. Additional bands are however seen at 2.2 kb and 1.5 kb (Table 4.), indicating multiple gene copies may be present, although confirmation of this would further investigation.

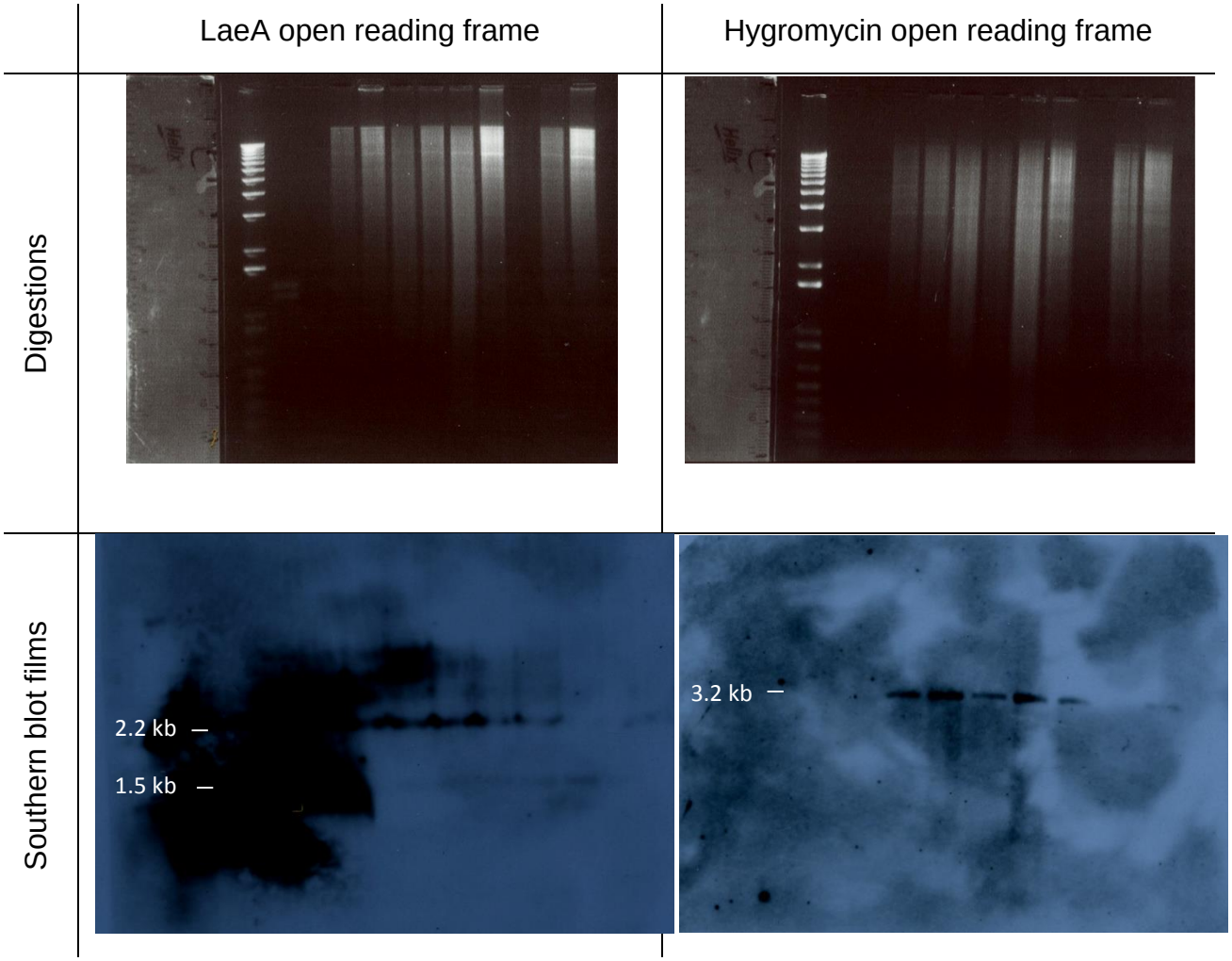


Table 4. Southern blot digestions and film development of the LaeA ORF and Hygromycin ORF.

For both LaeA ORF and Hygromycin ORF gel and film sample orders are as follows: Ladder, Positive control (40 ng of ORF or Hygromycin DNA), space, GD12 (control), B0407-1, B0407-2, C0407-1, C0407-2, A3110-2, C3110-3, C3110-5 and E3110-1.

3.2. *VeA* Results

3.2.1. VeA deletion cassette production using yeast plasmid recombination

Yeast recombination was used to construct VeA deletion cassette. These cassette were used in a standard split marker gene deletion method producing Δ*ThVeA*::*hph* mutants by the replacement of the VeA ORF with a hygromycin resistance cassette. PCR reactions were used to generate the VeA left flank (1 kb), VeA right flank (1.2 kb), and the trpC + hygromycin resistance cassette (1.5 kb) (Figure 5.). These products were gel purified and, with a linearized plasmid, added to *S. cerevisiae* cells to enable recombination (Figure 5.). Successfully recombinated plasmids – containing VeA left flank, trpC + hygromycin resistance and VeA right flank - were identified using colony PCR (Figure 3.) and miniprep digestions (NotI and *Hind*III, Figure 4.).

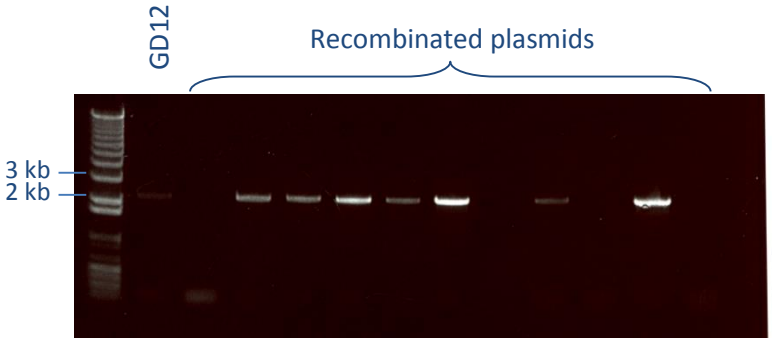


Figure 3. Colony PCR of GD12 and recombinated plasmid samples. Successful samples displayed a band size of 2.5 Kb.

3.2.2. Miniprep digestion (NotI and *Hind*III)

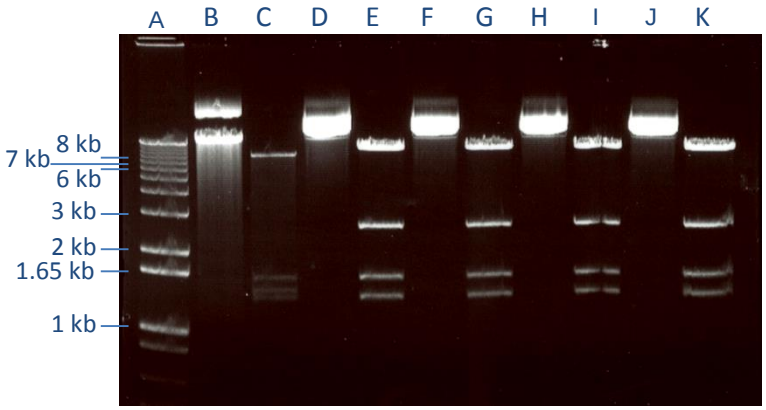
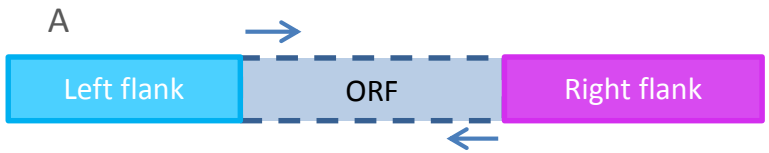
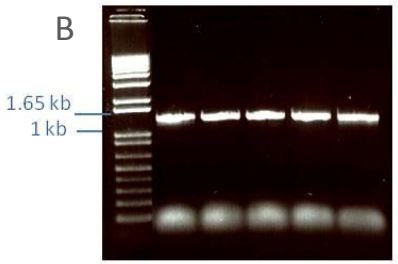


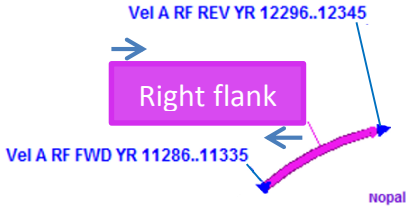
Figure 4. VeA recombinated plasmids
A) Ladder, B) GD12 undigested, C) GD12 digested, D) VeA 1 undigested, E) VeA 1

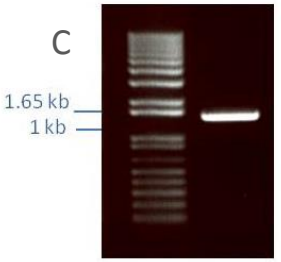
digested, F) VeA 2 undigested, G) VeA 2 digested, H) VeA 3 undigested, I) VeA 3 digested, J) VeA 4 undigested, K) VeA 4 digested.

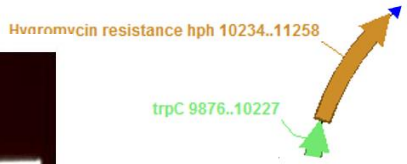
PCR was used to amplify the split marker cassette from the recombinated yeast plasmid to give LF + HY (2.4 Kb) and RF + YG (1.9 Kb) (Figure 5.). Gel purified product were then used to transform fungal protoplasts. If the LF + HY and RF + YG homogenously recombine, in the correct orientation, then the resistance cassette displaces the VeA ORF, leading to hygromycin resistance (Figure 5.).


A
Left flank
ORF
Right flank

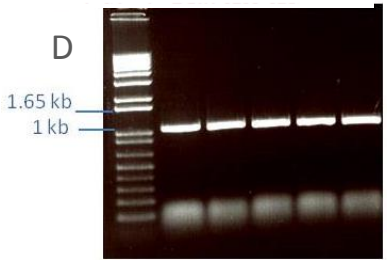

B
1.65 kb
1 kb

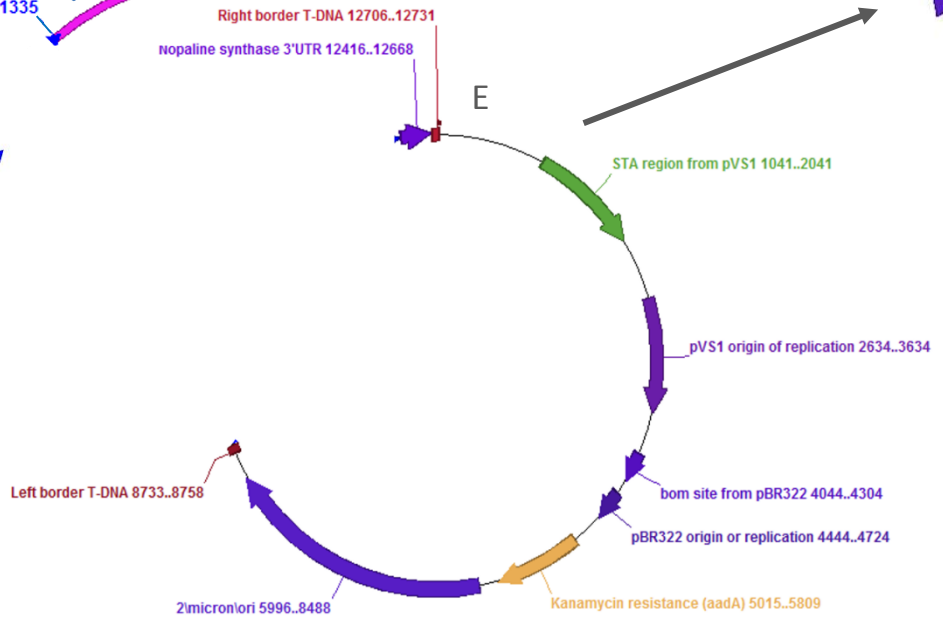

Vel A RF REV YR 12296..12345
Right flank
Vel A RF FWD YR 11286..11335

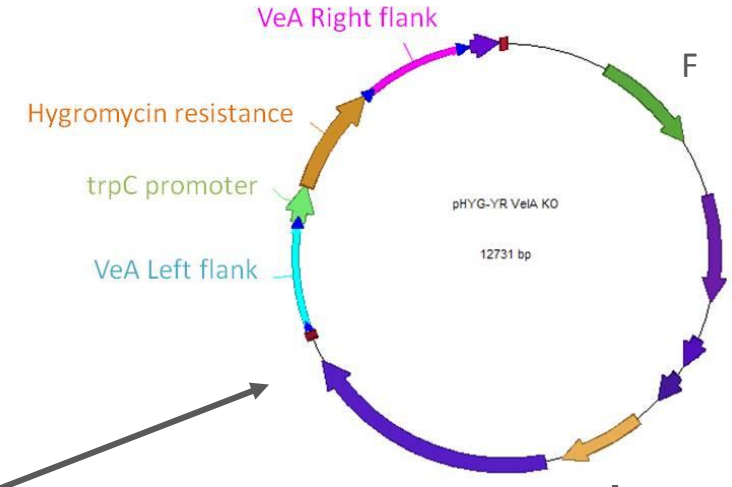

C
1.65 kb
1 kb

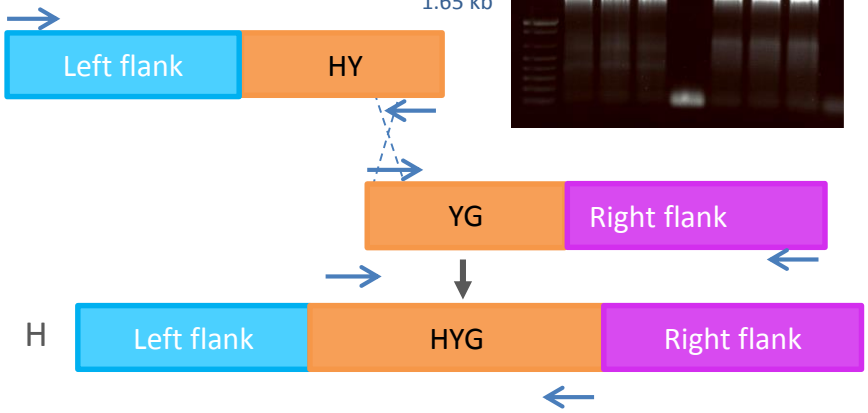

Hygromycin resistance hph 10234..11258
trpC 9876..10227


Vel A LF REV YR 9840..9889
Left flank
Vel A LF FWD YR 8830..8879


D
1.65 kb
1 kb


Right border T-DNA 12706..12731
nopaline synthase 3'UTR 12416..12668
E
STA region from pVS1 1041..2041
pVS1 origin of replication 2634..3634
bom site from pBR322 4044..4304
pBR322 origin or replication 4444..4724
Kanamycin resistance (aadA) 5015..5809
2\micron\ori 5996..8488
Left border T-DNA 8733..8758


VeA Right flank
F
Hygromycin resistance
trpC promoter
VeA Left flank
pHYG-YR VelA KO
12731 bp


Left flank
HY
YG
Right flank
H
Left flank
HYG
Right flank

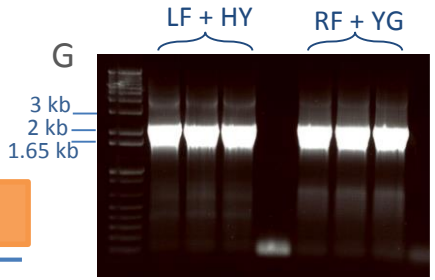

LF + HY
RF + YG
G
3 kb
2 kb
1.65 kb

Figure 5. VeA deletion strategy used to generate Δ*ThVeA*::*hph* mutants. A: VeA Left flank, VeA Right flank and VeA ORF in *T. hamatum* GD12 are graphically represented. VeA ORF forward and reverse primer locations for Southern blot analysis indicated by arrows. Left and right flanks were amplified from GD12 and PCR products used for yeast plasmid recombination. B: VeA right flank (1.2 kb - amplification from GD12), C: trpC + hygromycin resistance cassette (1.5 kb - amplification from Phyg-YR), D: VeA left flank (1 kb - amplification from GD12) and E: graphical representation of linearized Phyg-YR, F: graphical representation of successfully recombined yeast plasmid, G: Split marker cassette amplification from the successfully recombined yeast plasmid results in PCR products: LF (left flank) + HY (2.4 Kb) and RF (right flank) + YG (1.9 Kb), H: Homologous recombination of split marker cassette products results in the HYG resistance cassette replacing the VeA ORF. HYG forward and reverse primer locations for Southern blot analysis indicated by arrows. All far left hand lanes contain ladders (see appendix for ladder guides) to determine PCR products size (Kb). Some figure were created using modified images from ApE (plasmid editor).

3.2.3. Sequencing of recombinated *Saccharomyces cerevisiae* plasmid

One insertion was seen in the hygromycin resistance cassette within the recombinated yeast plasmid (Please see Figure A.9. (appendix)). Hygromycin resistance was still observed in mutants and therefore the insertion was deemed to not have an effect on resistance. No other mutations were observed indicating the successful recombination of the left flank, trpC promoter, hygromycin resistance cassette and right flank within the *S. cerevisiae* plasmid.

