Infection structure-specificity of β-1,3-glucan synthase is essential for pathogenicity of *Colletotrichum graminicola* and evasion of glucan-triggered immunity

Dissertation

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List of abbreviations

aa	Amino acid
AVC	Apical vesicle cluster
BLAST	Basic local alignment search tool
bp	Base pair
cDNA	Coding DNA
CHS	Chitin synthase gene
CM	Complete medium
CSPD	Chemiluminescent substrate for detection of phosphatat
cv	Cultivar
d	Days
DAI	Days after inoculation
dATP	Deoxyadenosin triphosphat
dCTP	Deoxycytidin triphosphat
dGTP	Deoxyguanosin triphosphat
DIC	Differential interference contrast
DIG dUTP	Digoxigenin labeled deoxyuridine triphosphate
DJ-PCR	Double joint PCR
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosid triphosphat
dsRNA	Double stranded RNA
dTTP	Deoxythymidin triphosphat
e.g.	Lat.: *exempli gratia*, for example
eGFP	Enhanced green fluorescent protein
E.R.	Endoplasmic reticulum
FDA	Fluorescein diacetate
Fw	Forward
G	Golgi apparatus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
GDP/GTP	Guanosine di/triphosphate
GLS	Glucan synthase gene
GT	Glucan transporter
h	Hour
HAI	Hours after inoculation
HDEL	(his-asp-glu-leu) C-terminal tertapeptide for retention in the E.R.

HIGS	Host induced gene silencing
hph	Hygromycin B phospotransferase gene
HR	Hypersensitive response (hypersensitive reaction)
i.e.	Lat.: *id est*, that is
IT	Intron
ITS	Internal transcript spacer (ribosomal sequence)
kb	Kilo base pairs
kDa	Kilo Daltons (protein mass unit)
KO	Knockout
KRE	Killer resistance gene
M	Molar
MPa	Mega Pascal
mRNA	Messenger RNA
NA	Numerical aperture
Nat-1	Nourseothricin acetyl transferase 1 gene
NCBI	National center for biotechnology information
oliC	Oligomycin resistance gene of *Aspergillus nidulans*
OMA	Oat meal agar
ON	Over night
ORF	Open reading frame
PAMPs/MAMPs	Pathogen/microbe associated molecular patterns
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
pH	Lat.: *potential hydrogenii*, hydrogen ion concentration
Pi	Inorganic phosphate
PM	Plasma membrane
PRR	Pattern recognition receptors (recognition system of plant)
qPCR	Quantitative PCR
qRT-PCR	Quantitative RT-PCR
RHO1	*RHO1* gene (regulator protein of glucan synthase)
RISC	RNA induced silencing complex
RM	Regeneration medium
RNA	Riboxyribonucleic acid
RNAi	RNA interference
rpm	Rotation per minute

RT	Room temperature
RT-PCR	Reverse transcriptase PCR
Rv	Reverse
SDH	Saccharopine dehydrogenase
SDS	Sodium dodecyl sulfate
siRNAs	Short interfering RNAs
SPEP	Secreted peptidase
sp	Species
Spk	Spitzenkörper
SSC	Sodium chloride/sodium citrate
ssp	Subspecies
ssRNA	Single stranded RNA
TAE	Tris/Acetat/EDTA
Taq	*Thermus aquaticus*
TE	Tris/EDTA
TM	Melting temperature
TMD	Transmembrane domain
toxB	Gene specific for toxin B of *Pyrenophora tritici-repentis*
Tris	Tris (hydroxymethyl) aminomethane
trpC	Tryptophan synthase C gene of *Aspergillus nidulans*
TS	Tween solution
tub1	α-Tubulin gene of *Botrytis cinerea*
U	Units
UDPGlc	Uredine diphosphate glucose
UDPGlc*N*Ac	Uredine diphosphate *N*-acetyglucosamide
UV	Ultraviolet
V	Vesicle
VSC	Vesicle supply center
v/v	Volume/volume (%)
WT	Wild-type
w/v	Mass/volume (%)
x	Times
YPD	Yeast pepton dextrose medium
YSCM	Yeast synthetic complete medium
Ø	Diameter

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Explanatory notes

The present work was written in English. Latin terms, species and genus names are in italics. Gene designations are written with capital letters in italics (e.g. *GLS1*). The name of the corresponding protein uses the same nomenclature, but in upright (e.g. GLS1). Deletion mutants carry a Δ and the gene name with lower-case letters in italics (e.g. Δ*rho1*). Genes, proteins and mutants, which are described in the literature, are named according to the nomenclature in the appropriate publication.

I Introduction

1.1 The importance of the fungal cell wall

Fungal cell walls merit detailed consideration because they have many essential roles (Martinez *et. al*., 2004). Cell walls are responsible for the fungal structure shape, because removal of the wall by lysing enzymes leads to spherical protoplasts (Bartinicki-Garcia and Lippan 1966; Werner *et al*., 2007). The cell wall is also the interphase between a fungus and its environment. The cell wall play indispensable roles for fungal adaptation in the environment: it protects against osmotic stress; it acts as molecular sieve controlling the passage of large molecules through the wall pore space; and when the wall contains pigments such as melanin it can protect against ultraviolet radiation or the lytic enzymes of other organisms. Furthermore, the wall can have several physiological roles. It can bind sites for enzymes, such as invertase and β-glucosidase. Some studies support also the hypothesis that fungal cell walls are involved in adhesion and cell-cell interaction (Peberdy, 1990; Ruiz-Herrera, 1992; Sentandreu *et al*., 1994; Deacon, 1997; Martinez *et. al*., 2004).

Pharmacological studies with fungi infecting humans suggest that cell wall polymers are required for pathogenicity (Wheeler *et al*., 2008; Tomazett *et al*., 2010). Indeed, the success of fungal infection depends of the ability to modify the cell wall during the infection process. For example, the ability of *Candida albicans* to switch between the yeast and filamentous forms is strongly associated with virulence. Filaments are distinct from yeast-form cells in cell wall arrangement, cell wall proteins and transcriptional programs. In addition, the hyphal form of the fungus has been shown to cause more tissue damage than the yeast-form in *ex vivo* models of candidiasis (Chaffin *et al*., 1998; Wheeler *et al*., 2008).

In plant pathogenic fungi, biogenesis of the cell wall and its modification are essential to meet changing structural requirements during infection-related morphogenesis and avoidance of surface exposition of elicitor-active cell wall polymers. Formation of rigid cell walls may be of prime importance in *ad planta* differentiated elaborate infection cells called appressoria, as an enormous turgor pressure of up to 8.0 MPa (80 bar) needs to be controlled (Howard *et al*., 1991b; Bastmeyer *et al*., 2002). Not only in appressoria, but also in infection hyphae formed in planta, cell wall polymers are essential structural compounds, as indicated

by severe hyphal distortions and virulence defects of mutants lacking these compounds in several pathogens (Madrid *et al*., 2003; Weber *et al*., 2006; Werner *et al*., 2007). However, functional analyses of the role of several cell wall compounds in pathogenesis are still lacking in phytopathogenic fungi.

1.2 Models of growth of fungal hyphae

Apical growth is the hallmark of fungi. A series of elegant models were created to explain apical growth of fungal hyphae including elasticity/rigidification (Robertson, 1965; Saunders and Trinci, 1979), plasticity (Bartnicki-Garcia, 1973), or polymer cross-linking (Wessels and Sietsma, 1981; Wessels, 1986), vesicle supply center and Spitzenkörper mobility (Bartnicki-Garcia, 1995 a,b).

Robertson (1965) performed early key experiments on apical growth, using simple methods but coupled with truly remarkable insight. He proved that the hyphal tips of *Fusarium oxysporum* and *Neurospora crassa* are plastic, deformable and show different growth patterns in different conditions of presence or absence of osmotic stabilization.

To interprete these results of the behavior of hyphal tips, Robertson (1965) proposes that apical growth can be divided into two different processes, i.e., extension of a plastic, deformable tip and rigidification of the wall in sub-apical regions. According to this model the turgor pressure is an essential factor in the growth of vegetative hyphae and indeed, re-adjustments of turgor pressure in the fungal cell wall are crucial for the germination of spores, and the penetration of plant surfaces by germ tubes (Robertson, 1965; Deacon, 1997).

The concept that the cell wall must be continuously loosened by lytic enzymes in order to expand is well established for intercalary wall growth in bacteria (Koch, 1988), plants (Cleland, 1981), and has also been suggested to occur during growth of some mushrooms. That wall-loosening enzymes play a role in apical extension of filamentous fungi has been advocated by Bartnicki-Garcia (1973) and Gooday and Trinci (1980). However, direct evidence for this concept, which presumes a “delicated balance between wall synthesis and wall lyses” (Bartnicki-Garcia, 1973) has not been obtained (Gooday *et al*., 1992; Sentandreu *et al*., 1994).

The consecutive “steady-stady model of apical growth” described by Wessels (1993) disregards the involvement of wall lytic enzymes. According to this model the newly formed wall at the extreme tip is viewed as being viscoelastic, so that it flows outwards and backwards as new components are added at the tips, then the wall rigidifies progressively by formation of extra bonds behind the tip. This is remarkably consistent with Robertson’s original idea of a plastic, deformable wall at the tip that is rigidified behind the apex, but based now on much more biochemical evidence for wall bonding (Wessels, 1993; Deacon 1997; Sietsma and Wessels, 1994).

Bartnicki-Garcia *et al*. (1995a,b) proposed the “Vesicle supply center (VSC) model of apical growth”. The VSC is localized at the extreme hyphal tip and plays an essential role in growth. The vesicles of the VSC care enzymes responsible for synthesis of new cell wall components. When these vesicles are fusioned to the plasma membrane at a most apical site, new cell wall polymers, synthesized in the plasma membrane, are transpoted by flow of new wall polymers to the outsite of the sub-apical wall at the hyphal tip. Two different options are propose to explain continuous tipward displacement of the VSC: a pushing mechanism and a pulling mechanism. A pushing mechanism would include anterograde force resulting from an attachment of the VSC with cytoskeletal components, or from bulk flow of the protoplasm towards the tip. A pulling mechanism implies a link between the apical plasma membrane/cell wall and the VSC. The VSC will be described in details in paragraph 1.3.

I.3 Apical organization of fungal hyphae

Fungi have an exclusive structure and organization, central to their mechanism of apical growth. There is a progressive change in the cytoplasm with distance behind the hyphal tip. The apical region is rich in organelles, including a dense zone of mitochondria immediately behind the growing apex. However, the hyphal apex (the terminal 1-5 µm) is filled with a cluster of small membrane-bound vesicles, the VSC or apical vesicle cluster (AVC), which plays a major role in growth. This was termed the Spitzenkörper (Spk) and it is thought to correspond to a central region of the AVC (Sentandreu *et al*., 1994; Deacon, 1997; Bartnicki-Garcia, 1990, 2003; Bartnicki-Garcia *et al*., 1995a,b).

The VSC model proposes the presence of a VSC at the hyphal tip that serves as a distribution center for plasma membrane and cell wall containing vesicles (Bartnicki-Garcia, 1990, 2003; Bartnicki-Garcia *et al*., 1995a,b). Vesicles are transported from distal parts of the cell to the VSC via the cytoskeleton, and are then distributed to the surface where they fuse with the plasma membrane. An apical microfilament-based system is involved in the transport of vesicles to the apex. In this context, the Spk can be perceived as a switching station where microtubule-based transport changes into microfilament-based transport (Riquelme *et al*., 2000; Bartnicki-Garcia *et al*., 1978; Bartnicki-Garcia, 2003). A scheme of the VSC model of fungal apical growth is shown in Figure 1 and some of its components are discussed below.

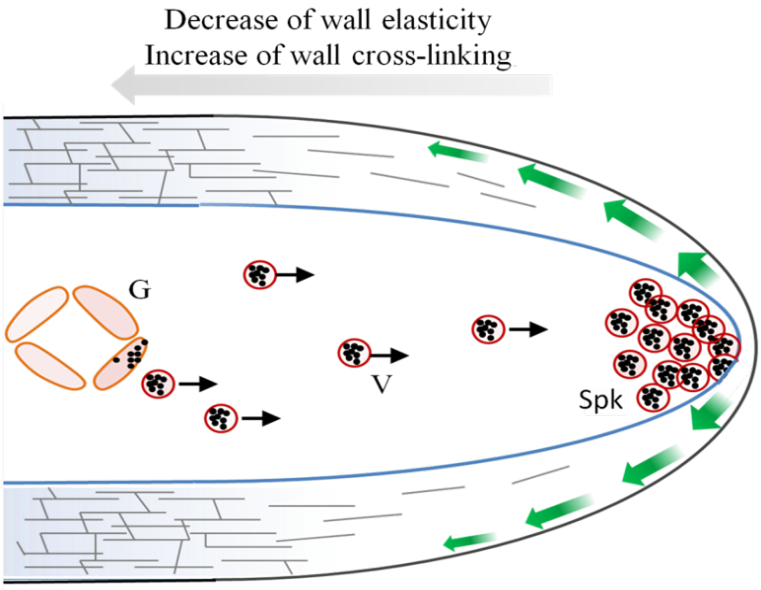

Fig. 1: Schematic representation of the organization of hyphal tip

A large number of vesicles (V), delivered from the Golgi apparatus (G), are transported to the hyphal tip. The vesicles in the extreme hyphal tip are organized in cluster, the apical vesicle cluster (AVC) or Spitzenkörper (Spk), which plays an essential role in growth. The vesicles are fusioned to the plasma membrane at a most apical site and new cell wall polymers, synthesized in the plasma membrane, are transpoted by flow of new wall polymers to the outsite of the sub-apical wall. There is little or no cross-linking of wall polymers in the hyphal tip. Behind the growing apex, the walls are gradually rigidified by cross-linking of walls polymers. The decrease thickness of arrows behind the tip signifies progressively reduced flow of new cell wall components (modified after Bartnicki-Garcia et al. 1990, 1995a; Wessels 1990; Deacon 1997).