3.2.4. DIG- Southern blot

Southern blot analysis was performed using a Digoxigenin-dUTP labelled probe of either the VeA ORF or Hygromycin ORF. Within the VeA ORF blot a band size of 4.3 Kb indicates a wild-type strain and an absence of a band signifies a lack of VeA ORF. For the Hygromycin ORF blot a band of 4.1 Kb size indicates a knockout strain and an absence of a band signifies a lack of Hygromycin ORF. Samples 1605-14, 1605-15, 1605-21 and 1605-22 were shown to be successful Δ*ThVeA*::*hph* mutants with a lack of bands in the VeA ORF blot and a single band at 4.1 Kb in the hygromycin ORF blot (Table 5.). Samples 1605-17 and 1605-28 showed multiple bands in the hygromycin ORF blot and for 1605-17 a band at 4.3 Kb in the VeA ORF blot for 1605-17 (Table

5.). These two samples therefore contain hygromycin ectopic insertions and are not successful Δ*ThVeA*::*hph* mutants.

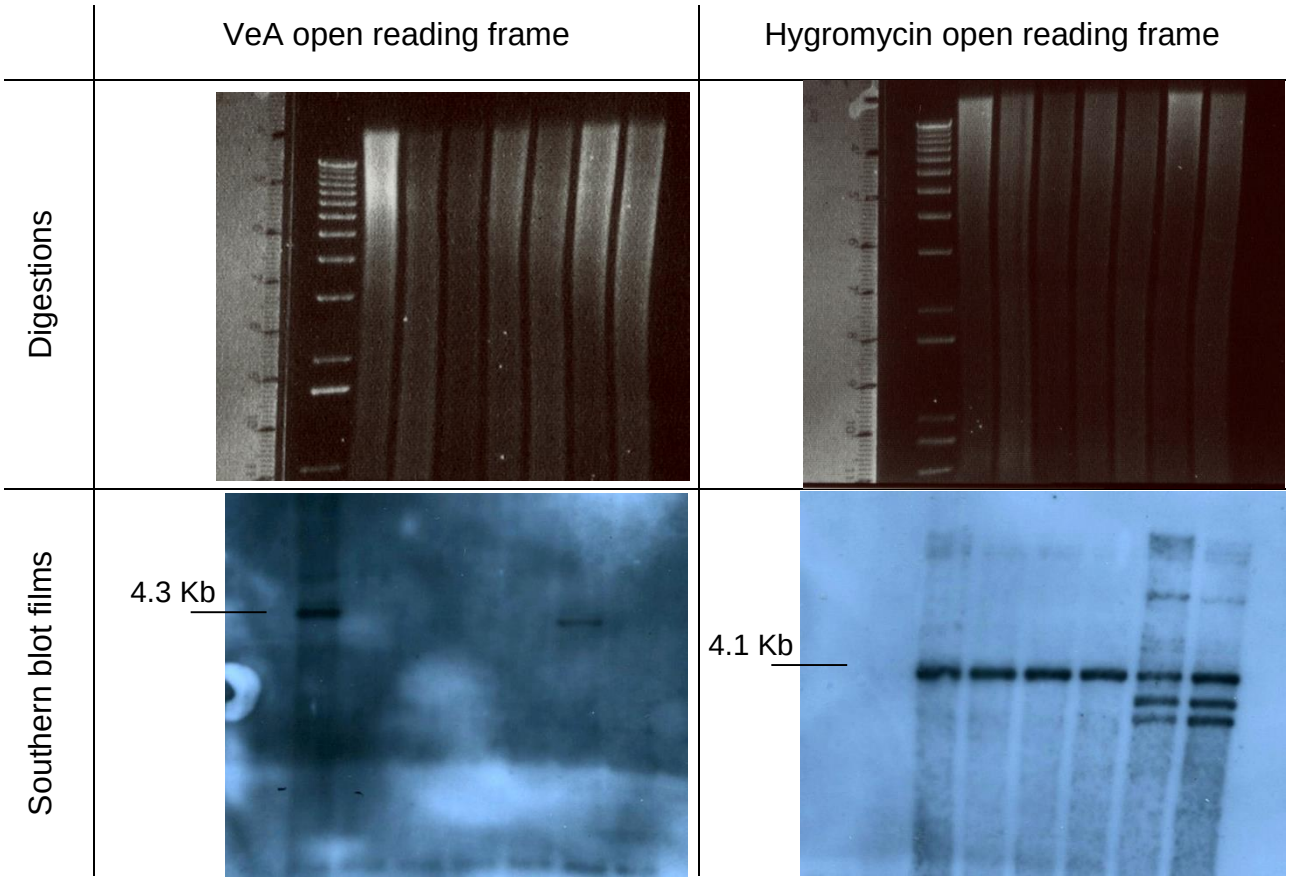


Table 5. Southern blot digestions and film development of the VeA ORF and Hygromycin ORF. For both VeA ORF and Hygromycin ORF gel and film sample orders are as follows: Ladder, GD12 (control), 1605-22, 1605-21, 1605-14, 1605-15, 1605-17, 1605-28

Samples 1605-14, 1605-21and1605-22 were chosen as three successful mutants and taken forward for phenotypic characterisation.

3.2.5. Phenotypic analysis

3.2.5.1. Growth curves + Spore count

Δ*ThVeA*::*hph* strains and GD12 were grown, on PDA plates, under light and dark conditions to view the effect of loss of VeA on mycelial growth. Plates were scanned daily for 7 d and the area (mm^2) was calculated using imageJ. In both light and dark conditions the mutants growth was slower and took 6 d to reach the edge of the petri dish rather than the 4 d taken by GD12 (Figure 7.). Spore counts were performed on these growth analyses plates. Δ*ThVeA*::*hph* mutants showed statistically significant different spore production from GD12, as determined by a one-way ANOVA (Light: $F(3,8)=51.37$, $p<0.01$ and dark: $F(3,8)=50.72$, $P<0.01$) (Figure 7.).

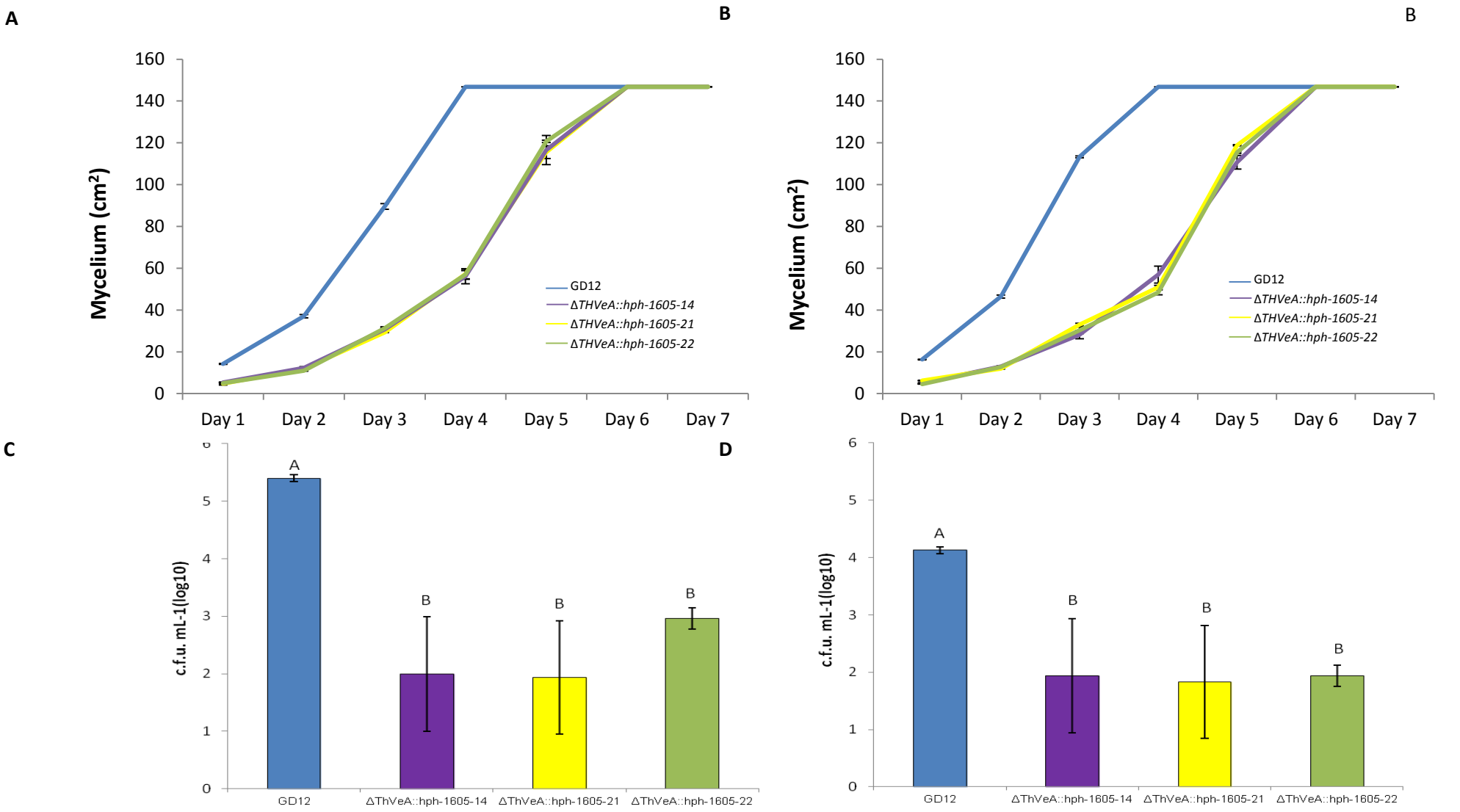


Figure 6. Growth of mycelium (cm^2) under both light (A) and dark (B) conditions and Log10 spore count of 7 d old petri dishes kept under both light (C) and dark (D) conditions. Samples with different letter are statistically significantly different from one another and ± bars represent standard error from the sample mean.

3.2.5.2. Confrontation assays with *Scelerotinia*.

T.hamatum GD12 and Δ*ThVeA*::*hph* mutants (top half of plates) were confronted against multiple S*clerotinia sclerotiorum* strains (base of plates) to determine the role of VeA in pathogenicity (Figure 8.). The faster growing wild type GD12 overgrew *S. sclerotiorum* strains, whereas the slower growing Δ*ThVeA*::*hph* mutants were incapable performing this action (Figure 8.). *S. sclerotiorum* showed visually increased sclerotia production against VeA deletion mutants compared to GD12 confrontations (Figure 8.).

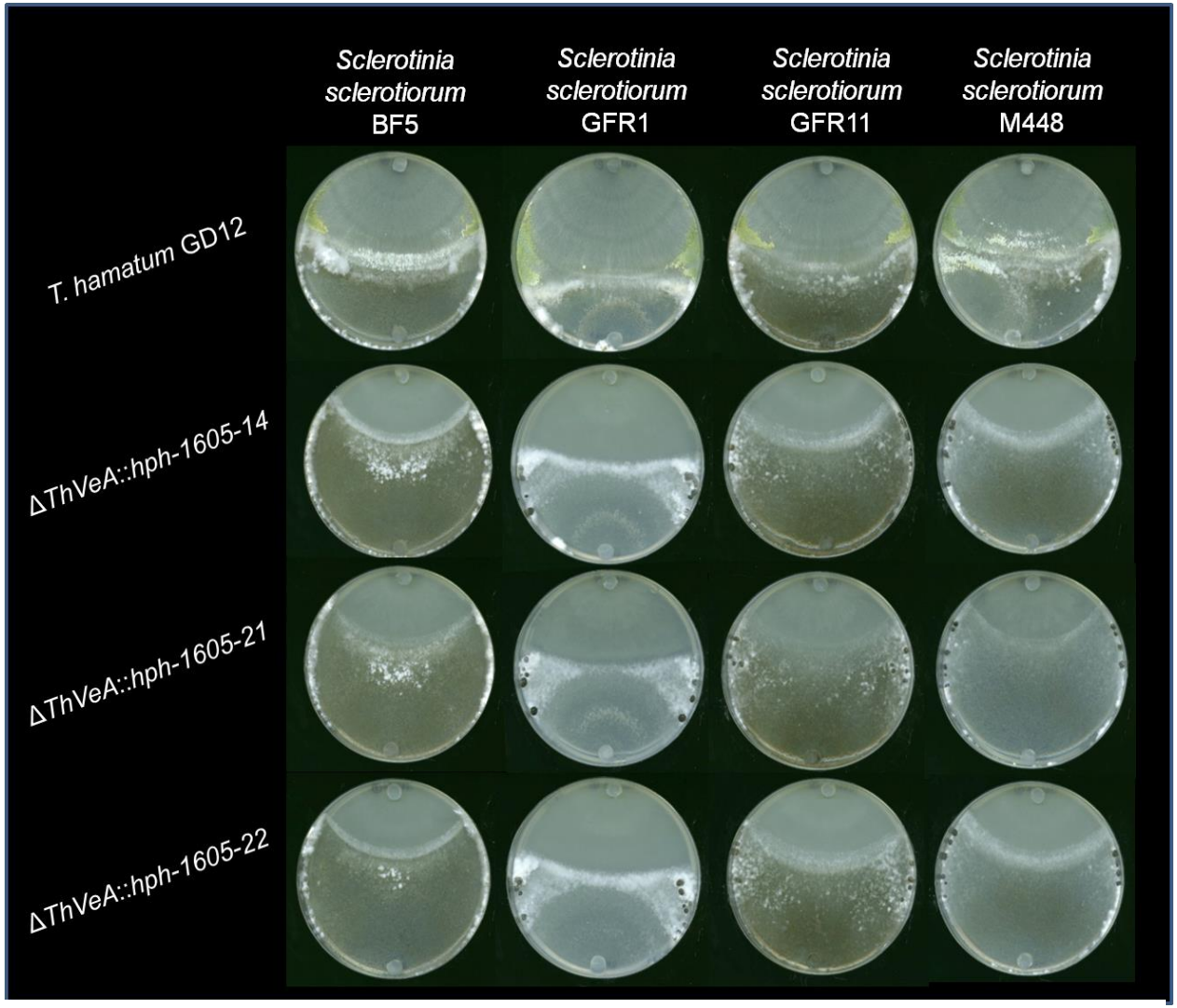


Figure 7. 6 d old confrontation plates of *T. hamatum* GD12 and mutants against various *Sclerotinia scletotiorum* strains.
All plugs at the top of plates are *T. hamatum* (GD12 or mutants) and all plugs at bottom of plates are from pathogens.

3.2.5.3. Sclerotia number and weight from confrontation assays with *Sclerotinia*

Confrontation plates of *T.hamatum* GD12 and Δ*ThVeA*::*hph* mutants against *Sclerotinia sclerotiorum* were grown for 12 d. The sclerotia from these plates were counted, dried and their final weight was taken. One way ANOVA determined that for each *Sclerotinia* species that the sclerotia number (BFS: $F(3,8)=87.03$, $P<0.01$; GFR1: $F(3,8)=20.23$, $P<0.01$; GFR11: $F(3,8)=9.34$, $P<0.01$; M448: $F(3,8)=28.45$, $P<0.01$) and weight (BFS: $F(3,8)=45.82$, $P<0.0$; GFR1: $F(3,8)=30.1$, $P<0.01$; GFR11: $F(3,8)=13.39$, $P<0.01$; M448: $F(3,8)=52.81$, $P<0.01$) were significantly different between groups. GD12 was shown to be significantly reduced in both sclerotia number and weight in comparison to Δ*ThVeA*::*hph* mutants (Figure 9. and Figure 10.).

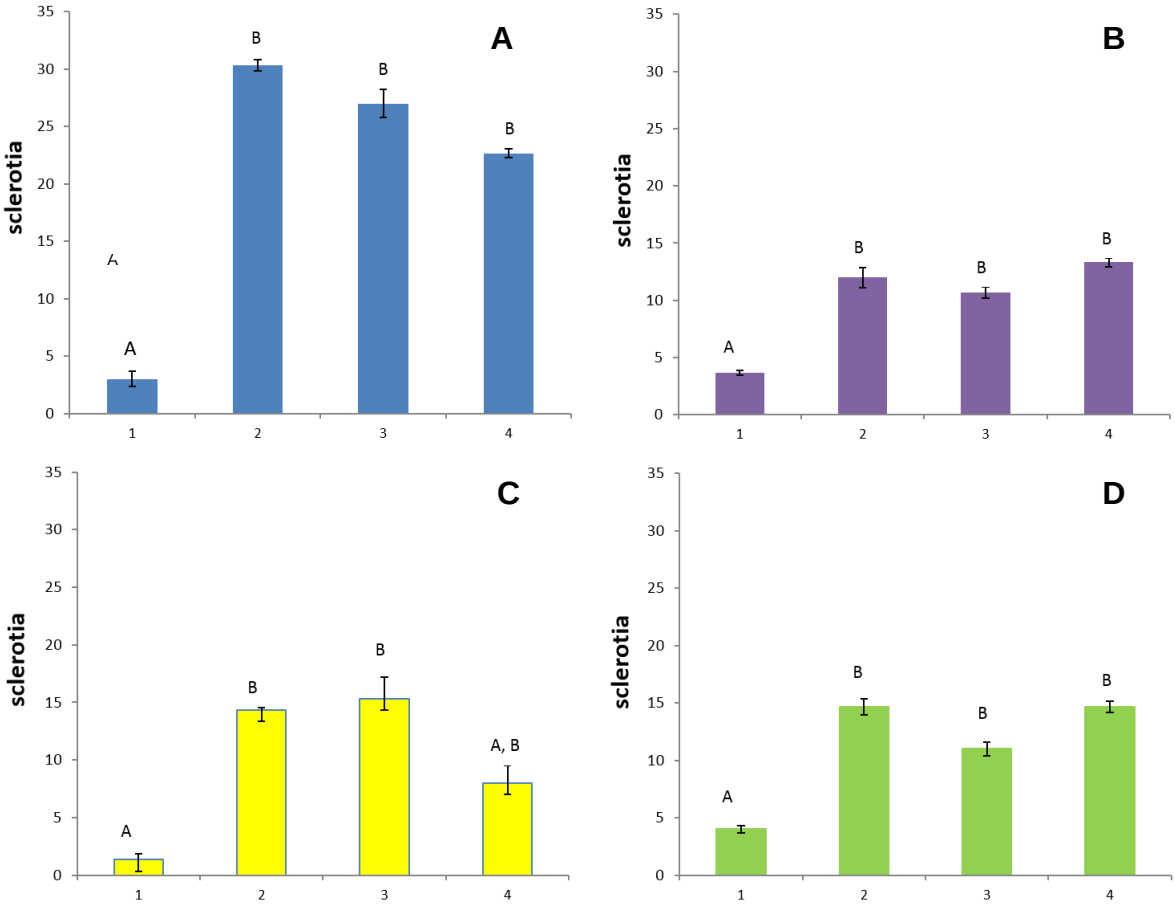


Figure 8. Average petri dish Sclerotia counts from 12 d old confrontation plates. Graphs: A) BFS, B) GRF1, C) GRF11 and D) M448. For each graph samples are as follows: 1) *T. hamatum* GD12, 2) *ΔThVeA::hph*-1605-14, 3) *ΔThVeA::hph*-1605-21 and 4) *ΔThVeA::hph*-1605-22. Within each graph samples with different letter are statistically significantly different from one another.

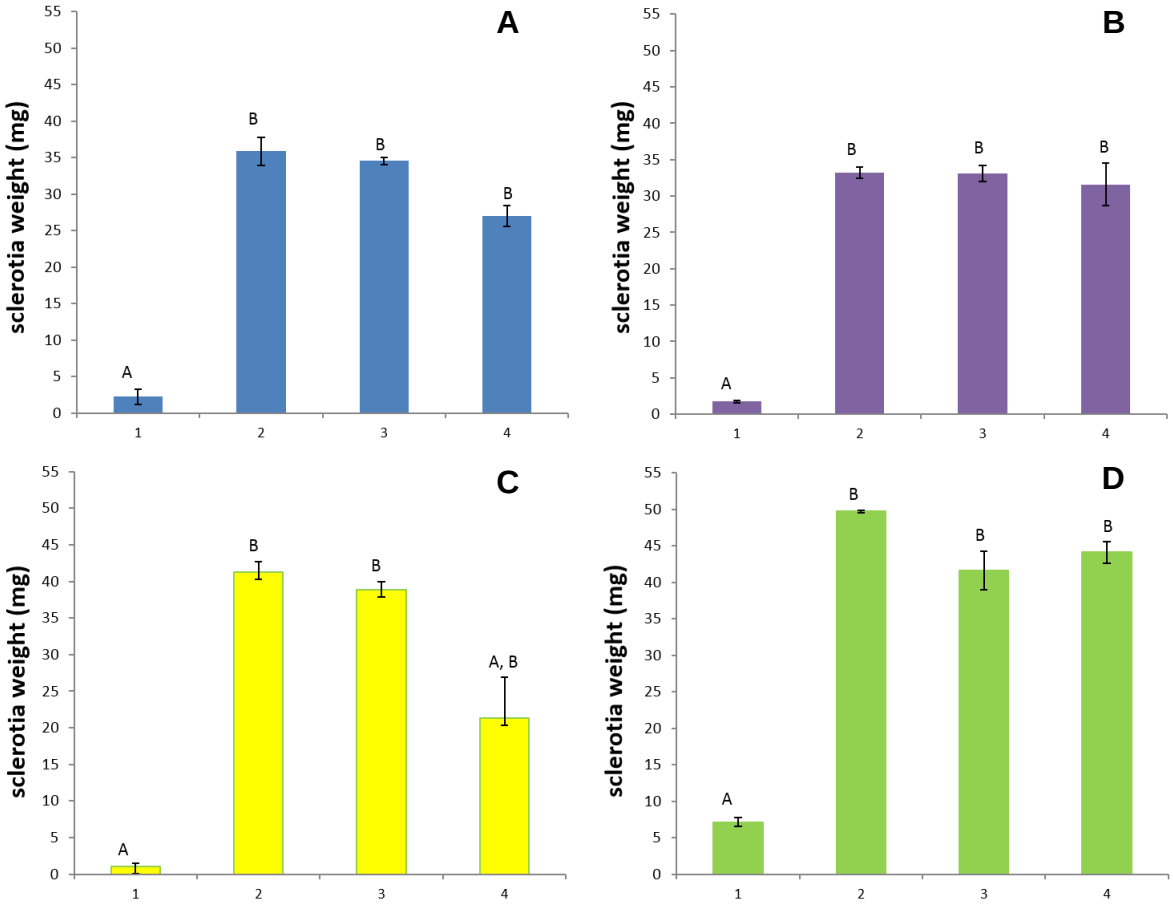


Figure 9. Average petri dish Sclerotia weights from 12 d old confrontation plates.
Graphs: A) BFS, B) GRF1, C) GRF11 and D) M448. For each graph samples are as follows: 1) *T. hamatum* GD12, 2) *ΔThVeA::hph*-1605-14, 3) *ΔThVeA::hph*-1605-21 and 4) *ΔThVeA::hph*-1605-22. Within each graph samples with different letter are statistically significantly different from one another.

4. Discussion

Two different deletion cassette construction methods were used to produce templates for the Velvet trimeric complex genes LaeA and VeA. These constructs were then used in split marker gene deletion with the aim of replacing the ORF with a hygromycin resistance cassette. LaeA and VeA in combination with VelB form a trimeric complex (VelA-VeB-LaeA) (Palmer and Keller, 2010). Studies in *Aspergillus nidulans* have shown within light conditions LaeA represses VeA and VelB, resulting in asexual development, whereas, under dark conditions levels of LaeA decrease allowing the proliferation of velvet proteins and the production of sexual spores (Bayram and Braus, 2012). The functions of trimeric aspects LaeA and VeA are not known within *Trichoderma hamatum* GD12. Here we set out to determine the roles played by these two velvet complex elements in biocontrol and PGP activity.

Multiple putative LaeA mutants were screened; all mutants showed ectopic or unsuccessful hygromycin insertions. Southern blot analysis showed a band at 3.14 Kb as expected for hygromycin presence. For the LaeA ORF a lack of expected band at 3.5 Kb should suggest deletion mutants; however additional 2.5 Kb and 1.5 Kb were seen. This could suggest the presence of multiple LaeA gene copies. Further analysis of the LaeA genome and mutants would be required to determine if this theory was correct. It is likely however that hygromycin insertions are ectopic and the LaeA ORF is still intact.

Attempts to delete the VeA ORF within *T. hamatum* GD12 were deemed, by southern blot, to be successful. Previous studies in *Aspergiullus* species (*A. nidulans A. Carbonarius*, *A. fumigatus*) have shown that VeAΔ mutants demonstrate a variety of the following phenotypes; continuous asexual spore production, inability to produce mycotoxins (i.e. aflatoxins or fusarins) and changes in hyphal growth (with variation depending on light stimulus) (Bayram & Braus, 2012; Crespo-Sempere *et al*., 2013; Park *et al*., 2012; Roberts, 2013; Bayram *et al*., 2010). Additional alterations have been shown in conidia production, either; increases (i.e. in *Aspergillus nidulans*) or decreases (i.e. *Aspergillus Carbonarius*) (Crespo-Sempere *et al*., 2013; Bayram and Braus, 2012). *T. hamatum* GD12 VeA mutants show similar qualities as demonstrated in other fungal deletion subjects, revealed by phenotypic analysis.

VeA and VelB are required for regular hyphal growth under light and dark conditions (Crespo-Sempere *et al*., 2013; Park *et al*., 2012). *Trichoderma virens* Vel1 deletion mutants have demonstrated light dependent alterations in radial growth (Mukherjee & Kenerley, 2010). Additionally *Aspergillus* VeA and VelB mutants have been shown to exhibit a significant – around 80% of the wild type - decrease in colony radial growth on solid media (Crespo-Sempere *et al*., 2013; Park *et al.*, 2012). *ΔThVeA::hph* mutants kept under both light and dark conditions showed similar reductions. The plate edge was reached by the control samples on 4 d and mutants on d 6, demonstrating around a 50% decrease in growth rate as a result of VeA deletion. These findings signify that VeA is required for normal fungal morphology under light and dark settings.

Fungi use external stimulation to discriminate between environments, within *Trichoderma* around 3% of all genes are light regulated (Park *et al*., 2012; Bayram and Braus, 2012). In *Aspergillus nidulans* signals from light receptors are sent to VeA, which acts as a bridging protein linking VelB to LaeA (Bayram *et al*., 2010). Disruption of VeA therefore affects the trimeric complex, allowing LaeA to continuously suppress sexual development, even in ideal dark conditions. *Trichoderma hamatum*, however, does not display sexual growth under standard laboratory conditions. Spore count analysis was therefore performed to view the effect of VeA deletion on asexual spore production. Previous studies in *Aspergillus*, *Penicillium*, *Fusarium* and *Trichoderma* species have shown VeA deletion results in a - species dependent - increase or decrease of conidia (asexual spores) (Calvo *et al*., 2004; Crespo-Sempere *et al*., 2013; Hoff *et al*., 2010; Krappmann, *et al*., 2005; López-Berges *et al*., 2013; Wiemann *et al*., 2013; Yang, Chen, & Ma, 2013; Mukherjee and Kenerley, 2010). Analysis of *ΔThVeA::hph* subjects, compared against GD12 controls, showed that mutants had significantly reduced asexual spore production under both light and dark conditions, indicating that standard *T. hamatum* GD12 asexual spore production requires VeA.

The necrotrophic fungus S*clerotinia sclerotiorum* causes harm to over 400, often economically important plant species (Troian, *et al*., 2014; Williams *et al*., 2011). Long term survival of this pathogen in the soil is achieved via the production of sclerotia (Kim and Knudsen, 2008). These hardy sclerotia can act as inoculum for plant infection and are traditionally difficult to treat (Troian *et al*., 2014; Kim and Knudsen, 2008). Therefore the removal or demolition of these structures is vital to protect crop plants

(Kim and Knudsen, 2008). These hard thick-walled structures can be colonized and destroyed by multiple *Trichoderma* species (Kim and Knudsen, 2008, 2011). Some studies indicate that this degradation is due to the production of serine and trypsin-like protrease allowing penetration and subsequent colonization by *Trichoderma* species (Kim and Knudsen, 2008).

Together the velvet proteins and LaeA contribute to fungal pathogenicity, i.e. in through aflatoxin production in *Aspergillus* species (Jain and Keller, 2013). Confrontation plate assays 5 dpi, of multiple *Scelerotinia* strains against GD12 and *ΔThVeA::hph*, were viewed. These demonstrated that whereas an unmodified *T. hamatum* was capable of growing over competitors' mycelium, mutant strains appeared to be fungistatic. This overgrowth of opposing pathogen mycelium has been seen in other S*clerotinia* and *Trichoderma* interactions (Troian *et al*., 2014). As demonstrated earlier mutants are slower growing, nevertheless even after 12 ds they were incapable of competitor over growth. Results indicate that deletion of VeA in *T.hamatum* GD12 reduces the biocontrol capacity of this fungus.

In confrontation a significantly increased number and overall weight of hard sclerotia were seen on mutant plates compared to wildtype GD12 plates. This suggests loss of VeA decreases the pathogenicity of GD12. Δ*ThVeA::hph* mutants could be incapable of parasitizing sclerotia structures and so unable to prevent their proliferation. As previously determined Δ*ThVeA::hph* mutants could not over grow the pathogens they were in confrontation with. Accordingly a lack of interaction is seen between mutant mycelia and sclerotia, meaning these structures cannot be physically parasitized. However the inability of mutants to grow over S*clerotinia* resulted in a larger surface area of the pathogen compared to wild type confrontations. This increase in sclerotia production may therefore be as a result of raised mycelium coverage. Further investigation would be required to determine the true cause of amplified sclerotia number and total weight in mutant confrontations.

Future work should focus upon microscopy studies to further understand the parasitism capabilities and interaction of both wild-type *T.hamatum* GD12 and *ΔThVeA::hph* mutants when physically interacting with scelerotia. Other characterisation work should also include effect of *VeA* deletion on growth promotion of monocots (i.e. wheat) and dicots (i.e. lettuce), as well as the extension of biological

control assays. *ΔThVeA::hph* mutants have shown reduction in hyphal growth, condia production, and alterations in over-growth capabilities when confronted with *Scerotinia* pathogens. This demonstrates that VeA within *T.hamatum* GD12 plays a vital role in regular morphological developments and spore production.

5. Appendix

5.1. Work conducted before September 2013

Work from Wills, O. (2013), 'Disruption of the Global Secondary Metabolism Regulator LaeA in *Trichoderma hamatum* GD12 (lab rotation project 2)'

Please note the following work (pages 42 - 55) has been taken directly from work previously conducted by myself during my MbyRes program and presented in: Wills, O. (2013), Disruption of the Global Secondary Metabolism Regulator LaeA in *Trichoderma hamatum* GD12 (lab rotation project 2). This work below details how the knockout construct was created for the attempted deletion of the *LaeA*. This information is included for reference only.

Materials & Methods

Trichoderma hamatum GD12 DNA extraction

Fungal cultures were grown on potato dextrose agar (PDA) for 3 d until a 16h fluorescent light regime at 26^{o}C. 4 x 5mm plugs of cultures, taken from the leading edge, were used to inoculate Potato dextrose broth (PDB) before being shaken for 3 d at 125 rpm under the same light and temperature conditions. Liquid cultures were filtered through Miracloth to collect the mycelium, which were washed with sterilized MilliQ H_2O (to eradicate excess PDB) prior to snap freezing in liquid N_2 and lyophilisation for 2 d.

Lyophilised mycelium was ground into a fine powder in a cooled sterile mortar, and added to 1 ml of SDS buffer (SDS 1%, EDTA 0.025 M, NaCl 5 M, Tris-HCl 0.2 M and sterile H_20) before being incubated at 65^{O}C for 30 minutes. Samples were centrifuged at 14,000 rpm (~16,000 x *g*) for 10 min, the supernatant transferred to fresh tubes containing 800 µl of phenol, vortexed to mix, and centrifuged for a further 5 min at 14,000 rpm. This process was repeated using 800 µl of phenol:chloroform

and then 800 µl of chloroform:pentanol (24:1 vol/vol). The supernatant was transferred to a fresh eppendorf tube before 0.6 volume of ice-cold isopropanol was added to aid DNA precipitation. Samples were vortexed and incubated on ice for 5 min before being centrifuged at 14,000 rpm for 10 minutes at 4^{O}C. The supernatant was decanted and tubes were inverted on blue towel to dry, before 500 µl of ice cold 70% ethanol was added and samples were centrifuged at 14,000 rpm for 5 min. The supernatant was removed before samples were inverted on blue towel and allowed to dry at room temperature for 20 min. Pellets containing DNA were re-suspended in 28 µl sterile MilliQ water and 2 µl RNAase and left for 30 min at room temperature before being stored at -20^{O}C. The DNA quantity and quality was determined by using gel electrophoresis and a Nanodrop spectrophotometer (Thermo scientific). Samples containing RNA were further treated with 2 µl RNAase.

Polymerase chain reaction (PCR) was performed using fungal ITS specific primers to verify that the DNA obtained was from the Kingdom Fungi. Promega GoTaq® green master mix was used at 12.5 µl per sample along with 1µl forward Primer (ITS 1: (5'-CCGTAGGTGAACCTGCGG-3'), 1µl reverse Primer (ITS4: 5'-TCCTCCGCTTATTGATATGC-3'), 1µl of DNA, and 9.5 µl nuclease-free H_2O. PCR was performed as follows: one cycle of initial denaturing was undertaken for 3 min at 95^{O}C, followed by 35 cycles of: denaturing (30 s at 95^{O}C); annealing (30 sec at 55^{O}C); and extension (2 min at 72^{O}C); finally one 10 min cycle at 72^{O}C was performed, before samples were held at 4^{O}C until use. PCR was undertaken using a GeneAmp PCR system 9700 version 3.05. (Applied Biosystems). PCR products were separated at 100v for 1.5 hours on 0.8% (w/v) gels, and a 1kb ladder was used to determine molecular weights.

Deletion of the encoding gene (laeA)

Gene disruption was performed by using a split marker method of homologous recombination, based upon the method described in Catlett *et al*, 2002. Using the *Trichoderma hamatum* GD12 genome that had previously been sequenced in-house (Le Cocq, 2012), the NODE_6819 was identified to contain the *laeA* ortholog in the

fungus. Primers were created to flank the *laeA* open reading frame (ORF) with the left flank section at 1 kb upstream and right flank at 550 downstream, so as to avoid missing regions of nucleotide sequence (Table A.1.; Figure A.1.).