The observed or predicted gradients of wall properties in the hyphal apex (Robertson, 1965, Saunders and Trinci, 1979; Bartnicki-Garcia, 1968; Wessels and Sietsma, 1981, Wessels, 1986) are probably not the cause of hyphal morphogenesis but, rather, a reflection of the pattern of vesicle discharge, which generates a graded distribution of biochemical/biophysical activities on the cell surface. Accordingly, the key to hyphal morphogenesis probably does not lie in the apex per se, as believed previously (Robertson, 1965, Bartnicki- Garcia, 1968, Gooday and Trinci, 1980) but in the mechanisms that cause the linear displacement of the VSC.

I.4 Fungal cell walls polymers

Studies of cell walls of members belonging to different taxonomic groups of fungi have shown an evident variability, not only in the nature of the polissaccharides present, but also in the arrangement between them (Bartnicki-Garcia, 1968; Peberdy, 1990; Ruiz-Herrera, 1992; Wessels and Sietsma, 1981; Sietsma and Wessels, 1994).

Fungal cell wall polymers can be classified mainly into two groups, concerning to their functions: the so-called structural polymers that are responsible for rigidity of the cell wall, and the matrix polymers, in which they are embedded, that give resistance to compression. The types of polysaccharide are found to differ between the major fungal groups, as shown in Table 1. The chytridiomycota, ascomycota and basidiomycota typically have chitin and glucan (polymers of glucose) as their major wall polysaccharides. The zygomycota typically have a mixture of chitin, chitosan and polymers of uronic acids such as glucuronic acid instead of glucans (Wessels and Sietsma, 1981; Sietsma and Wessels, 1994).

Table 1: Main wall constituents found in each division of fungi (modified after Gooday, 1995)

Phylum	Fibrous	Gel-like Polymer
Basidiomycota	chitin, β-1,3, β-1,6 glucan	α-1,3 glucan, xylomannoproteins
Ascomycota	chitin, β-1,3, β-1,6 glucan	α-1,3 glucan, galactomannoproteins
Zygomycota	chitin, chitosan	Polyglucuronic acid, glucuronomannoproteins, polyphosphate
Chytridiomycota	chitin, glucan	glucan

The polysaccharides, composing 90% of the cell wall have been simplistically separated into a fibrillar skeleton that is alkali-insoluble and amorphous cement that is alkali-soluble. Fibrillar polysaccharides are located close to the plasma membrane to be able to perform their mechanical function, whereas amorphous polysaccharides are present in the entire cell wall but especially in the outer face. However, this clear-cut separation is only theoretical since it is technically difficult to analyse the cell wall polymers without prior chemical treatment of the cell wall. In addition, the way the amorphous and fibrillar cell wall components are linked is to date unclear since linkages removed by NaOH remain unknown. A better understanding of the spatial association of the cell wall components would involve the development of antibodies recognizing specific linkages between individual carbohydrates as previously used to define plant cell wall structures (Knox, 2008; Latgé, 2010).

Mannoproteins are also characterized as important components of fungal cell walls. Glucan and or chitin have been generally endowed with the structural function, although some mannoproteins, normally considered as typical matrix polymers, may also play significant roles in maintaining the overall architecture of the walls (Ruiz-Herrera, 1992; Wessels and Sietsma, 1981; Sietsma and Wessels, 1994; Sentandreu *et al*., 1994).

I.4.1 Glucans

Glucans are the most prominent polysaccharides present in the cell wall of ascomycetes. The term glucan applies to several polysaccharides made up of glucose units and can be divided according to their type of bonding into α- and β-glucans. Both types contain 1,3- and/or 1,4-linkages, but only β-1,3-glucans also contain 1,6-bounds (Figure 2). Both α- and β-linked glucans are present in the most fungal taxa, although β-glucans are more abundant. Ruiz-Herrera (1992) has described six different types of β-glucans, based on whether they are linear or branched, and the type and size of their branches. Cosidering this heterogeneity, it is not surprising that the structure of glucans is extremely variable, from soluble to gel-forming, and insoluble microfibrilar polymers (Ruiz-Herrera, 1992; Sietsma and Wessels, 1994; Sentandreu *et al*., 1994; Latgé, 2010).

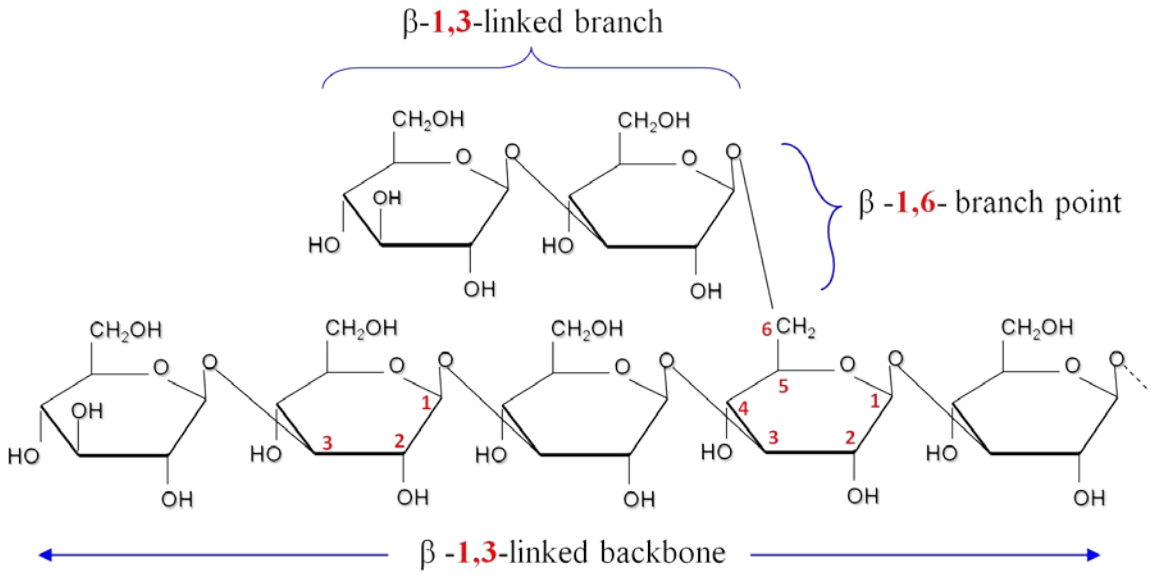

Fig. 2: Scheme of a branched fragment of β-1,3-glucan containing a β-1,6-bond
(modified after Ruiz-Herrera, 1991; Roemer *et al*., 1994)

Glucans play various roles in fungal physiology. Some are important for virulence such as the β-1,3-glucans from the human pathogens *Paracoccidioides brasiliensis* (Tomazett *et al.*, 2010) and *C. albicans* (Wheeler *et al*., 2008), but their most important role is structural. Thus, interference with glucan synthesis by drugs leads to severe morphological modifications (Perez *et al.*, 1981) and changes in glucan structure have been found correlated with mutations inducing cell weakness in *Saccharomyces cereviseae* (Blagoeva *et al*., 1991) and *Aspergillus nidulans* (Borgia and Dodge, 1992).

Considering that all naturally occurring β-glucans are branched polysaccharides (Wessels and Sietsma 1981), their biosynthesis must involve several steps: initiation, elongation of the β-1,3-linked chain, branching, and branch elongation (Ruiz-Herrera, 1992; Latgé, 2010).

Moust of our knowledge on glucan synthesis concerns chain elongation, although all data have been collected with crude enzyme preparations. A second point to be noticed is that, in contrast to chitin synthases, the properties for the different β-glucan sythases are extremely variable among the different systems analysed (Ruiz-Herrera, 1992; Ruiz-Herrera and Sentandreu, 1989; Latgé, 2010).

An intelligent approach to study branching of β-glucans has come from the analysis of *S. cereviseae* mutants resistant to a killer toxin. Some of these mutants are deficient in the natural toxin receptor, a branched β-1,6-linked glucan. These results clearly showed that these genes are normally involved in the synthesis of β-1,6-glucan. The genes *KRE1* and *KRE5* have been cloned (Boone *et al*., 1990) and a functionally homologus gene of *KRE1* has been isolated from *C. albicans* (Boone *et al*., 1991). *KRE1* codes for a 32 kDa protein which is probably glycosylated and secreted. *KRE5* codes for a protein containing a HDEL signal for retention in the endoplasmic reticulum, and an additional gene, *KRE6*, encodes a protein involved in both β-1,6 and β-1,3-glucan synthesis (Roemer *et al.*, 1994). Subsequently, additional *KRE* genes have been detected (Brown and Bussey, 1993) that extended our knowledge of the route of β-glucan synthesis. These results suggested that glucan branching is possibly an early event happening intracellulary, even though final branching might occur within the domains of the cell wall (Sentandreu *et al*., 1994).

I.4.2 Chitin

Chitin is an unbranched polysaccharide exclusively containing *N*-acetylglucosamine (Glc*N*Ac) residues (Figure 3). This polysaccharide is composed by units of Glc*N*Ac joined through β-1,4 linkages and has a important structural function. Chitin chains have the tendence to associate by hydrogen bonding in arrays of fibrils or sheets which are stabilized by C=O … H-N bonds (Sentandreu *et al*., 1994). Chitin and β-1,3-glucan are linked in fungal cell walls by covalent bounds (Latgé, 2010).

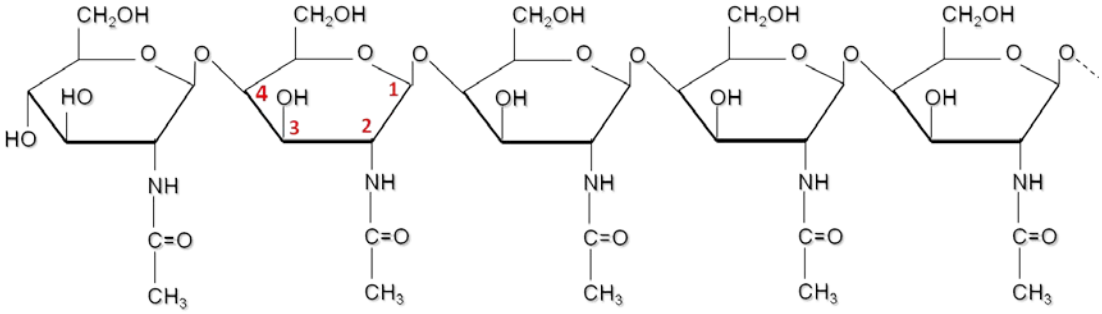

Fig. 3: Scheme of a linear fragment of chitin, *N*-acetylglucosamine containing β-1,4-bonds

From a biochemical point of view, chitin biosynthesis can be considered as transglycolase reaction where acetylglucosamyl residues are transferred from a donor to an acceptor. The universal donor for the reaction is the nucleotide uredine-diphosphate-*N*-acetyglucosamide (UDP Glc*N*Ac), while the acceptor is the growing chitin chain (Latgé, 2010).

There is no general model describing the catalytic reaction carried out by chitin sythases. Several details remain unresolved. The first reflects to the initiation to the reaction. The prevailing opinion is that no acceptor is necessary for initiation of chitin chain, although some reports have suggested that either a polypeptide or a glucolipid were ultilized as an acceptor for chain inbiciation (Bartnicki-Garcia *et al*., 1978).

Chitin can also be converted in chitosan. Chitin deacetylase, the enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of *N*-acetyl-D-glucosamine residues, was first identified and partially purified from extracts of the fungus *Mucor rouxii* (Araki and Ito, 1975). Since then, the presence of this enzyme activity has been reported in several other fungi (Siegrist and Kauss, 1990; Trudel and Asselain, 1990).

I.5 Synthesizing enzymes of structural cell walls compounds

I.5.1 Glucan synthase

Normal growth and morphogenesis of fungi are dependent upon the activity of plasma membrane-localized β-1,3-glucan synthase (GLS). This enzyme forms the most prominent structural cell wall polymer, which confer form and rigidity to vegetative hyphae and to specialized infection structures such as appressoria and infection hyphae growing inter- or intracellularly in their host (Wessels, 1993 and 1994). β-1,3-glucan is synthesized by a plasma membrane associated glucan synthase complex, which uses UDP-glucose as a substrate and extrudes linear chains of β-1,3-glucans through the membrane (Beauvais, *et al*., 2003). Once these glucans are extended into the extracellular cell wall space, they become β-1,6-branched before they can serve as an anchor for other polysaccharides (Gastebois *et al*., 2010).