	Product	Product size	Primer name	Primer sequence (5' to 3')	DNA template
First round PCR	Left flank	1 kb	LaeA_LF_F	GAATCGTCGCTTATCCTAAC	GD12 DNA
			LaeA_LF_R (+M13)	GTCGTGACTGGGAAAACCCTGGCG CGAGTCCTATAGGAGCTT	
	Right flank	0.55 kb	LaeA_RF_F (+M13)	TCCTGTGTGAAATTGTTATCCGCTA CCTGCATACAGACACAC	GD12 DNA
			LaeA_RF_R3	CTGCGTATATGGAGAACCTT	
	'HY'	1.4 kb	HYsplit	GGATGCCTCCGCTCGAAGTA	PHYG template
			M13 F	GTCGTGACTGGGAAAACCCTGGCG	
	'YG'	0.8 kb	YGsplit	CGTTGCAAGACCTGCCTGAA	PHYG template
			M13 R	TCCTGTGTGAAATTGTTATCCGCT	
Second round PCR	LF+HY	2.4 kb	LaeA_LF_F	GAATCGTCGCTTATCCTAAC	First round PCR product: 'left flank' and 'HY'
			HY Nested primer (Nst1 HYsplit)	CGTCTGCTGCTCCATACAAGCCAA	
	RF+YG	1.35 kb	YG split	CGTTGCAAGACCTGCCTGAA	First round PCR product: 'right flank' and 'YG'
			LaeA_RF_R3	CTGCGTATATGGAGAACCTT	

Table A.1. Primer sequences used to create the first round and second round PCR products.

TGAATCGTCGCTTATCCTAACCCTCGGCATCTGAGCGGCGTACTCTTGTACAACGTACAA
TTATATCTGTGTCACCTCCTTGGGTTTCAACCCGCGAAATATCTCCGTGTAAGTCACCAT
CTATCTTTGTTTTGTACGTCTGGTTACCCAACATCGTCCCTCCCGTCAGTTCGGTTATAT
GCTATTCTCCACACACCTATCTCTCCTGTCTTACCATGGTTTTGCGTATTGAAAGGCGCC
ATGTATGATACTGTGTACGTGCGGCTACGACTTGGTGTCATGTTCTACTAGTCAACATGG
CTTCTTTTCTTGGTAGCCTATCAATCACATCAGTCTGCTCCATCCCAAGTCTTACCTTTT
TACCTCTTCTGGGATAGCCATGTGTATCTCTTGTCTCACTATATTTCCTTGATTAAATCC
TGCTTGCTCTCCCTGATTTTGTGTTTCTTTTCTCTCTCTTCTCGTCTTTTTGTTTTGTTT
CTACCAACTCCCACCTGCGATCTTGTCCAAATTTCATAGGTGCTCCAATACCAAGCCTGA
AGCTTAGTATACTCTCCGGGAAAGCCCCCGGTTTTCAGTAAGAGTGAGAGACGTCCATTC
TTATTTGGAGAATAGAGATTTACCCCCCTGTCGTCATATATGGCAGTCAACCATCTTCTC
TTCGTTTTTACCTCTACTACTCTGCCCCTGGTTCTTTTTATTTCATCTTTTGTTCTTTTT
TATATCCCATCTCAGGACCAATTAGTATCATTGGCTATATGCAGAGCAGAAGAATGGGGT
TTCCCTCGCCCTTGCCTGAAGACATACACAATTACACATTCTTGCACTAAATTTCTTGTT
ACTTTCCGCTTCTTTTTTTTTATCCAATACTTTCTTTCTGTAATTCTTTTTACTAATCTT
AGACTCTAGTTAATCTGATAATATTCACATTGGTTGATTTTCGCGTCTACCTGCTCGTAA
TTGCTGACAGCCATTTATTTCCTGGATTCCTATTTCCGTGCTACCTGCCTGGACAGCCCT
TGGGACCCCCATTATTTTTCTCGAAGGCTTTCAAGCTCCTATAGGACTCGCCATACTCGC
C**ATGTCGTCTCGAAACGCTCCAAGCGGGTGCGTTGCACCCTCCCCAGCCACTGCTGCTCC**

GCCTTCGCCCACGAATCTGCGACTAACTGTTGGGCAACCCGTCAGTGAGTCAGCCAATGA
ACCAGGAAGTGAATCAGAGGAACGAATTCTCCAGGACGGATATTGGGAGTACGGTCGATT
TTATGGTAGCTGGAAGAAAGGGAAATACAATTTTCCAATTGACAAGGTTTGCCTCCGCTT
GGCCACAGTATATGCGACATCTATTTTGACATCATTGCTTAGGAGGAGCTCAGTAGGCTG
GATATTCTCCACAAATACTTTGTCGTAGAAACCGAAGATCGCGTCACTTCCGTTCCTTTG
GATAAAGAAGGACGACCGAAAATCATGGACCTCGGAACGGGCACAGGTATTTGGGCTTTC
CATGTGGTGGAAGGGTAAGTTTTACCATGTCACCTGGTCCAAACTTGTTGCGTATCATCG
CGCTAATAATCACCAGTTACATCCCAAATGCCCAGATTATGGCCGTTGATCTCAATCAGA
TTCAGCCAGCTCTGTAAGTAGATATTCAGAGACTTGAGTTGTGCCGACTATCTAACGCGC
CACCACAGCATCCCTCCAGGTGTAACTACAAAGCAGTTTGACATTGAGGAGCCTTCATGG
GAACCACTTCTCCGTGACTGCGACTTGATCCATCTTCGGCTGCTCTACGGCAGTATCAAA
GACGATATGTGGGCCGGTATTTACCGCAAAATCTTCGAGTGCGTATCTAATATTCTTGAC
AAGCTTCTTCCCATCTGACGTGTTTAGGCACTTGGCCCCTGGAGGCTACGTCGAGCATTT
AGAAATAGATTGGACACCACAATGGGATGGCGACGACCGTCCCACGCACTCGGCTATTCG
TGAGTGGTCCCAGCAATTTCACCGGGCCATGCATCGATACCGCCGCGGTGTCAAAGTATC
GACCGAAGACACCAAGCGCATGATAGAAGCAGCTGGCTTCACTGATTTTAAAGAAACTAC
GATCCGGTGCTATGTTAATCCGTGGTCTACTGATCGTCACCAGCGAGAGGCCGCTCGCTG
GTTCAATCTGGCTTTAGGCCTAGGTCTTGAGGCCATGAGCTTGATGCCTATGATTGACAT
GCTGGGTATGAAAAAAGATGATGTTGTAGACCTCTGCAAACGGGTTAAAGCCGAAACTTG
CGTTCTGCGCTACCACGCCTACTTTACTCTGTAAGACCCAACCAACACGACTTTGAAACG
TTTTCTTCAATCAGTATTAACCTGCATACAGACACACCTGGACGGCCAAAAAACCCGCTA
GTCCGCCGCAGTAAGATGCCTAAGTGTTCAGTATCTTCGTCACTATTTAGGACTCTCAAG
CATTTGCATCCATAATTCATAGGATGCAGACTGTGTTATTGACCAAGATCACTGATCATA
CAAGGGTAAAGCAACAATACGATTCCTCGGGGATCATCTTAACGGCGCTCGGCGTCGAAG
GACCTCGGCCTCCTAAGGCGACTGCGGCCTGATACCCACCAATTTGGCTGGGGCTGATGT
CTGAGTATGGTTAATGACGGGCACTATAAACGGATTTGCATGAAGCAAGGAAGATCCAAG
GGCTAGATCCCATGGCGGCTTCATCTTCCGGTGCCAGTAGATGAGTTTATTTACCGGATA
TCACGGCTTTGAGAGTATGGTTCATGTTTATGGGATAGTCATGTGTGCAATTTAGCGTTT
GGAAGTAACAATTTAGAATTAAAGAAAGAATGGTTCTTCCCCCTTACAATATCCTCTTTG
TCCTTGCCCCTTCTTGTGTAGCTATGAGGTTTTTTTTTTTTTTTTTTAACGNNNNNNNNN
NAGGGCCACTGATGGTTTGGTGAAGTCTATCATAGCTATGCTGCCATAAAACAGCCCAAT

Figure A.1. LaeA GD12 construct primers.
A section of the *Trichoderma hamatum* GD12 NODE_6819 is presented. The *lae* ORF is shown in dark blue text. The light blue highlighted primers encompass the left flank and the green highlighted primers encompass the right flank. The yellow highlighted section 'NNNNNNNNNN' denotes missing nucleotide sequence. Underlined sequences denote stop or start codons.

Construction of the LF + HY and RF + YG cassettes

PCR reactions were carried out using a GeneAmp PCR system 9700 version 3.05. (Applied Biosystems) or Pelitier thermal cycle 200 (MJ research) and gradient PCR was performed using Sure cycler 8800 (Agilent technologies, Berkshire) both using GoTaq® Green Master Mix. Unless stated otherwise, samples contained: 12.5 µl GoTaq® Green Master Mix, 1 µl forward primer, 1 µl reverse primer, 50 ng of DNA template and double processed tissue culture water (Sigma-Aldrich) to give a total sample volume of 25 µl. PCR products, unless otherwise stated, were separated

electrophoretically by using 0.8% (w/v) agarose gels at 100 v for 1.5 hours, and a 1 kb ladder was used to determine molecular weights.

Left flank (LF) and right flank (RF) amplification

PCR was performed using the following cycling parameters: one cycle of initial denaturing for 3 min at 94^0C, followed by 35 cycles of: denaturing (94^OC for 30 s; annealing (50^OC for 30 s); and extension (72^OC for 1 min); then a final extension of 10 min at 72^OC, before samples were held at 15^OC until use.

Amplification of 'HY' and 'YG'

'HY' and 'YG' PCR amplification was carried out using the following cycling parameters: one cycle of initial denaturing at 92^OC for 5 min, followed by 35 cycles of: denaturation (30 s at 92^OC); annealing (30 sec at 60^OC); extension (1 min at 72^OC); a final extension step at 72^OC 10 min, before samples were held at 15^OC until use.

Fusion of LF+'HY' and RF+'YG'

Fusion PCR

Amplification was performed using Phusion® High-Fidelity PCR kit. Unless stated otherwise, samples contained: 5 µl Phusion® HF, 0.5 µl dNTPs, 1.25 µl forward primer, 1.25 µl reverse primer, 0.25 µl Phusion® DNA polymerase, 40-55 ng/ml DNA templates of LF+HY or RF+YG, and double processed tissue culture water (Sigma-Aldrich) to make a final sample volume of 25 µl.

Fusion PCR of the LF + 'HY' and RF + 'YG'

The PCR conditions were as follows: initial denaturation at 98^OC for 30 s, 35 cycles of denaturation for 10 s at 98^OC; annealing (30 s at 62^OC); extension of 45 s for LF +

'HY' or 30 s for RF + 'YG' at 72^{O}C, followed by a final extension at 72^{O}C for 10 min. Samples were held at 15^{O}C until use or were stored at -20^{O}C.

Cloning of LF + 'HY' and RF + 'YG'

Bacterial transformation

For bacterial transformation StrataClone Blunt PCR Cloning Kit (category number: #240207) was used. Ligation reactions contained: 3 µl of StrataClone cloning buffer, and 2 µl of Fusion PCR product of concentration 5-50 ng/µl. This solution was incubated at room temperature for 5 min before being chilled on ice. One µl of the ligation mixture was added to thawed StrataClone SoloPack competent cells (*Escherichia coli* strain XL1 Blue (Stratagene) – genotype: *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*$^{-}$[F′ *pro* AB^{+} *lac*I^{q} *lac*ZΔM15 *Tn*10 (*tet*^{r})]) and the tubes mixed by inversion before being placed on ice for 20 min. The transformation mixture was then heat shocked at 42^{O}C for 45 sec before being chilled on ice for a further 2 min. Two hundred and fifty µl of 42^{O}C pre-warmed Luria-Bertani broth (LB; tryptone, 10 g L^{-1}, yeast extract, 5 g L^{-1}, NaCl, 10 g L^{-1}, pH 7.5) was added to the transformation reaction material. Competent cells were allowed to recover by incubation at 37^{O}C with gentle agitation, plated as 5 µl or 100 µl volumes on colour screening plates (LB (tryptone, 10 g L^{-1}, yeast extract, 5 g L^{-1}, sodium chloride, 10 g L^{-1}, (pH 7.5), agar 18 g L^{-1}) and ampicillin (10 mg/ml) with 40 µl of 2% X-gal spread on to each plate), and held at 37^{O}C for 16 h. Colonies which were white or light blue were picked and used in further analysis.

Colony PCR

PCR reactions were performed using GoTaq® green master mix and DNA obtained by touching selected colonies with a pipette tip and inserting these into reaction tubes. The following cycling parameters were used: an initial cycle of denaturation for 5 min at 94^{O}C, 35 cycles of; denaturation (94^{O}C for 30 s); annealing (50^{O}C for 30 s); and extension (72^{O}C) for 150 s for LF+HY or 90 s for RF+YG; then a final extension step of 72^{O}C cycle for 10 min, before samples were held at 15^{O}C until use.

Bacterial DNA quick preparations

Bacterial colonies were used to create small-scale plasmid DNA preparations. Single bacterial colonies were picked and used to inoculate LB containing the antibiotic amplicillin (10mg/ml). Cultures were grown at 37^{O}C overnight at 200 rpm and kept at 4^{O}C. Long term storage of cells was achieved by adding 600 µl of bacterial culture to 400 µl sterile 50% (v/v) glycerol. Suspensions were vortex rapidly, snap frozen in liquid N_2 and subsequently stored at -80^{O}C. 1.5 ml of the aforementioned bacterial culture solution was pelleted by centrifugation at 13,000 rpm for 1 min. Cells were lysed by the addition of 100 µl of lysis solution (250 µl 1M Tris (pH 8.0), 200 µl 0.5M EDTA, 170 µl 60% (v/v) sucrose, 2.38 mL H_2O), the mixture vortexed, and, following the addition of 200 µl of alkaline solution (100 µl NaOH, 500 µl 10% SDS, 4.4 ml H_2O). were chilled on ice for 10 min with occasional shaking. 150 µl of 3M sodium acetate (pH 5.2) was added and solutions were mixed by inversion before incubation on ice for 10 min. Tube contents were then centrifuged at 13,000 rpm for 10 min at 4^{0}C, the supernatant mixed with 1 ml of 100% ethanol, and samples chilled on ice for 10 min. Solutions were centrifuged at 13,000 rpm for 5 min at room temperature and 1 ml of 70% (v/v) ethanol was added to the pelleted material. Supernatants were removed and the tubes were drained on blue towel. 30 µl of MilliQ water and 2 µl RNase was added to each sample and solutions were stored at -20^{O}C prior to use.

Digestion of plasmid DNA with restriction enzymes

Digestion of DNA was carried out using restriction endonucleases and corresponding buffer solutions from Promega UK Ltd (Southampton, UK). Six µl of DNA was added to 3 µl of buffer (buffer H), 1 µl of restriction enzyme (*Eco*RI) and 20 µl of sterile water. Either 3-4 h or overnight incubations were performed at 37^{O}C.

Preparation of high quality plasmid DNA (Midiprep method)

The Promega PureYield^{TM} plasmid Midiprep System (A2492) was used to obtain transformed bacterial plasmids containing desired DNA. Overnight cultures were established by incubating 10 µl of *E. coli* colonies (grown first in LB which contained

the desired insert, confirmed by quick preparations and digestion PCR) in 50 ml LB containing ampillicin (10 mg/ml). Cultures were grown at 37^{O}C with shaking (200 rpm). Solutions were centrifuged at 5000 x *g* for 10 min and cells re-suspended in 1.5 ml of re-suspension solution. Cell lysis solution (1.5 ml) was added to the cell suspension, tubes were inverted 3-5 times for mixing, and were then incubated at room temperature for 3 min. Samples then received 2.5 ml of neutralization solution and were inverted a further 5-10 times to enable mixing prior to centrifugation for 15 min at 15,000 x *g*. A column stack was assembled by placing a Binding Column^{TM} onto a vacuum manifold and nesting a Cleaning Column^{TM} on top. The lysate supernatant was poured into the cleaning column and the vacuum applied to allow DNA to bind. The cleaning column was removed and 5 ml of endotoxin removal wash and then 20 ml of column wash were added in turn to the binding column with the vacuum applied each time. The membrane was dried by applying the vacuum for approximately 60 s. The binding column was then removed and placed into a 50 ml Falcon tube, 600 µl of nuclease free water was added and incubated for 1 min before spinning at 2,000 x *g* for 5 minutes to elute the DNA. DNA quantity and quality was determined using a Nanodrop spectrophotometer and solutions were stored at -20^{O}C for long term storage.

Obtained Midiprep plasmid DNA was used as a template in PCR reactions to amplify desired DNA using Promega GoTaq® green master mix. PCR reactions were performed using the following cycling parameters: one cycle of initial denaturation for five min at 94^{O}C, followed by 35 cycles of: denaturation (94^{O}C for 30 sec); annealing (50^{O}C for 30 sec); and extension, 72^{O}C for 150 s for LF+HY or 90 sec for RF+YG; then a final extension of 10 min at 72^{O}C, before samples were held at 15^{O}C. Products were separated on a 0.8% (w/v) agarose gel and gel purified before use.