Mutations that alter intracellular levels of substrate of these enzymes result in cells with aberrant morphologies (Ha *et al*., 2006). Mutants with strongly reduced levels of GLS activity are non-viable, unless cells are grown in medium providing osmotic support (Douglas, 2007). Chemically, the fungal cell wall consists of 80 to 90% polysaccharides (Bartnicki-Garcia, 1968). β-glucans seem to be the principal polysaccharides elaborated by the fungus *Phytophthora cinnamomi*. The two different β-glucans, β-1,3 and β-1,4-glucan, comprise nearly 90 % of the mycelia walls of this oomycete (Bartnicki-Garcia, 1966). Beta glucans constitute the most important structural polysaccharides of the yeast cell wall (Lopez-Romero and Ruiz-Herrera, 1977). The *Saccharomyce*s cell wall model helped understanding the mechanism of glucan biosynthesis; e.g. fibrillogenesis *in vitro* (Larriba *et al*., 1981), the role of nucleotides (Notario *et al*., 1982; Szaniszlo *et al*., 1985), and the composition of the GLS of catalytic and regulatory subunits (Kang and Cabib, 1986).

The enzyme involved in β-1,3-glucan synthesis in yeast is the GLS, which uses UDP-glucose as substrate, producing a linear β-1,3-glucan (Cabib *et al*., 1998). The activity requires at least two components, a catalytic and a regulatory subunit (Kang and Cabib, 1986; Ribas *et al*., 1991). The latter is the RHO1 GTPase, which activates GLS (Arellano *et al*., 1996; Drgonová *et al*., 1996; Qadota *et al*., 1996). This GTPase plays a fundamental role in many morphogenetic processes, particularly in the organization of the actin cytoskeleton (Arellano *et al*., 1999; Cabib *et al*., 1998; Cabib *et al*., 2001). Although β-1,3-glucan biosynthesis has been the subject of intensive research efforts for the last 30 years, the β-1,3-glucan biosynthetic pathway is not fully understood (Beauvais *et al.*, 2001).

In contrast, the vast majority of filamentous ascomycetes sequenced so far have only a single *GLS* gene in their genomes (Latgé, 2007; Mouyna *et al*., 2004). β-1,3-glucan serves as the main structural polymer, comprising between 65 and 90% of the fungal cell wall, to which other polymers such as chitin or glycoproteins are covalently linked, and this network of polymers confers mechanical strength and integrity (Bowman *et al*., 2006). β-1,3-glucan is vectorially synthesized across the plasma membrane, and purification of the large glucan synthase complex of *Aspergillus fumigatus* by product entrapment allowed the identification of four proteins, i.e. the catalytic subunit FKS1, the regulatory small G-protein RHO1, a membrane H^+-ATPase, and an ABC-type glucan transporter (Beauvais *et al*., 2001). A scheme of fungal glucan synthase complex is shown in Figure 4.

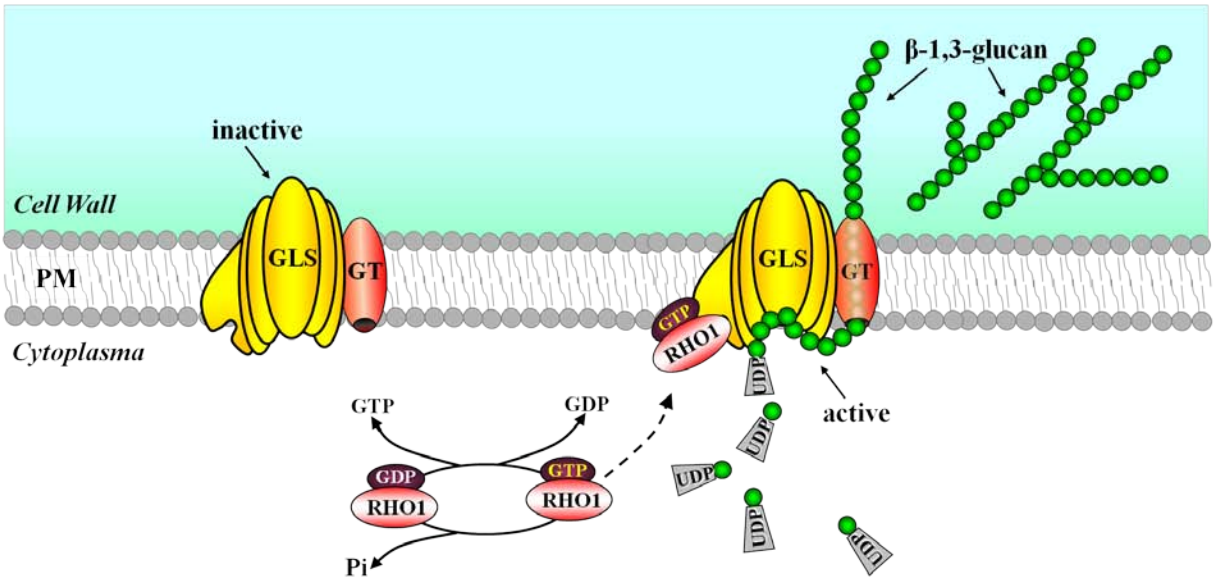

Fig. 4: The fungal β-1,3-glucan synthase complex

The RHO1 GTPase, after phosphorylation, activates GLS. Subsequently, GLS, which uses UDP-glucose as substrate, synthesizes linear fragments of β-1,3-glucan and the ABC-type glucan transporter (GT) mediates the transport into the fungal cell wall. GLS, β-1,3-glucan synthase; PM, plasma membrane; GDP/GTP, guanosine di/triphosphate; Pi, inorganic phosphate.

Attempts to delete *GLS* genes of filamentous fungi have so far not been successful, suggesting that *GLS*s are essential viability factors. Indirect evidence supporting this assumption comes from pharmacopathological studies. The echinocandin class of antimycotic drugs, e.g. caspofungin, are potent GLS inhibitors efficiently controlling invasive *Aspergilloses* and skin infections caused by the maize pathogen *C. graminicola* (Serfling *et al.*, 2007, and references therein). The fact that drug research focuses on development of novel GLS inhibitors (Kondoh *et al.*, 2005; Kondoh *et al.*, 2002) further indicates that GLS is a key enzyme in fungal cell wall biogenesis and highlights the suitability of this enzyme as a target in disease control.

While GLS in filamentous fungi is encoded by a single gene, three genes (*FKS1; FKS2; FKS3*) exist in the yeast *S. cereviseae*. In budding yeast, two highly homologous genes, *FKS1* and *FKS2*, encode two putative GLS catalytic subunits, which are differentially expressed, depending on the cell cycle or growth conditions (Inoue *et al.*, 1995; Mazur *et al.*, 1995; Zhao *et al.*, 1998). Single disruptants of either *FKS1* or *FKS2* are viable, but the double

deletion is lethal (Mazur *et al*., 1995). A third *FKS* gene of *S. cerevisiae* appears to be non-functional (Mazur *et al*., 1995). Disruption of *FKS1* in *S. cerevisiae* leads to significant reduction in β-1,3-glucan synthase activity *in vitro*. Also in *Cryptococcus neoformans* the *FKS1* gene is essential for viability (Douglas *et al*., 1994; Mazur *et al*., 1995; Thompson, 1999).