Gene clean of PCR products

The Wizard ® SV gel and PCR clean-up system (Promega, Southampton, UK) was used for gene clean cleaning to obtain purified DNA. Following electrophoresis, DNA bands were excised and weighed, 1µl of membrane binding solution was then added for each 1mg of gel slice. Samples were vortexed and then incubated at 65^{O}C to dissolve the gel slice. The contents were then transferred to a mini-column inside a

collection tube. Samples were incubated for 1 min at room temperature, centrifuged for 1 min at 16,000 x *g*, and the flow-through discarded. Seven hundred µl of membrane wash solution was added, followed by a further 500 µl, and the mini-column centrifuged at 16,000 x *g* for 1 min and 5 min respectively. Samples were subsequently centrifuged at 16,000 x *g* for 1 min to enable evaporation of residual ethanol. Fifty µl of nuclease free water was added and samples were incubated for 1 min at room temperature before centrifugation at 16,000 x *g* for 1 min. The eluted fluid containing DNA was retained was assessed for DNA quantity and quality by using a Nanodrop spectrophotometer and products were stored at -20^{O}C until used.

Transformation of *Trichoderma hamatum* GD12

Trichoderma conidia were obtained from 2-wk-old petri-dish cultures and used to inoculate potato dextrose broth (PDB) at a concentration of approximately 10^6 conidia/ml. Cultures were placed in petri dishes under a 16-h fluorescent light regime at 26^{o}C for 48 h. Fungal biomass was harvest via filtration through sterile miracloth, the material rinsed with sterile dH_2O and blotted dry using paper towel. 0.5-0.6g of the washed biomass was added to a 50-ml Falcon tube (Becton Dickinson) containing 15 ml of sterile filtered enzyme solution (1.2mg chitinase (Sigma), 7mg lyticase (Sigma), 44mg cellulase (Sigma) in osmoticum (5ml of 1M $CaCl_2$, 9.11g mannitol, 1.06g MES, 95ml dH_2O, adjusted to pH 5.5 with KOH)) and shaken at 250rpm for 25 min at 21^{O}C. Protoplasts concentration was determined using a haemocytometer. Digested fungal matter was passed through miracloth and rinsed with 5 ml of osmoticum and the obtained supernatant was spun at 5000 rpm for 5 min at 4^{0}C. Two washes were performed; samples were centrifuged – 5000 rpm 4^{O}C for 5 minutes - supernatant was removed and pellets were re-suspended in 500 µl and samples combined to leave only two eppendorfs. The samples were then centrifuged again at 5000 rpm and 4^{O}C for 5 min and a final volume of 300 µl sterile osmoticum was added.

Transformations were performed through the addition of 10µl of DNA (4ng/ µl) to the ‘test’ tube and 10 µl of osmoticum to the ‘control’ tube, before incubation on ice for 20 min. Next, 130 µl of PEG (40% PEG 8000 osmoticum) was added to the

samples, mixed by inversion, and a further 130 µl of PEG added with mixing. On each occasion the samples were incubated at room temperature for 30 min. Treated samples were added to 150 ml of PDA containing 0.8 M sucrose at 42^{O}C, samples were mixed gently and poured into petri dishes. Plates were incubated in the dark for 24 h at 26^{O}C before a selective top agar, PDA containing hygromycin B (0.22 µl/ml), was added. Successfully transformed protoplasts, whose hyphae emerged through the selective overlay, were isolated and sub-cultured onto PDA plus hygromycin and then on to V8 medium.

Results

Assembly of the deletion construct to enable disruption of the laeA-encoding gene

The split marker method was used to generate Δ*ThlaeA*::*hph* mutants by replacing the laeA ORF with a hygromycin resistance cassette. PCR reactions and gel electrophoresis were performed to successfully produce the first round products LF (1 kb), RF (0.55 kb), HY (1.4 kb) and YG (0.8 kb) (Figure A.2). Left and right flank primers were designed to contain an added region -M13- on the 5' and 3' inner regions which are complementary to the outer 5' to 3' regions of the *hph* (hygromycin) resistance gene (Table A.1). This extension enables the fusion of first round PCR products with the 'HY' and 'YG' (these two aspects together form HYG (hph)) during the second round PCR to give two products; LF + HY (2.4 kb; LF 1 kb + HY of 1.4 kb) and RF + YG (1.35 kb; RF 0.55 kb + YG 0.8 kb) (Figure A.2). Gel purified second round PCR products were used to transform fungal protoplasts. Where both homologous recombination events occur, for correct orientation of the hph gene, the laeA ORF is displaced resulting in hygromycin resistance (Figure A.2).

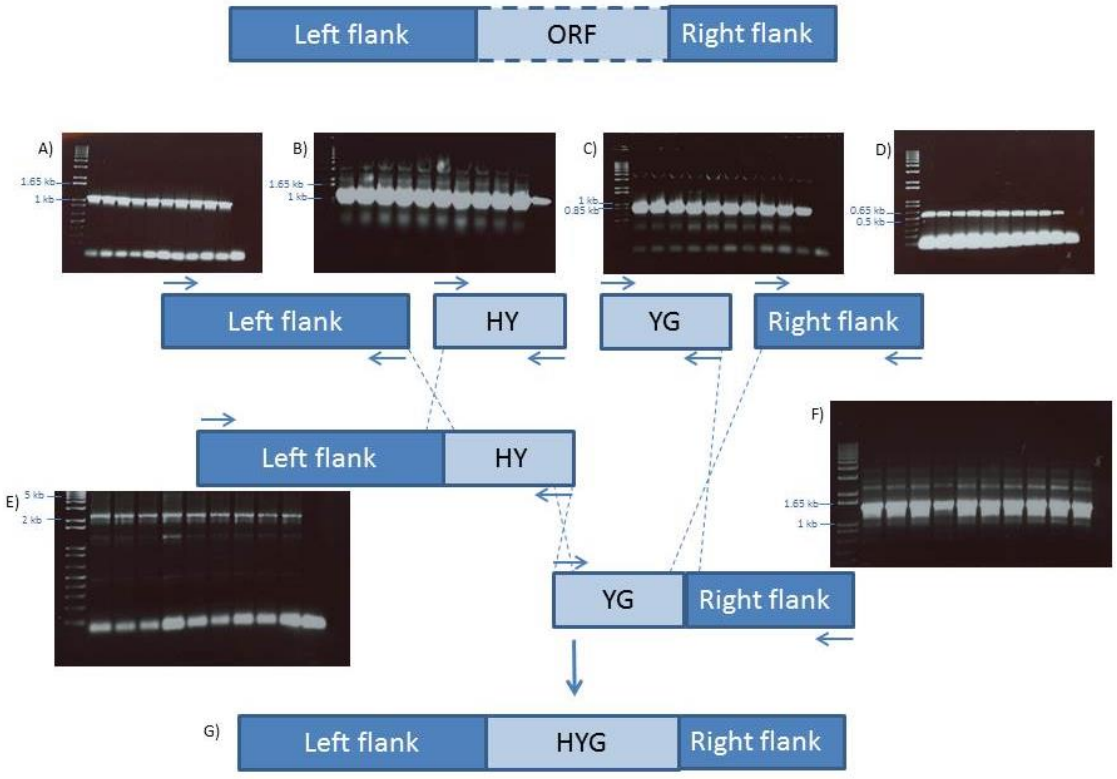
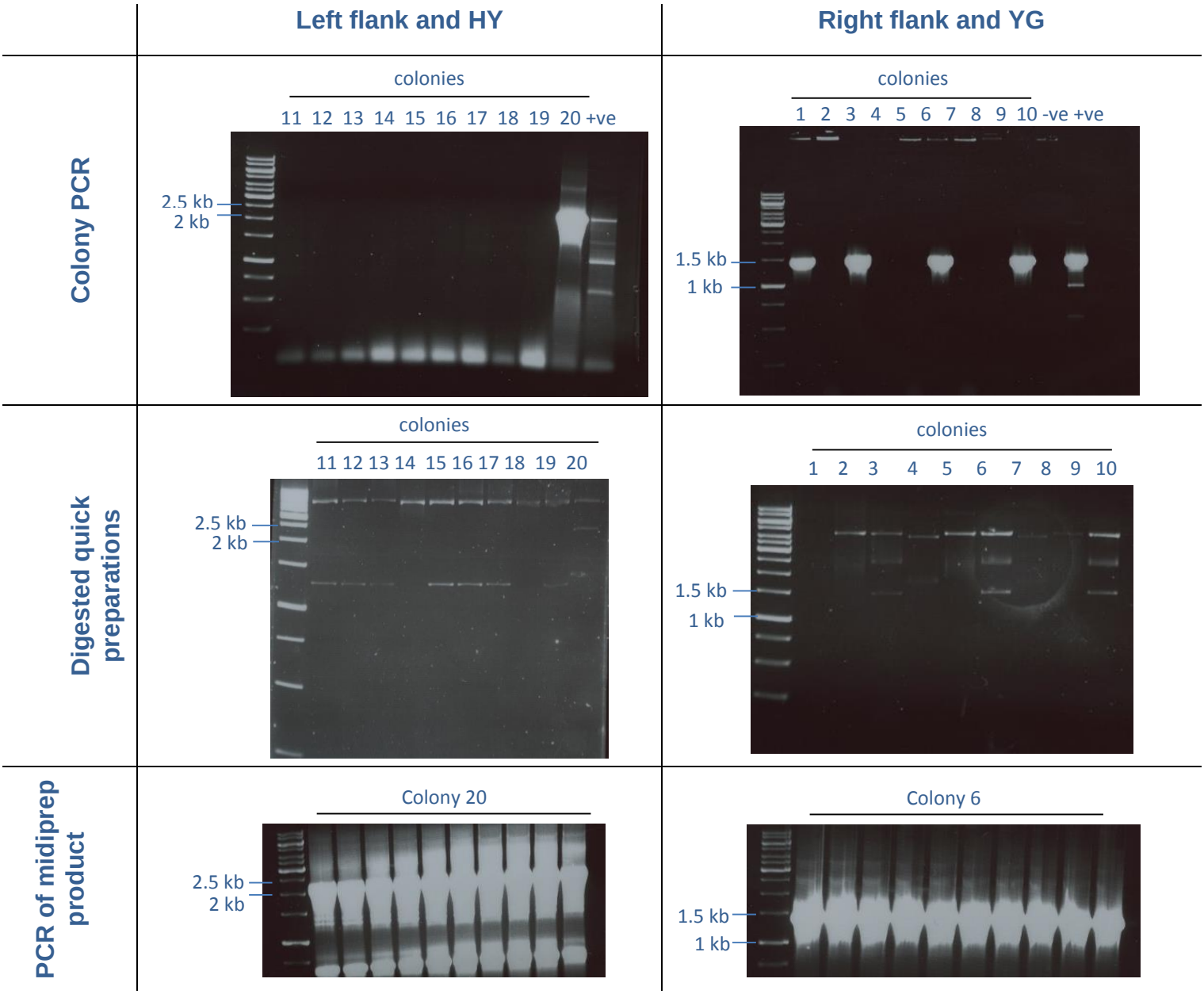


Figure A.2. Split marker method used to generate Δ*ThlaeA*::*hph* mutants. Agarose gel electrophoresis showing PCR amplifications for: first round products of: A) Left flank first round – 1 kb; B) HY – 1.4 kb; C) YG – 0.8 kb, D) Right flank first round – 0.55 kb; and second round products, created by the use of fusion PCR : E) Left flank first round product fused to HY, resulting in a 2.4 kb split marker product; F) Right flank first round product fused to YG, giving a 1.35 kb split marker product. G) Three independent homologous recombination events results in HYG resistance cassette replacing the laeA ORF. All far left hand lanes contain standard ladders used to determine size of PCR products in kb (see appendix for ladder guides).

Cloning of LF + HY and RF + YG through bacterial transformations



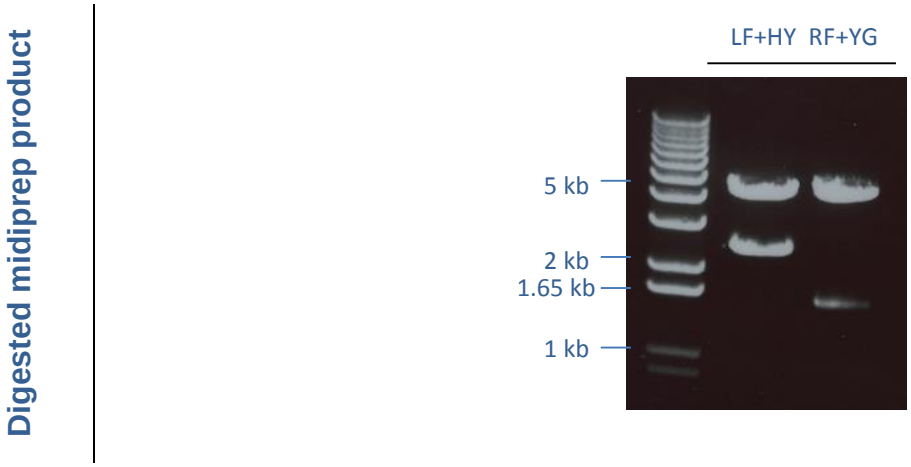


Figure A.3. Gel electrophoresis of bacterial transformation stages. Lane 1 of all images contain standard ladders to determine molecular weight (see appendix for ladder guides).

Colony PCR and quick DNA digest preparations were used to determine which cultures to take forward to the midiprep stage.. For these two procedures, the LF + HY amplicons of colony 20 were of the molecular weight 2.4 kb and for the RF + YG amplicons colonies 3, 6 and 9 were of the molecular weight 1.35 kb, indicating that these bacterial transformations were successful in taking up their designated DNA (Figure A.3). Colony 20 for the LF + HY and colony 6 for the RF + YG were taken forward and used within midipreps, which enabled the generation of high quality plasmid DNA. Midiprep products were amplified using PCR and the product separated by gel electrophoresis to acquire DNA products for use in laeA transformations of *Trichoderma hamatum* GD12 (Figure A.3). Midiprep products were also digested to confirm that the correct band sizes of 2.4 kb and 1.35 kb had been obtained for the LF + HY and RF + YG respectively (Figure A.3).

Transformation of *Trichoderma hamatum* GD12

LaeA transformations of *Trichoderma hamatum* GD12 resulted in the production of eleven putative Δ*ThlaeA*::*hph* mutants:

- A0407-1
- A0407-2
- B0407-1

- B0407-2
- C0407-1
- C0407-2
- C0407-3
- C0407-4
- C0407-5
- D0407-1
- A1007-1

5.2. Work conducted from September 2013 onwards

LaeA

PCR screening of putative Δ*ThlaeA*::*hph* to determine samples to be taken forward to southern blotting

PCR was undertaken as an initial screening to determine whether putative Δ*ThlaeA*::*hph* were true mutants.

(A)

ACCATCCCACCTGCTGCTTGACTCGCACACCCGTTCCTCCCTAGTTAGCCGCTGCTTCCT
ATTCCTTACGAACTGGTGGTAAAGGTGACCGCGGTGACCGGAGCTGAGATTTTCAAACCT
CTTTTCAGGATCCTCAGGAGCTTCGGGTTTACCACCGCCTGGGATAATAAAGGCAAGAGT
TGAATCGTCGCTTATCCTAACCCTCGGCATCTGAGCGGCGTACTCTTGTACAACGTACAA
TTATATCTGTGTCACCTCCTTGGGTTTCAACCCGCGAAATATCTCCGTGTAAGTCACCAT
CTATCTTTGTTTTGTACGTCTGGTTACCCAACATCGTCCCTCCCGTCAGTTCGGTTATAT
GCTATTCTCCACACACCTATCTCTCCTGTCTTACCATGGTTTTGCGTATTGAAAGGCGCC
ATGTATGATACTGTGTACGTGCGGCTACGACTTGGTGTCATGTTCTACTAGTCAACATGG
CTTCTTTTCTTGGTAGCCTATCAATCACATCAGTCTGCTCCATCCCAAGTCTTACCTTTT
TACCTCTTCTGGGATAGCCATGTGTATCTCTTGTCTCACTATATTTCCTTGATTAAATCC
TGCTTGCTCTCCCTGATTTTGTGTTTCTTTTCTCTCTCTTCTCGTCTTTTTGTTTTGTTT
CTACCAACTCCCACCTGCGATCTTGTCCAAATTTCATAGGTGCTCCAATACCAAGCCTGA
AGCTTAGTATACTCTCCGGGAAAGCCCCCGGTTTTCAGTAAGAGTGAGAGACGTCCATTC
TTATTTGGAGAATAGAGATTTACCCCCCTGTCGTCATATATGGCAGTCAACCATCTTCTC
TTCGTTTTTACCTCTACTACTCTGCCCCTGGTTCTTTTTATTTCATCTTTTGTTCTTTTT
TATATCCCATCTCAGGACCAATTAGTATCATTGGCTATATGCAGAGCAGAAGAATGGGGT
TTCCCTCGCCCTTGCCTGAAGACATACACAATTACACATTCTTGCACTAAATTTCTTGTT
ACTTTCCGCTTCTTTTTTTTTATCCAATACTTTCTTTCTGTAATTCTTTTTACTAATCTT
AGACTCTAGTTAATCTGATAATATTCACATTGGTTGATTTTCGCGTCTACCTGCTCGTAA
TTGCTGACAGCCATTTATTTCCTGGATTCCTATTTCCGTGCTACCTGCCTGGACAGCCCT
TGGGACCCCCATTATTTTTCTCGAAGGCTTTCAAGCTCCTATAGGACTCGCCATACTCGC
C**ATG**TCGTCTCGAAACGCTCCAAGCGGGTGCGTTGCACCCTCCCCAGCCACTGCTGCTCC
GCCTTCGCCCACGAATCTGCGACTAACTGTTGGGCAACCCGTCAGTGAGTCAGCCAATGA
ACCAGGAAGTGAATCAGAGGAACGAATTCTCCAGGACGGATATTGGGAGTACGGTCGATT
TTATGGTAGCTGGAAGAAAGGGAAATACAATTTTCCAATTGACAAGGTTTGCCTCCGCTT
GGCCACAGTATATGCGACATCTATTTTGACATCATTGCTTAGGAGGAGCTCAGTAGGCTG
GATATTCTCCACAAATACTTTGTCGTAGAAACCGAAGATCGCGTCACTTCCGTTCCTTTG
GATAAAGAAGGACGACCGAAAATCATGGACCTCGGAACGGGCACAGGTATTTGGGCTTTC
CATGTGGTGGAAGGGTAAGTTTTACCATGTCACCTGGTCCAAACTTGTTGCGTATCATCG
CGCTAATAATCACCAGTTACATCCCAAATGCCCAGATTATGGCCGTTGATCTCAATCAGA
TTCAGCCAGCTCTGTAAGTAGATATTCAGAGACTTGAGTTGTGCCGACTATCTAACGCGC
CACCACAGCATCCCTCCAGGTGTAACTACAAAGCAGTTTGACATTGAGGAGCCTTCATGG
GAACCACTTCTCCGTGACTGCGACTTGATCCATCTTCGGCTGCTCTACGGCAGTATCAAA
GACGATATGTGGGCCGGTATTTACCGCAAAATCTTCGAGTGCGTATCTAATATTCTTGAC
AAGCTTCTTCCCATCTGACGTGTTTAGGCACTTGGCCCCTGGAGGCTACGTCGAGCATTT
AGAAATAGATTGGACACCACAATGGGATGGCGACGACCGTCCCACGCACTCGGCTATTCG
TGAGTGGTCCCAGCAATTTCACCGGGCCATGCATCGATACCGCCGCGGTGTCAAAGTATC