I.5.2 Chitin synthase

The chitin synthases (CHSs) that are responsible for the synthesis of linear chains of β-1,4-*N*-acetylglucosamine from the substrate UDP-*N*-acetylglucosamine are a family of membrane integral proteins with molecular weights of 100–130 kDa (Roncero, 2002).

It is now generally accepted that CHSs in fungi are located in two compartments: chitosomes (Bartnicki-Garcia *et al*., 1978), which are microvesicles involved in the transfer of the enzyme to the cell surface, and the plasma membrane, where the final synthetic reaction takes place (Bartnicki-Garcia *et al*., 2003).

Fungal genomes harbor between one (e.g. the ancestral fungus *Encephalitozon cuniculi*) and more than 20 *CHS* genes (e.g. the rice pathogen *Rhizopus oryzae*) (Latgé, 2007). Steinberg and co-workers investigated the function of the entire repertoire of *CHS* genes of the maize pathogen and basidiomycete *Ustilago maydis* by targeted deletion of individual and combinations of *CHS* genes and showed that a class V CHS is essential for in planta development and pathogenicity (Weber *et al*., 2006). Also in the ascomycetes *F. oxysporum* and *Colletotrichum graminicola*, class V CHSs are indispensable for in planta development and pathogenicity (Madrid *et al*., 2003; Werner *et al*., 2007). Δ*CgChsI* and Δ*CgChsIII* mutants did not differ significantly from the wild-type isolate with respect to hyphal growth and pathogenicity. As functions of *CHS*s may overlap, gene deletions are not lethal if the mutants are osmotically stabilized (Madrid *et al*., 2003; Weber *et al*., 2006; Werner *et al*., 2007; Kong *et al*., 2012).

Chitin biosynthesis is understood best in the model yeast *S. cerevisiae*. Three chitin synthases (*CHS1–3*) are responsible for the synthesis of the *S. cerevisiae* chitin (Cabib *et al*., 2001). The Chs3p is responsible for the synthesis of the bulk chitin of the cell wall and for the increase in chitin synthesis as a response to cell wall stress. The transport of Chs3p from

chitosomes to the plasma membrane or activity requires Chs4-6p (Ziman *et al*., 1998). Chs2p is responsible for synthesis of septal chitin. Chs2p acts in the formation of the primary septum. Its function depends directly on the formation of the actin-myosin ring (Schmidt *et al*., 2002). Chs1p acts as a repair enzyme during cell separation (Smits *et al*., 2001).

I.6 Structural fungal cell walls modifications avoid plant recognition

The ability of plants to detect pathogens is crucial for their survival (Nürnberger *et al*., 2004). This is made possible by the ability of the plant to recognize non-self structures through pattern recognition receptors (PRR) and the structures recognized by these receptors are termed pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs) (Nürnberger *et al*., 2004).

Cell wall modifications may be important especially in biotrophic and hemibiotrophic fungi to establish a compatible interaction with their hosts. These fungi, when invading the plant cell, invaginate its plasma membrane, leading to sealing of the *in planta* formed biotrophic hyphae and the establishment of an intimate interaction. The narrow interfacial matrix layer separating the fungal hypha from the plant plasma membrane, however, may represent a detrimental environment for the pathogen, with β-1,3-glucanases and chitinases challenging the integrity of hyphal walls, giving rise to elicitor-active chitin and β-1,3-glucan fragments (Deller *et al*., 2011). Indeed, PAMPs such as β-1,4-*N*-acetyl glucosamine oligomers are recognized by corresponding plasma membrane-localized PRRs such as the two LysM receptors CEBiP and OsCERK1, which cooperatively mediate chitin elicitor signaling and immunity in rice (Shimizu *et al*., 2010). In tomato, sub-nanomolar concentrations of *N*-acetylchitooligosaccharides are sufficient to induce defense responses (Felix *et al.*, 1993).

Plant pathogenic fungi have developed different mechanisms to compromise chitin detection and initiation of defense responses. For example, secretion of LysM domain-containing effectors proteins leads to sequestration of polymeric chitin and fragments thereof (de Jonge and Thomma, 2009). Furthermore, conversion of surface-exposed chitin to its non-

acetylated derivative chitosan is also thought to contribute to circumventing PAMP-triggered immunity (El Gueddari *et al.*, 2002).

Not only chitin, but also linear or branched β-1,3-glucan fragments are known as highly potent elicitors of defense responses in plants (Cosio *et al.*, 1996; Klarzynski *et al.*, 2000; Shetty *et al.*, 2009). However, enzymatic modifications or sequestration of β-1,3-glucan polymers, or fragments thereof, has not been reported, and mechanisms leading to evasion of β-1,3-glucan-triggered immune responses are unknown. The treatment of a susceptible wheat cultivar with purified β-1,3-glucan fragments from cell walls of *Septoria tritici* gave complete protection against disease and this was accompanied by increased of β-1,3-glucanase gene expression and the accumulation of callose (Shetty *et al*., 2009).

I.7 The importance of the maize pathogen *Colletotrichum graminicola*

I.7.1 *Colletotrichum* as a model for fungal biology and genetic studies

Colletotrichum graminicola (Cesati) Wilson [teleomorph *Glomerella graminicola* (Politis)], a representative of the Phylum Ascomycota (Pyrenomycetidae, Phyllachorales), is the causal agent of the worldwide-distributed maize (*Zea mays* L.) anthracnose and stalk rot disease (Wilson, 1914). *Colletotrichum* is one of the most common and important genera of plant-pathogenic fungi. Virtually every crop plants grown throughout the world are susceptible to one or more species of *Colletotrichum*. Industry estimates that stalk rots causes maize yield losses in the range of 6% annually (Bergstrom and Nicholson, 1999). *C. graminicola* is also an excellent model for fungal genetic studies. *C. graminicola* is among the best characterized and most tractable of the *Colletotrichum* fungi (Perfect *et al*., 1999). The complete genome of *C. graminicola* was recently sequenced (*Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT, http://www.broadinstitute.org; O'Cornel *et al*., 2012) and it facilitates genetic manipulation. The transformation and gene deletion are routine methods for this fungus. *C. graminicola* allows performing sexual crosses; it can be easily cultured and stored. Detached leaf assays can be easily performed at laboratory conditions and pathogenicity assays are straightforward.