GACCGAAGACACCAAGCGCATGATAGAAGCAGCTGGCTTCACTGATTTTAAAGAAACTAC
GATCCGGTGCTATGTTAATCCGTGGTCTACTGATCGTCACCAGCGAGAGGCCGCTCGCTG
GTTCAATCTGGCTTTAGGCCTAGGTCTTGAGGCCATGAGCTTGATGCCTATGATTGACAT
GCTGGGTATGAAAAAAGATGATGTTGTAGACCTCTGCAAACGGGTTAAAGCCGAAACTTG
CGTTCTGCGCTACCACGCCTACTTTACTCTGTAAGACCCAACCAACACGACTTTGAAACG
TTTTCTTCAATCAGTATTAACCTGCATACAGACACACCTGGACGGCCAAAAAACCCGCTA
GTCCGCCGCAGTAAGATGCCTAAGTGTTCAGTATCTTCGTCACTATTTAGGACTCTCAAG
CATTTGCATCCATAATTCATAGGATGCAGACTGTGTTATTGACCAAGATCACTGATCATA
CAAGGGTAAAGCAACAATACGATTCCTCGGGGATCATCTTAACGGCGCTCGGCGTCGAAG
GACCTCGGCCTCCTAAGGCGACTGCGGCCTGATACCCACCAATTTGGCTGGGGCTGATGT
CTGAGTATGGTTAATGACGGGCACTATAAACGGATTTGCATGAAGCAAGGAAGATCCAAG
GGCTAGATCCCATGGCGGCTTCATCTTCCGGTGCCAGTAGATGAGTTTATTTACCGGATA
TCACGGCTTTGAGAGTATGGTTCATGTTTATGGGATAGTCATGTGTGCAATTTAGCGTTT
GGAAGTAACAATTTAGAATTAAAGAAAGAATGGTTCTTCCCCCTTACAATATCCTCTTTG
TCCTTGCCCCTTCTTGTGTAGCTATGAGGTTTTTTTTTTTTTTTTTTAACGNNNNNNNNN
NAGGGCCACTGATGGTTTGGTGAAGTCTATCATAGCTATGCTGCCATAAAACAGCCCAAT
AAACATATATGGTTCGGCTAATTGCAAACCATCCAACGCATCGTCTCGGCATAGTATTTT
ATGATTGTCTTGTGGCGTTGGTCGACAGTACTACTACGCAGTGGCATGAGCATCCTGGTC
TGAATACTCGTATGTGACGAGTAATATGAGTTAAGAGAAAAGCGATAAATGGGGTTCCGA
TATGCACGCCATGTAACCTGGCCAGATCCGCCAGCCTGTCCAGGGTGTGTAAGTAGACCG

(B)

TGATATTGAAGGAGCATTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACACATCAATGC
TATTTTGGTTTAGTCGTCCAGGCGGATCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCACTTGTAAGCAGTAGCGGCGGCGCTCGAAGTGTGA
CTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAAC
TTGTGCGTTTGTCAAGCAAGGTAAGTGAACGACCCGGTCATACCTTCTTAAGTTCGCCCT
TCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCCTCGATCATGAA
AAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGT
CTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGG
AGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTA
TGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGA
ATTCACGGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGA
CCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGAT
CGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGG
TCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTG
GCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGAT
GCTTTGGGCCGAGGACTGCCCCGAAGTCCACCTCGTGCACGCGGATTTCGGCTCCAACAA
TGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGG
GGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGA
GCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCG
GGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTT
CGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGAC
TGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGA
AGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAGAG
TAGATGCCGACC

Figure A.4. LaeA PCR knockout confirmation primers. (A) A part of NODE_6819 (*Trichoderma hamatum* GD12) is displayed. Dark blue text denotes the *lae* open reading (ORF). (B) Hygromycin vector sequence (not including TRPC promoter). Primers are denoted by: LF primers, RF primers, LF knock out confirmation primers, RF knock out confirmation primers, HY and YG. Underlined sequences denote stop or start codons.

Primer name	Sequence
KOC_LaeA_preLF	TTCAGGATCCTCAGGAGCTT
KOC_LaeA_inORF_LF	TTAGTCGCAGATTCGTGGGC
KOC_LaeA_preRF	GCGTAGTAGTACTGTCGACC
KOC_LaeA_inORF_RF	AGACCTCTGCAAACGGGTTA
KOC_inHY	TACCCGCAGGACATATCCAC
KOC_inYG	TTAGCCAGACGAGCGGGTTC

Table A.2. Primer sequences used to determine whether *laeA* transformations of GD12 were successful.

PCR of the LaeA open reading frame and Hygromycin

(A)

ACCATCCCACCTGCTGCTTGACTCGCACACCCGTTCCTCCCTAGTTAGCCGCTGCTTCCT
ATTCCTTACGAACTGGTGGTAAAGGTGACCGCGGTGACCGGAGCTGAGATTTTCAAACCT
CTTTTCAGGATCCTCAGGAGCTTCGGGTTTACCACCGCCTGGGATAATAAAGGCAAGAGT
TGAATCGTCGCTTATCCTAACCCTCGGCATCTGAGCGGCGTACTCTTGTACAACGTACAA
TTATATCTGTGTCACCTCCTTGGGTTTCAACCCGCGAAATATCTCCGTGTAAGTCACCAT
CTATCTTTGTTTTGTACGTCTGGTTACCCAACATCGTCCCTCCCGTCAGTTCGGTTATAT
GCTATTCTCCACACACCTATCTCTCCTGTCTTACCATGGTTTTGCGTATTGAAAGGCGCC
ATGTATGATACTGTGTACGTGCGGCTACGACTTGGTGTCATGTTCTACTAGTCAACATGG
CTTCTTTTCTTGGTAGCCTATCAATCACATCAGTCTGCTCCATCCCAAGTCTTACCTTTT
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TGCTTGCTCTCCCTGATTTTGTGTTTCTTTTCTCTCTCTTCTCGTCTTTTTGTTTTGTTT
CTACCAACTCCCACCTGCGATCTTGTCCAAATTTCATAGGTGCTCCAATACCAAGCCTGA
AGCTTAGTATACTCTCCGGGAAAGCCCCCGGTTTTCAGTAAGAGTGAGAGACGTCCATTC
TTATTTGGAGAATAGAGATTTACCCCCCTGTCGTCATATATGGCAGTCAACCATCTTCTC
TTCGTTTTTACCTCTACTACTCTGCCCCTGGTTCTTTTTATTTCATCTTTTGTTCTTTTT
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TTCCCTCGCCCTTGCCTGAAGACATACACAATTACACATTCTTGCACTAAATTTCTTGTT
ACTTTCCGCTTCTTTTTTTTTATCCAATACTTTCTTTCTGTAATTCTTTTTACTAATCTT
AGACTCTAGTTAATCTGATAATATTCACATTGGTTGATTTTCGCGTCTACCTGCTCGTAA
TTGCTGACAGCCATTTATTTCCTGGATTCCTATTTCCGTGCTACCTGCCTGGACAGCCCT
TGGGACCCCCATTATTTTTCTCGAAGGCTTTCAAGCTCCTATAGGACTCGCCATACTCGC
C**ATGTCGTCTCGAAACGCTCCAAGC**GGGTGCGTTGCACCCTCCCCAGCCACTGCTGCTCC
GCCTTCGCCCACGAATCTGCGACTAACTGTTGGGCAACCCGTCAGTGAGTCAGCCAATGA
ACCAGGAAGTGAATCAGAGGAACGAATTCTCCAGGACGGATATTGGGAGTACGGTCGATT
TTATGGTAGCTGGAAGAAAGGGAAATACAATTTTCCAATTGACAAGGTTTGCCTCCGCTT
GGCCACAGTATATGCGACATCTATTTTGACATCATTGCTTAGGAGGAGCTCAGTAGGCTG
GATATTCTCCACAAATACTTTGTCGTAGAAACCGAAGATCGCGTCACTTCCGTTCCTTTG
GATAAAGAAGGACGACCGAAAATCATGGACCTCGGAACGGGCACAGGTATTTGGGCTTTC
CATGTGGTGGAAGGGTAAGTTTTACCATGTCACCTGGTCCAAACTTGTTGCGTATCATCG
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CACCACAGCATCCCTCCAGGTGTAACTACAAAGCAGTTTGACATTGAGGAGCCTTCATGG
GAACCACTTCTCCGTGACTGCGACTTGATCCATCTTCGGCTGCTCTACGGCAGTATCAAA
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GACCGAAGACACCAAGCGCATGATAGAAGCAGCTGGCTTCACTGATTTTAAAGAAACTAC
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GCTGGGTATGAAAAAAGATGATGTTGTAGACCTCTGCAAACGGGTTAAAGCCGAAACTTG
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GTCCGCCGCAGTAAGATGCCTAAGTGTTCAGTATCTTCGTCACTATTTAGGACTCTCAAG
CATTTGCATCCATAATTCATAGGATGCAGACTGTGTTATTGACCAAGATCACTGATCATA
CAAGGGTAAAGCAACAATACGATTCCTCGGGGATCATCTTAACGGCGCTCGGCGTCGAAG
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CTGAGTATGGTTAATGACGGGCACTATAAACGGATTTGCATGAAGCAAGGAAGATCCAAG
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TCACGGCTTTGAGAGTATGGTTCATGTTTATGGGATAGTCATGTGTGCAATTTAGCGTTT
GGAAGTAACAATTTAGAATTAAAGAAAGAATGGTTCTTCCCCCTTACAATATCCTCTTTG
TCCTTGCCCCTTCTTGTGTAGCTATGAGGTTTTTTTTTTTTTTTTTTAACGNNNNNNNNN
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AAACATATATGGTTCGGCTAATTGCAAACCATCCAACGCATCGTCTCGGCATAGTATTTT
ATGATTGTCTTGTGGCGTTGGTCGACAGTACTACTACGCAGTGGCATGAGCATCCTGGTC
TGAATACTCGTATGTGACGAGTAATATGAGTTAAGAGAAAAGCGATAAATGGGGTTCCGA
TATGCACGCCATGTAACCTGGCCAGATCCGCCAGCCTGTCCAGGGTGTGTAAGTAGACCG

(B)

TGATATTGAAGGAGCATTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACACATCAATGC
TATTTTGGTTTAGTCGTCCAGGCGGATCACAAAATTTGTGTCGTTTGACAAGATGGTTCA
TTTAGGCAACTGGTCAGATCAGCCCACTTGTAAGCAGTAGCGGCGGCGCTCGAAGTGTGA
CTCTTATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAAC
TTGTGCGTTTGTCAAGCAAGGTAAGTGAACGACCCGGTCATACCTTCTTAAGTTCGCCCT
TCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCCTCGATCATGAA
AAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGT
CTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGG
AGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTA
TGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACATTGGGGA
ATTCACGGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGA
CCTGCCTGAAACCGAACTGCCCGCTGTTCTGCAGCCGGTCGCGGAGGCCATGGATGCGAT
CGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGG
TCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTG
GCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGAT
GCTTTGGGCCGAGGACTGCCCCGAAGTCCACCTCGTGCACGCGGATTTCGGCTCCAACAA
TGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGG
GGATTCCCAATACGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGA
GCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCGGCTCCG
GGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTT
CGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGAC
TGTCGGGCGTACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGA
AGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAGAG
TAGATGCCGACC

Figure A.5. Primers for LaeA DIG southern blot probe creation. (A) A part of NODE_6819 (*Trichoderma hamatum* GD12) is displayed. Dark blue text denotes the *lae* ORF. (B) Hygromycin vector sequence (not including TRPC promoter). Primers are denoted by: LF primers, RF primers, LeaA open reading frame southern knockout confirmation primers, HY, YG and hygromycin southern knockout confirmation primers. Underlined sequences denote stop or start codons.

Primer name	Sequence
SKOC_LaeA_ORF_F	CGTCTCGAAACGCTCCAAGC
SKOC_LaeA_ORF_R	TAGGCGTGGTAGCGCAGAAC
SKOC_LaeA_HYG_F	TTGGGCTTGGCTGGAGCTAG
SKOC_LaeA_HYG_R	TTTGCCCTCGGACGAGTGCT

Table A.3. Primer sequences used to amplify the LaeA open reading frame and the hygromycin open reading frame

DIG- Southern blot

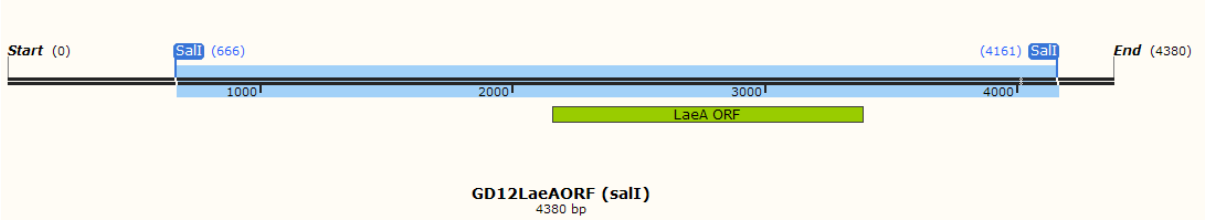

Figure A.6. Digestion of putative Δ*ThlaeA*::*hph* by SalI.
The continued presence of the LaeA ORF is shown by a band size of 3.5 Kb

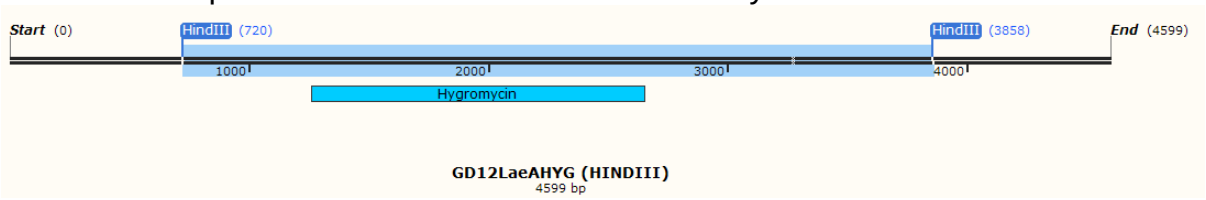

Figure A.7. Digestion of putative Δ*ThlaeA*::*hph* by *Hind*III.
A band size of 3.14 Kb indicates the deletion of the LaeA ORF and replacement with the Hygromycin ORF.

VeA

Product name	Band size	Primer name	Primer sequence (5’ to 3’)	DNA template
VeA left flank	1 kb	VeA_LF_FWD_YR	cctaggccaccatgttgggcccggcgcgcc AGTACCAATAGCACCCTGCT	GD12 DNA
		VeA_LF_REV_YR	ctccttcaatatcagttgggtaccgagctcG GTATCGCGTGAAATAAATG	
VeA right flank	1.2 kb	VeA_RF_FWD_YR	ggatcctctagagtcgacctgcaggcatgc GCCTCTCGGCATGCTACAAA	GD12 DNA
		VeA_RF_REV_YR	agtcagatctaccatggtggactcctcttaG TCACAGTCCGACAAACTAG	
trpC and Hygromycin resistance marker	1.5 kb	trpC_FWD_Ecor1	gagctcggtacccaactgatattga	Phyg-YR
		Hyg_Rev_HindIII	gcatgcctgcaggtcgactctagag	
Left flank +HY	2.4 kb	VeA_LF_FWD_YR	cctaggccaccatgttgggcccggcgcgcc AGTACCAATAGCACCCTGCT	PHYG-YR with VeA inserted
		HYSplit	GGATGCCTCCGCTCGAAGTA	
Right flank + YG	1.9 kb	YGsplit	CGTTGCAAGACCTGCCTGAA	PHYG-YR with VeA inserted
		VeA_RF_REV_YR	agtcagatctaccatggtggactcctcttaG TCACAGTCCGACAAACTAG	

Table A.4. Primers used to generate constructs for VeA knockout.