I.7.2 The biology of the hemibiotrophic fungus *Colletotrichum graminicola*

C. graminicola exhibits an interesting infection biology. This fungus is a facultative pathogen. The ability to grow on dead plant material enables the fungus to survive the winter and this is the starting point for the formation of primary inoculums in spring. Asexual spores, also called conidia, are formed in a special structure called acervulus. The conidia are spread carried by splashing water (Figure 5). Often, the adesion of conidia occours within 30 minutes after the contact with the host surface. Conidia are able to secrete glycoproteins, enabling them to stick to hydrophobic surfaces (Mercure *et al*., 1994).

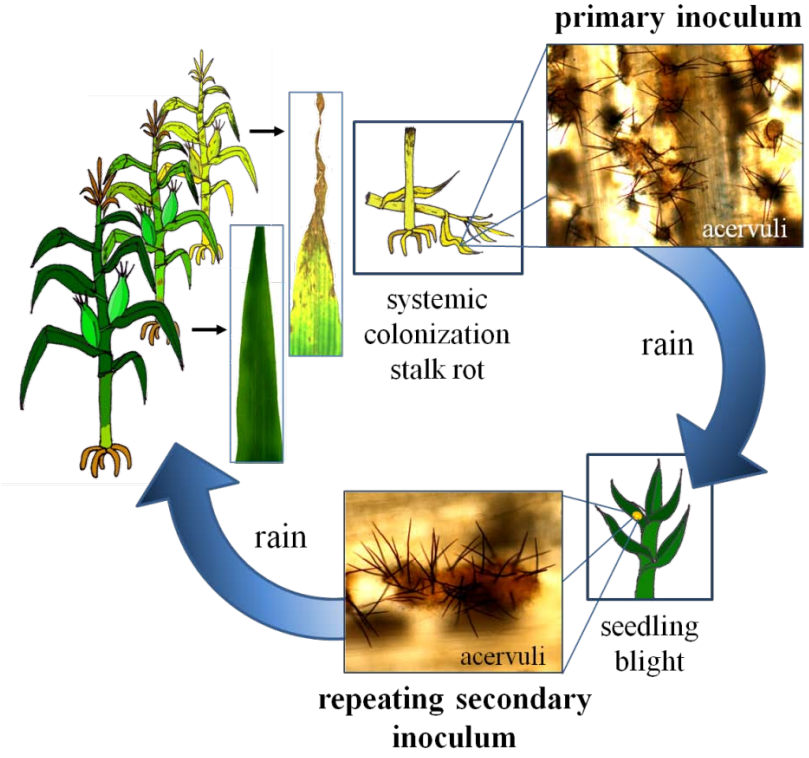

Fig. 5: Corn anthracnose cycle

The corn anthracnose cycle starts when the primary inoculum arises from overwintered corn debris and is moved by splashing rain onto newly emerged seedlings, which then become blighted. Repeated cycles of conidia production on blighted leaves provide secondary inoculum for season-long foliar infection as well as later-season stalk infection. Foliar lesion development becomes more pronounced as plants begin to senesce. Living stalks are systemically infected through wounds. Basal stalk rot, often accompanied by stalk lodging and a black discoloration of the stalk rind, occurs in plants undergoing senescence. The fungus survives as a saprophyte in corn debris above the soil surface. Conidia are produced on the overwintered maize plants in an extracellular mucilage that protects them from desiccation until new maize plants are available (modified after Bergstrom and Nicholson, 1999).

Five to six hours later, a germ tube develops from the two-celled septated conidium. Usually, only one of the two cells of the conidium germinates, in rare cases; both cells germinate and form appressoria. Often, the differentiation of appressorium occurs immediately adjacent to the conidium, with formation of only a very short germ tube (Figure 6A) (Bergstrom and Nicholson, 1999). The appressoria serve for the direct penetration of the pathogen into the host. Due to the deposition of melanin into the appressorial cell wall and the synthesis of osmolytes inside the appressorium, a high turgor pressure is generated (Figure 6 B). The appressorial pressure is directed to the base of the appressorium and the penetration peg grows into the cell wall of the host epidermis (Howard *et al*., 1991a,b; Howard and Valent, 1996; Deising *et al*., 2000). Additionally, cell wall-degrading enzymes such as cellulases, polygalacturonases, xylanases and pectinases probably play a role during penetration of the plant epidermis (Nicholson *et al.*, 1976; Wernitz, 2004). In *Colletotrichum* sp, which infect dicotyledonous plants, this has already been shown (Wattad *et al.*, 1995; Wijesundera *et al.*, 1989).

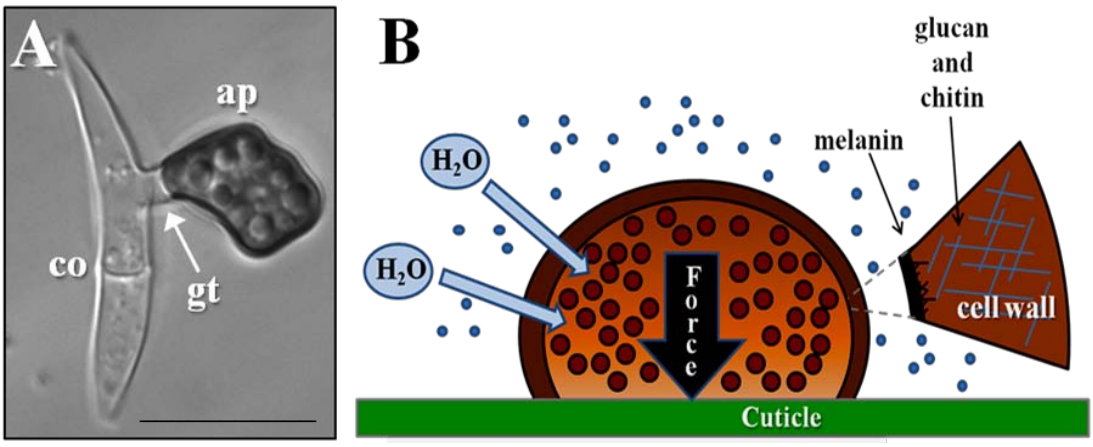

Fig. 6: The function of the appressorium of the hemibiotropic fungus *C. graminicola*

(A) The appressorium of *C. graminicola*. co, two-celled septate conidium; gt, germ tube (arrow); ap, appressorium. Bar is 10 µm.

(B) The appressoria has a strong cell wall composed mainly of chitin and glucan. These appressoria adhere to the plant surface and develop a penetration peg at their basis. The incorporation of melanin into the cell wall and synthesis of intracellular osmotically active substances allow generating hydrostatic pressure. Force is exerted vertically and can be efficiently directed to the cuticle. Red balls represent osmolytes; blue lines in the cell wall represent glucan and chitin (modified after Bastmeyer *et al.*, 2002).