CTACTGCTGCTCACGGAGCGGCAAGCAGTAGGTGCTGCCACCACCGTTACAGGTGCTACG
AGTGGTGCAGGTACAGTACCTGTGCTACGGTACAGCGCCGCAAATGCATCAAGTGCTGTG
TCTGGCCCACCAATTGCGTGGCAGCCGGTGTCTTTTCTTTTCCTTTTGTTCCATCCTCTT
TTTTTGAAACCACCGCATCGCACATCATCCTTGCTCGTGCTATTAGCCCATTGCCTGACT
CGAGCCATGCATTTGCCCGCTCGCTTTGCCTGGTCCTTCTTTTTGCCCGCAACGCCGGCC
GCCATTTTTTTTTTTCTTCTTTCATTTTTAACGTTAAACACGGGAACGGGGGGCTGCAGT
AGCAGCACAAGCACAAGCATCAGTGCTGTCAAAAGCTACTGCTGGAGAAGCAAACATTGC
TGCGACCGACCGTCTGGAACCCGCTGGGGAACCAAGCAAAGGCCAAGCGTGGAGAAGCCG
GGTCTGCCACCCCCTCAGTCTGTGCCGCAGCCAATAGACTTCTAGTCGCCAGGGCTGGGC
TGCAGCCTCTCATCAGCACGAAGCTCAAGCGACCAGTGCTTTTCTTTTTTTGGCTTTTCT
GCTTCCTCCCATGTGCTCCCATGTGTGCCTGTGCTGGTGAGGCGAGAGCAATGAGTCCAT
GCACCTGAACACAAAAGAGGCACGGCACTTGTGCTTGGTGGGTTGGGTACCTTGGTGGTG
AGCTGTCTCGTCGCTTACAAAGTACATTGGCGTGGACAGCACTGTACTGTACAGTATAGC
ACCAGCCAGACGGCAATACCAACACAGGGGCCCGGGCTACACTAAGCAGGCTAGCTTATC
CCATTAATAGGTGCAGCTCGGCACGAGCACTCGTACACACAAGTACTGTACATGGCAGCA
GGGTCCTAGGAGCGGTCCGTCCTGGCATAGTACCAATAGCACCCTGCTGGCATGAGCATT
GCAGCGCGGTACCTAGTGGGCATTTTAGTGCCTTGTACCGTATAACTCAGACAAGCCCTC
GGTAGCGGTACCAGTACCAGAAAGTCACCCGTGAAGAAGCTGGGGGAGAAGATCCTTCCT
GCTCTTTCTCCTCCCGAACGAACCCAACCGCCATCCGGGCTCTCCAATCTCCACCCCTCT
GCCATCATCTGCTCTGCTGCATTCACCCCCAGCGCTCACCCCTCTCCACGCTCCTTTAAA
AAGAAAGAAAAAAAGGGAAAAAACGAAACCTTTCGCGTTCTTTCGGCCAAGGCTTACCTG
ATTGGACCTGGCCCAAGTCCTGGACATGCCAGGCTCCCTCAGCTGCGAGCCCCGCCCTCT
AGTCCCAGATTCCCGGGGCCCTCCCTCAATAATTGTGGCCCTGGCTCGAGACGGAACAGC

GTTGCCCAGCCTGGCCAGCATCTCTCAACTGCTACTACTACCTACACGGTCGCACATCCA
CCTCTTCGTCCTCCCGCTGGTTTCTCTCCTCTTGCACCTTCCAAGCACACAACCTCGATA
TACCCCCAAGGCCTGAGAGACGATAGACGAGACCAGAGAAGGAGAATACAAAACAAGACG
ATCAACTCAGTCACTCTCGACACAACCCGTCGTCGCTGCTATACATGATTAAACGACTCT
CGATTACTCGCGTACTGTAGTCTGGGCTGAACAGCGGGCTCCTTGCTCTCCCGCTCTGAC
GGTTTGGTGGCTGGAGAGAGTGCCTCGAGTCATTTGAAGGTGAGTTGATCATTTCTCATC
AAACACAGCTGCATTGCTTCTTTTTGCAGCTTCAAGTCTCGCCTCTTTACTGTCGCCGCC
ACTGCCTCATCTTCGATCTATTCGATGATCGCAGCCAAATGAAAAACTGTAGCACTAACA
TGGGCAAAACAGCCTCTTTTTGATACCTTCGTCTTCTTCATTCAATTACATTTATTTCAC
GCGATACCATGGCGACACCTTCCAGTGTGGCCTCCTCAGCAGGCCAGCGGGACATGGTCC
AACGCATTCACCGTGTGACTAGAGAGAACCGTCACCTTTGGTACCAGTTGACGGTTCTGC
AGCAGCCAGAGCGAGCTCGTGCATGTGGTTCGGGCATGAAAGGTACGGTTTTCATATTGA
CGGATGGATGATGCGTCGCTCTTCTCCATCTTTTCTATGCGAAAGTCTTCTTTGCTCACT
CCCTTCGTAGCTAATAGCGACCGACGACCCGTTGACCCGCCACCTGTTGTTGAACTTCGC
ATTATCGAGGGTCCTTCCGTTGAGGAAGGCAAGGACATCACTTTCGACTACAACGCCAAT
TTCTTCCTCTATGCGAGCTTGGAGCAGGCCCGTCCCATTGCCCATGGCCGTGTTCAAAAT
GGAGCTACTAACAACCCTCCCATCCTGACTGGTGTTCCTGCATCTGGCATGGCTTATCTC
GACCGACCTACTGAGGCTGGATACTTCATCTTCCCCGATCTCTCCGTCCGACACGAGGGC
TACTTCCGCCTTTCATTCAGCTTGTTCGAGACGACCAAGGAGGCCAAGGACTTTGACCTG
GAGCCTTCCGATTCCGATCTCCCCCCGGGTGTTGACTGGAGAATGGAAATCAAGACGCAG
CCCTTCAACGTCTTCAGTGCCAAGAAGTTCCCTGGACTGATGGAAAGCACTCCCCTCAGC
AAGACTGTTGCCGACCAAGGATGCCGAGTCCGTATCCGCCGCGATGTGCGTATGAGGAAG
CGTGATGGTAAGGGTAGCGGCTATGACCGACGCGAAGAAGAGTATGCTCGGCGTCGGACC
GTCACGCCGGCTCCCGCCGATGATCCTCACGGCCTGCGAGCCAGATCCGCCAGTAACGCG
AGCGAACACCGTGCGCCTTACATGCCACAGGAGCCATCACGGCGGCCATCTGCTGCTGAT
AGCTACCATGCTCCCTCATTGCACCCGCCTCCCCCGCCTCCGCCTCCCTCTTATGACGCA
CCTCGGCCATCCGCTCCTCCTGGCCATCTTGCATTCGGCGACCCAGCAATTCCGCAATAT
GGAGCGCCCGCTCCTCCTCGCCCATATGCTCACCAGCAAAGCGCCCCGATTCCTCCTGCA
ACACCTACTGGGCCATACCCAACAGCAGCTTCGCCATCTCCGTATGCCAAGACAGACTCG
CAGCCGTACAACTACAGTGCTCGACCTCCGGCGCCTAGCCCATCTCCAATTCCGCCTATG
AGGCACGCCATTTACGACAGTCGACCCTCAGAACCGTACTCATCATCTGAGAGGCGACCT
TCGCATGCTCCTTACCCTCCTTCTGGGCCGGCGGCACCATACTCTGCACCCTCTCTTCCG
CCTCTGCCTAGACGAGAGTCGACTCAATCATCATTGGCACCCCTCAGGATTGCTTCTTTG
GTGTCGCCTCTTCCGCCTATCGAGGCCCAGACAGAGCCTCTTCCTCCTCCACCAATGATG
TCGACAGGTGGCAAGAGAAAGCACGACTACGTCTTCTCCCAAAGTACCAAGCCGTTGTAC
AATGGCCAGAGGCAACTTGATGCACACTTTAGTCACGGCTACCGTGGAGTCACTCCCGAG
CCTGATCAGGGTGTCTACTCAAGAGCCGACGGTCATATCGGCGTCGTTACCTTTAACCAA
TACCAGGTATAAGCCTCTCGGCATGCTACAAATTCGACTCCGATGTATGAGTGACTCAAA
AATATTGGAGTATTATCTGCATTTCATTTTGATGCGTGATAGATTTCTCATGGGCGTTTT
GGGGCTTGTATCGACTATTAAATACCTCTTTTATTATTATTTCGCAAGAGGGCAACATGA
ATGGGCTAGTGATTTCTGGAGTTGGGGCACGACAAGTAGCTTGAAGCTTATATATAAAGC
AAGATGGCTCGACGGAACACAGCAACTTTATTGTGTTTTTTTTCCTTTTATATACATAT
ATATATCATGGTTATTCTTGATTCTTTCCTCCGACAGGTTGCACCACGCATAGATTGCCG
AAACAAAAAGAGAGACGTGAATGCGATGAAAATACCATTTACGCTCTACGTTGAATTTAG
TACTACCATATCTTGTGTTAATTTGGGTGGCATCACTGTTTCGCTGAAACTATCATTCCT
TGTTGGTTTTTTTTNNNNNNNNNNCTGATTCAACATTTGTGGTCGGTAGCTTTTGTTTCT
TATGTCTTTCTTGAAAGATGGTTTATAATTGATACATGAGGTTGAGAACCATTCTATATT
CGACTATTCTTTTCTTTTTCTATCTAACTGGTGAATTGAATTGTGATATATAATGCGCCC
TGAAATGCATAACTCTTGGATAACTCGGCAGCACATCACACAACTTGGTAGCCGGCACTG
TAGCTCGTGCTCTGATCGTTTCAGGCCAGTACAGCCGCCAAGCGTAGTGTCAGTCCTGCC
AGGTAGTTGCCGCACCCAAGTAATATAACTTCAAGTTGATAAACCTCTCTACTTGCTTCA
GGCTAAAAAAGAGCCGGAGCGAGATTCCTAAAGAGACTATAAATCGTATGGCATGAGCAA
TTAAAACCCTCAAAAAGAGAGGGAAGGAGGAGACTGAGTGTGGAAAAAAAAAATTAAAAG
AAGAGATGGGTGAAATACTACCCTCGGGTCGGCTAGTTTGTCGGACTGTGACTGTATTTT
CGAGCACAGAGTGGCTTTTAGGCCCGTTAAATGAGTAGGTGAGAGACCAGGGCTGGATGC
GCTAGAGGCGCTCTCTTGTGACCTGGGTATATATTGTGTGGCTGGCGCGAAGGAAACTCA

GTTCCCTATCGAGCTTGGAGTCCAGCTGGTCATGTGTGCTTCTTGGGTTCTACTGTATTG
TCATTGTCTCGTTTGTGCTGCGAGCTGGCGGCTAGTGTAGACTTGGCTGTCTTTGCCTCC
GCATTTTCTTCATCATCCCTTGGATACCAGCGTGCAGCAGCGTGCAGATGCTCGTGGCCC
TGATCATGCCAGCACGGAGTAGGCGCCGGCAGGCAAGTGCTAGCCCGTCCGGCCAGCGTC

Figure A.8. VeA GD12 construct primers. *Trichoderma hamatum* GD12 NODE_4214. A section of NODE_4214 from *Trichoderma hamatum* GD12 is presented. The *VeA* ORF is written in blue text. The primers highlighted dark blue encompass the left flank and the primers highlighted pink encompass the right flank. The 'NNNNNNNNNN' yellow highlighted segment signifies missing nucleotides. Sequences that are underlined represent stop or start codons.

Sequencing

Name	Primer sequence	Area sequenced
LF DNA border	tggcaggatatattgtggtgtaaaca	Left flank
VeA Forward	gagctcggtacccaactgatattga	TrpC and Hyromycin resistance cassette (forward)
VeA Reverse	gcatgcctgcaggtcgactctagag	Hygromycin resistance cassette (reverse)
RF DNA border	taaacgctcttttctcttaggtttac	Right flank

Table A.5. Primers used to sequence the *Saccharomyces cerevisiae* plasmid recombinated with VeA.
the Plasmid contains the inserted left and right flanks in addition to the trpC promoter and hygromycin resistance cassette.

A)

ATGTACTGAATTAACGCCGAATTAATTCCTAGGCCACCATGTTGGGCCCGGCGCGCCAGTACCAATAG
CACCCTGCTGGCATGAGCATTGCAGCGCGGTACCTAGTGGGCATTTTAGTGCCTTGTACCGTATAACT
CAGACAAGCCCTCGGTAGCGGTACCAGTACCAGAAAGTCACCCGTGAAGAAGCTGGGGGAGAAGATCC
TTCCTGCTCTTTCTCCTCCCGAACGAACCCAACCGCCATCCGGGCTCTCCAATCTCCACCCCTCTGCC
ATCATCTGCTCTGCTGCATTCACCCCCAGCGCTCACCCCTCTCCACGCTCCTTTAAAAAGAAAGAAAA
AAAGGGAAAAAACGAAACCTTTCGCGTTCTTTCGGCCAAGGCTTACCTGATTGGACCTGGCCCAAGTC
CTGGACATGCCAGGCTCCCTCAGCTGCGAGCCCCGCCCTCTAGTCCCAGATTCCCGGGGCCCTCCCTC
AATAATTGTGGCCCTGGCTCGAGACGGAACAGCGTTGCCCAGCCTGGCCAGCATCTCTCAACTGCTAC
TACTACCTACACGGTCGCACATCCACCTCTTCGTCCTCCCGCTGGTTTCTCTCCTCTTGCACCTTCCA
AGCACACAACCTCGATATACCCCCAAGGCCTGAGAGACGATAGACGAGACCAGAGAAGGAGAATACAA
AACAAGACGATCAACTCAGTCACTCTCGACACAACCCGTCGTCGCTGCTATACATGATTAAACGACTC
TCGATTACTCGCGTACTGTAGTCTGGGCTGAACAGCGGGCTCCTTGCTCTCCCGCTCTGACGGTTTGG
TGGCTGGAGAGAGTGCCTCGAGTCATTTGAAGGTGAGTTGATCATTTCTCATCAAACACAGCTGCATT
GCTTCTTTTTGCAGCTTCAAGTCTCGCCTCTTTACTGTCGCCGCCACTGCCTCATCTTCGATCTATTC
GATGATCGCAGCCAAATGAA

B)

ATGAATGCCTATTTTGGTTTAGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGT
TCATTTAGGCAACTGGTCAGATCAGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTT
ATTAGCAGACAGGAACGAGGACATTATTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGT
CAAGCAAGGTAAGTGGACGACCCGGTCATACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATT

CAATCTGACTTACCTATTCTACCCAAGCATCCAAATGAAAAAGCCTGAACTCACCGCGACGTCTGTCG
AGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCT
CGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTT
CTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGTGCTTGACA
TTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGTGCACAGGGTGTCACGTTGCAAGAC
CTGCCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCGGAGGCCATGGATGCGATCGCTGCGGC
CGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAATACACTACATGGC
GTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTGATGGACGACACCGTC
AGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCA
CCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTCATTG
ACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGGCCGTGG
TTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCGCG
CCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGGTTGACGGCAATTTCG
ATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGT
ACACAAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGG
AAACCGACGCCCCAGCACTCGTCCGAGGGCAAAGGAATAGAGTAGATGCCGA

C)

AACATAGATGACACCGCGCGCGATAATTTATCCTAGTTTGCGCGCTATATTTTGTTTTCTATCGCGTA
TTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTACATGT
TAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGA
TTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAACGATCGGGGAAATTCGAGCTGGTCACCTGTAA
TTCACACGTGGTGGTGGTGGTGGTGGCTAGCGTTAACACTAGTCAGATCTACCATGGTGGACTCCTCT
TAGTCACAGTCCGACAAACTAGCCGACCCGAGGGTAGTATTTCACCCATCTCTTCTTTTAATTTTTTT
TTTCCACACTCAGTCTCCTCCTTCCCTCTCTTTTTGAGGGTTTTAATTGCTCATGCCATACGATTTAT
AGTCTCTTTAGGAATCTCGCTCCGGCTCTTTTTTAGCCTGAAGCAAGTAGAGAGGTTTATCAACTTGA
AGTTATATTACTTGGGTGCGGCAACTACCTGGCAGGACTGACACTACGCTTGGCGGCTGTACTGGCCT
GAAACGATCAGAGCACGAGCTACAGTGCCGGCTACCAAGTTGTGTGATGTGCTGCCGAGTTATCCAAG
AGTTATGCATTTCAGGGCGCATTATATATCACAATTCAATTCACCAGTTAGATAGAAAAAGAAAAGAA
TAGTCGAATATAGAATGGTTCTCAACCTCATGTATCAATTATAAACCATCTTTCAAGAAAGACATAAG
AAACAAAAGCTACCGACCACAAATGTTGAATCAG

Figure A.9. VeA recombinated plasmid sequencing. A) VeA left flank, B) Trpc + hygromycin resistance gene and C) VeA right flank (later half). One insertion was seen.

PCR of the VeA open reading frame and Hygromycin

A)

CTACTGCTGCTCACGGAGCGGCAAGCAGTAGGTGCTGCCACCACCGTTACAGGTGCTACG
AGTGGTGCAGGTACAGTACCTGTGCTACGGTACAGCGCCGCAAATGCATCAAGTGCTGTG
TCTGGCCCACCAATTGCGTGGCAGCCGGTGTCTTTTCTTTTCCTTTTGTTCCATCCTCTT
TTTTTGAAACCACCGCATCGCACATCATCCTTGCTCGTGCTATTAGCCCATTGCCTGACT
CGAGCCATGCATTTGCCCGCTCGCTTTGCCTGGTCCTTCTTTTTGCCCGCAACGCCGGCC
GCCATTTTTTTTTTTCTTCTTTCATTTTTAACGTTAAACACGGGAACGGGGGGCTGCAGT
AGCAGCACAAGCACAAGCATCAGTGCTGTCAAAAGCTACTGCTGGAGAAGCAAACATTGC
TGCGACCGACCGTCTGGAACCCGCTGGGGAACCAAGCAAAGGCCAAGCGTGGAGAAGCCG

GGTCTGCCACCCCCTCAGTCTGTGCCGCAGCCAATAGACTTCTAGTCGCCAGGGCTGGGC
TGCAGCCTCTCATCAGCACGAAGCTCAAGCGACCAGTGCTTTTCTTTTTTTGGCTTTTCT
GCTTCCTCCCATGTGCTCCCATGTGTGCCTGTGCTGGTGAGGCGAGAGCAATGAGTCCAT
GCACCTGAACACAAAAGAGGCACGGCACTTGTGCTTGGTGGGTTGGGTACCTTGGTGGTG
AGCTGTCTCGTCGCTTACAAAGTACATTGGCGTGGACAGCACTGTACTGTACAGTATAGC
ACCAGCCAGACGGCAATACCAACACAGGGGCCCGGGCTACACTAAGCAGGCTAGCTTATC
CCATTAATAGGTGCAGCTCGGCACGAGCACTCGTACACACAAGTACTGTACATGGCAGCA
GGGTCCTAGGAGCGGTCCGTCCTGGCATAGTACCAATAGCACCCTGCTGGCATGAGCATT
GCAGCGCGGTACCTAGTGGGCATTTTAGTGCCTTGTACCGTATAACTCAGACAAGCCCTC
GGTAGCGGTACCAGTACCAGAAAGTCACCCGTGAAGAAGCTGGGGGAGAAGATCCTTCCT
GCTCTTTCTCCTCCCGAACGAACCCAACCGCCATCCGGGCTCTCCAATCTCCACCCCTCT
GCCATCATCTGCTCTGCTGCATTCACCCCCAGCGCTCACCCCTCTCCACGCTCCTTTAAA
AAGAAAGAAAAAAAGGGAAAAAACGAAACCTTTCGCGTTCTTTCGGCCAAGGCTTACCTG
ATTGGACCTGGCCCAAGTCCTGGACATGCCAGGCTCCCTCAGCTGCGAGCCCCGCCCTCT
AGTCCCAGATTCCCGGGGCCCTCCCTCAATAATTGTGGCCCTGGCTCGAGACGGAACAGC
GTTGCCCAGCCTGGCCAGCATCTCTCAACTGCTACTACTACCTACACGGTCGCACATCCA
CCTCTTCGTCCTCCCGCTGGTTTCTCTCCTCTTGCACCTTCCAAGCACACAACCTCGATA
TACCCCCAAGGCCTGAGAGACGATAGACGAGACCAGAGAAGGAGAATACAAAACAAGACG
ATCAACTCAGTCACTCTCGACACAACCCGTCGTCGCTGCTATACATGATTAAACGACTCT
CGATTACTCGCGTACTGTAGTCTGGGCTGAACAGCGGGCTCCTTGCTCTCCCGCTCTGAC
GGTTTGGTGGCTGGAGAGAGTGCCTCGAGTCATTTGAAGGTGAGTTGATCATTTCTCATC
AAACACAGCTGCATTGCTTCTTTTTGCAGCTTCAAGTCTCGCCTCTTTACTGTCGCCGCC
ACTGCCTCATCTTCGATCTATTCGATGATCGCAGCCAAATGAAAAACTGTAGCACTAACA
TGGGCAAAACAGCCTCTTTTTGATACCTTCGTCTTCTTCATTCAATTACATTTATTTCAC
GCGATACCATGGCGACACCTTCCAGTGTGGCCTCCTCAGCAGGCCAGCGGGACATGGTCC
AACGCATTCACCGTGTGACTAGAGAGAACCGTCACCTTTGGTACCAGTTGACGGTTCTGC
AGCAGCCAGAGCGAGCTCGTGCATGTGGTTCGGGCATGAAAGGTACGGTTTTCATATTGA
CGGATGGATGATGCGTCGCTCTTCTCCATCTTTTCTATGCGAAAGTCTTCTTTGCTCACT
CCCTTCGTAGCTAATAGCGACCGACGACCCGTTGACCCGCCACCTGTTGTTGAACTTCGC
ATTATCGAGGGTCCTTCCGTTGAGGAAGGCAAGGACATCACTTTCGACTACAACGCCAAT
TTCTTCCTCTATGCGAGCTTGGAGCAGGCCCGTCCCATTGCCCATGGCCGTGTTCAAAAT
GGAGCTACTAACAACCCTCCCATCCTGACTGGTGTTCCTGCATCTGGCATGGCTTATCTC
GACCGACCTACTGAGGCTGGATACTTCATCTTCCCCGATCTCTCCGTCCGACACGAGGGC
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CGTGATGGTAAGGGTAGCGGCTATGACCGACGCGAAGAAGAGTATGCTCGGCGTCGGACC
GTCACGCCGGCTCCCGCCGATGATCCTCACGGCCTGCGAGCCAGATCCGCCAGTAACGCG
AGCGAACACCGTGCGCCTTACATGCCACAGGAGCCATCACGGCGGCCATCTGCTGCTGAT
AGCTACCATGCTCCCTCATTGCACCCGCCTCCCCCGCCTCCGCCTCCCTCTTATGACGCA
CCTCGGCCATCCGCTCCTCCTGGCCATCTTGCATTCGGCGACCCAGCAATTCCGCAATAT
GGAGCGCCCGCTCCTCCTCGCCCATATGCTCACCAGCAAAGCGCCCCGATTCCTCCTGCA
ACACCTACTGGGCCATACCCAACAGCAGCTTCGCCATCTCCGTATGCCAAGACAGACTCG
CAGCCGTACAACTACAGTGCTCGACCTCCGGCGCCTAGCCCATCTCCAATTCCGCCTATG
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TCGCATGCTCCTTACCCTCCTTCTGGGCCGGCGGCACCATACTCTGCACCCTCTCTTCCG
CCTCTGCCTAGACGAGAGTCGACTCAATCATCATTGGCACCCCTCAGGATTGCTTCTTTG
GTGTCGCCTCTTCCGCCTATCGAGGCCCAGACAGAGCCTCTTCCTCCTCCACCAATGATG
TCGACAGGTGGCAAGAGAAAGCACGACTACGTCTTCTCCCAAAGTACCAAGCCGTTGTAC
AATGGCCAGAGGCAACTTGATGCACACTTTAGTCACGGCTACCGTGGAGTCACTCCCGAG
CCTGATCAGGGTGTCTACTCAAGAGCCGACGGTCATATCGGCGTCGTTACCTTTAACCAA
TACCAGGTATAAGCCTCTCGGCATGCTACAAATTCGACTCCGATGTATGAGTGACTCAAA
AATATTGGAGTATTATCTGCATTTCATTTTGATGCGTGATAGATTTCTCATGGGCGTTTT
GGGGCTTGTATCGACTATTAAATACCTCTTTTATTATTATTTCGCAAGAGGGCAACATGA
ATGGGCTAGTGATTTCTGGAGTTGGGGCACGACAAGTAGCTTGAAGCTTATATATAAAGC

AAGATGGCTCGACGGAACACAGCAACTTTATTGTGTTTTTTTTCCTTTTATATACATAT
ATATATCATGGTTATTCTTGATTCTTTCCTCCGACAGGTTGCACCACGCATAGATTGCCG
AAACAAAAAGAGAGACGTGAATGCGATGAAAATACCATTTACGCTCTACGTTGAATTTAG
TACTACCATATCTTGTGTTAATTTGGGTGGCATCACTGTTTCGCTGAAACTATCATTCCT
TGTTGGTTTTTTTTNNNNNNNNNNCTGATTCAACATTTGTGGTCGGTAGCTTTTGTTTCT
TATGTCTTTCTTGAAAGATGGTTTATAATTGATACATGAGGTTGAGAACCATTCTATATT
CGACTATTCTTTTCTTTTTCTATCTAACTGGTGAATTGAATTGTGATATATAATGCGCCC
TGAAATGCATAACTCTTGGATAACTCGGCAGCACATCACACAACTTGGTAGCCGGCACTG
TAGCTCGTGCTCTGATCGTTTCAGGCCAGTACAGCCGCCAAGCGTAGTGTCAGTCCTGCC
AGGTAGTTGCCGCACCCAAGTAATATAACTTCAAGTTGATAAACCTCTCTACTTGCTTCA
GGCTAAAAAAGAGCCGGAGCGAGATTCCTAAAGAGACTATAAATCGTATGGCATGAGCAA
TTAAAACCCTCAAAAAGAGAGGGAAGGAGGAGACTGAGTGTGGAAAAAAAAAATTAAAAG
AAGAGATGGGTGAAATACTACCCTCGGGTCGGCTAGTTTGTCGGACTGTGACTGTATTTT
CGAGCACAGAGTGGCTTTTAGGCCCGTTAAATGAGTAGGTGAGAGACCAGGGCTGGATGC
GCTAGAGGCGCTCTCTTGTGACCTGGGTATATATTGTGTGGCTGGCGCGAAGGAAACTCA
GTTCCCTATCGAGCTTGGAGTCCAGCTGGTCATGTGTGCTTCTTGGGTTCTACTGTATTG
TCATTGTCTCGTTTGTGCTGCGAGCTGGCGGCTAGTGTAGACTTGGCTGTCTTTGCCTCC
GCATTTTCTTCATCATCCCTTGGATACCAGCGTGCAGCAGCGTGCAGATGCTCGTGGCCC
TGATCATGCCAGCACGGAGTAGGCGCCGGCAGGCAAGTGCTAGCCCGTCCGGCCAGCGTC

B)

GATATTGAAGGAGCATTTTTTGGGCTTGGCTGGAGCTAGTGGAGGTCAACAATGAATGCCTATTTTGGTTT
AGTCGTCCAGGCGGTGAGCACAAAATTTGTGTCGTTTGACAAGATGGTTCATTTAGGCAACTGGTCAGATC
AGCCCCACTTGTAGCAGTAGCGGCGGCGCTCGAAGTGTGACTCTTATTAGCAGACAGGAACGAGGACATTA
TTATCATCTGCTGCTTGGTGCACGATAACTTGGTGCGTTTGTCAAGCAAGGTAAGTGGACGACCCGGTCAT
ACCTTCTTAAGTTCGCCCTTCCTCCCTTTATTTCAGATTCAATCTGACTTACCTATTCTACCCAAGCATCC
AAATGAAAAAGCCTGAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCC
GACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGT
CCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGATCGTTATGTTTATCGGCACTTTGCATCGGCCG
CGCTCCCGATTCCGGAAGTGCTTGACATTGGGGAGTTCAGCGAGAGCCTGACCTATTGCATCTCCCGCCGT
GCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCTGTTCTCCAGCCGGTCGCGGAGGC
CATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCG
GTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTATCACTGGCAAACTGTG
ATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGCTCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCC
CGAAGTCCGGCACCTCGTGCATGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAG
CGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATACGAGGTCGCCAACATCCTCTTCTGGAGG
CCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCC
GCGCCTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAGCTTGTTGACGGCAATTTCGA
TGATGCAGCTTGGGCGCAGGGTCGATGCGACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACAC
AAATCGCCCGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGA
CGCCCCAGCACTCGTCCGAGGGCAAAGGAATAG

Figure A.10. Primers for VeA DIG southern blot probe creation. (A) A part of NODE_4214 (*Trichoderma hamatum* GD12) is displayed. Dark blue text denotes the *lae* ORF. (B) Hygromycin vector sequence (not including TRPC promoter). Primers are denoted by: LF primers, RF primers, VeaA open reading frame southern knockout confirmation primers, and hygromycin southern knockout confirmation primers. Underlined sequences denote stop or start codons.

Primer name	Sequence
SKOC_VeA_ORF_F	ACACCTTCCAGTGTGGCCTC
SKOC_VeA_ORF_R	GGTTAAAGGTAACGACGCCG

SKOC_LaeA_HYG_F	TTGGGCTTGGCTGGAGCTAG
SKOC_LaeA_HYG_R	TTTGCCCTCGGACGAGTGCT

Table A.6. Primer sequences used to amplify the VeA open reading frame and the Hygromycin open reading frame

DIG- Southern blot

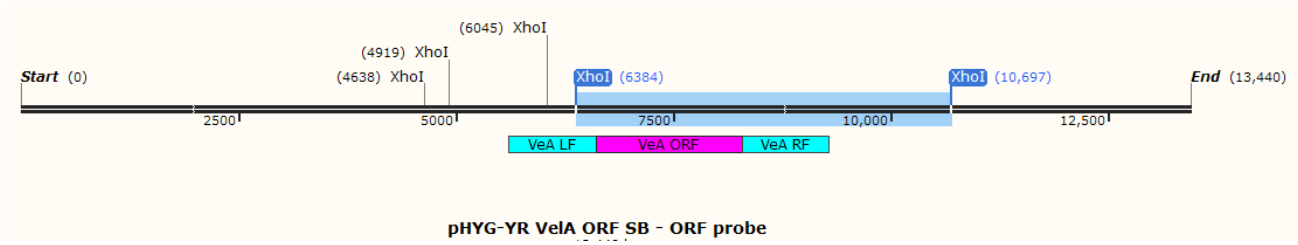


Figure. A.11. Digestion of putative Δ*ThVaeA*::*hph* by XhoI, indicates the continued presence of the LaeA ORF is shown by a band size of 4.3 Kb

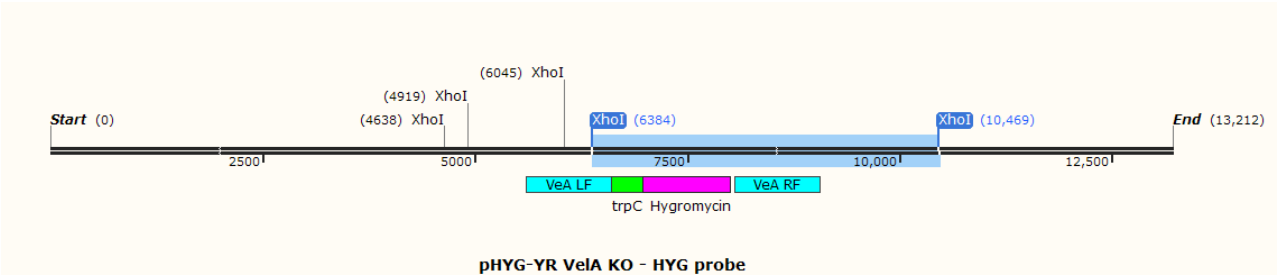
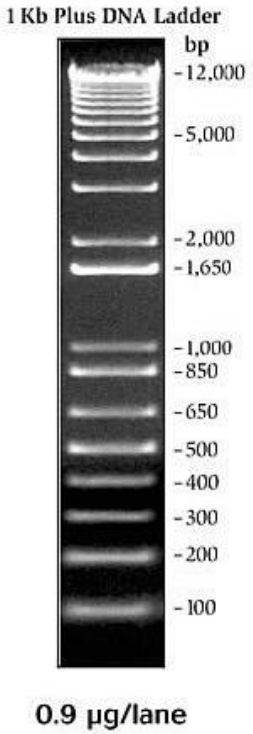
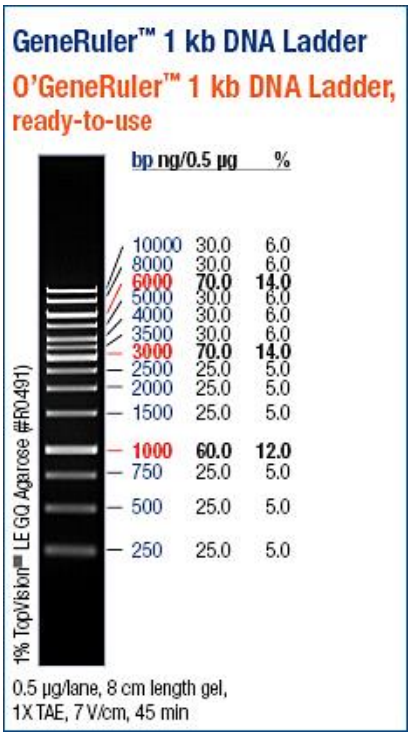


Figure. A.12. Digestion of putative Δ*ThVeA*::*hph* by XhoI, a band size of 4.1 Kb indicates the deletion of the LaeA ORF and replacement with the Hygromycin ORF.

Ladders

1 kb plus ladder (Invitrogen) and 1kb DNA ladder (GeneRuler):





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