After penetration into the host cell, most *Colletotrichum* species establish a biotrophic interaction (Figure 7, light green structures). It is assumed that different strategies to avoid defense responses, i.e. masking of invading hyphae or active suppression of defense, are essential for a biotrophic parasitic lifestyle (Münch *et al*., 2008).

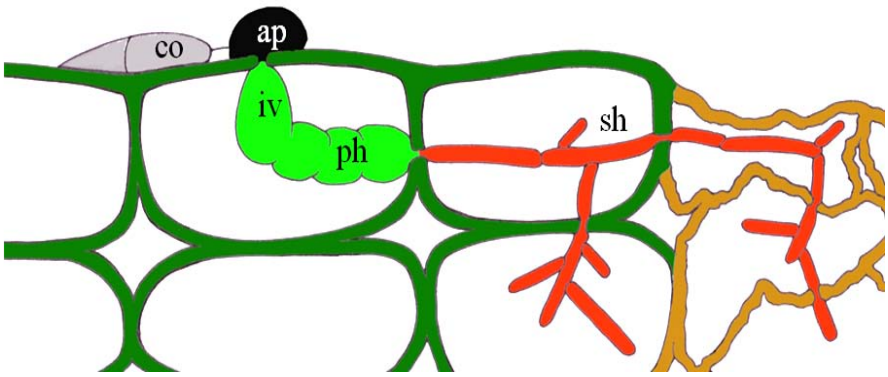

Fig. 7: The hemibiotropic life style of the maize pathogen *C. graminicola*

When a conidium germinates, it forms a strong appressorium. When mature, the wall of the appressorium is heavily melanized. The appressorium is attached to the leaf surface by an adhesive compound secreted by the fungus. Penetration of the host cell occurs through the appressorial pore. If penetration is successful, the fungus forms an infection vesicle that invaginates the host plasma membrane. In this condition, the fungus is growing as a biotrophic pathogen. Primary hyphae emerge from the infection vesicle and also grow biotrophically. Next, the fungus breaks through the host plasma membrane, invades surrounding host cells, and grows as a necrotroph. co, conidium; ap, appressorium; iv, infection vesicle; ph, primary hypha; sh, secondary hypha (modified after Bergstrom and Nicholson, 1999; drawing by Daria Deising).

During initial invasion and biotrophic development, the pathogen masks its surface by converting the hyphal surface-exposed chitin by deacetylation. This mechanism helps avoiding degradation of chitin by plant chitinases, recognition of chitin fragments and elicitation of defense responses (El Gueddari *et al*., 2002). Interestingly, apposition of proteins may also be involved in masking of chitin. An avirulence gene product, Avr4, of the tomato leaf-mold fungus *Cladosporium fulvum* has a chitin binding domain (Rivas and Thomas, 2005). The Avr4 protein could function in binding to and shielding of the fungal

cell wall so that chitin is not recognized by plants as a pathogen-associated molecular pattern (Van den Burg *et al*., 2003). Both conversion of chitin to chitosan as well as apposition of a new protein layer would reduce the concentration of elicitor-active fungal cell wall fragments and reduce, delay or prevent initiation of defense responses. In addition and possibly in order to allow invasive growth into adjacent cells, proteins capable of suppressing defense responses may be secreted into the host tissue (Münch *et al*., 2008).

The typical biotrophic structures for this fungus are infection vesicles and primary hyphae (Figure 7, light green structures). In the biotrophic life stages, the fungus starts to take up nutrients from the apoplast surrounding the plant cell. Fungal hyphae are here in close contact with the plant plasma membrane without affecting the plant cell integrity. The transition to necrotrophic lifestyle takes 24 to 36 hours after penetration. In this phase, the fungus forms secondary hyphae (Figure 7, red structures), which disrupt the plasma membranes of the host (Bergstrom and Nicholson, 1999). After the spread of the pathogen in host plant tissues, the first symptoms can be seen as chlorotic leaf areas. In the course of colonization necrotic spots appear (Figure 5, leaf picture showing antrachnose symptoms). In this dead tissue, the fungus again forms acervuli and conidia for further spread (Figure 5; acervuli and primary inoculum).

The infection of the stalk basically follows the same pattern. The disease occours potentially in fields infestated by the stalk-boring insect *Ostrinia nubilalis*, which wounds the corn stalk. The colonization of xylem by infectious mycelium leads to a systemic infection (Bergstrom and Nicholson, 1999; Bergstrom and Nicholson, 2000).

I.8 The aims of this work

The overall aim of this study was to functionally characterize the β-1,3-glucan synthase (*GLS1*) gene of the causal agent of leaf anthracnose and stalk rot of maize, *C. graminicola*, during vegetative and pathogenic development. Glucan is the most prominent cell wall component in ascomycetes. Several studies on fungal chitin synthases showed strong morphological distortions and defects in virulence/pathogenic in several fungal *CHS* mutants. Therefore, the evaluation of the role of β-1,3-glucan in fungal cell walls during vegetative and pathogenic development is of significant interest.

In order to understand the role of β-1,3-glucan in different vegetative and pathogenic hyphae, the expression of *GLS1* and synthesis of the polymer must be recorded. As this aim can be best accomplished, I decided to construct *GLS1*:*eGFP* replacement strains of *C. graminicola*. Furthermore, promoter replacement studies represent an excellent tool to further understand infection structure-specificity of *GLS1* expression.

As described mainly for human pathogens, the *RHO1* gene, which encodes the regulatory protein of the GLS1 protein, is essential for fungal morphogenesis and pathogenesis. Δ*rho1* strains show strongly reduced GLS1 protein activity. Thus, the investigation of the role of the *RHO1* gene of *C. graminicola* in cell wall biogenesis and pathogenic development, as well as the confirmation of results of *GLS1* studies, can complement studies on synthesis of β-1,3-glucan and confirm data obtained with *GLS1*.

Cell wall modifications are important mainly in biotrophic and hemibiotrophic fungi to establish a compatible interaction with their hosts. Biotrophic structures are non-destructive and should avoid recognition by plant receptor. Alternatively, avoidance of defense responses can occur by secretion of effectors by these pathogenic hyphae. Glucan fragments are recognized by corresponding plasma membrane-localized PRRs, and recognition is likely inducing defense responses. Therefore, another goal of this study was to investigate how β-glucan-triggered immunity can be avoided.

II Materials and methods

II.1 Fungal strains, culture and growth conditions, *in vitro* differentiation of infection structures and infection assays

II.1.1 Fungal strains

In this study, the wild-type (WT) strain M2 of *C. graminicola* (Ces.) G.W. Wilson (teleomorph *Glomerella graminicola* D.J. Politis) was used to characterize the single copy *GLS1* gene encoding a GLS1 protein and the *RHO1* gene encoding a RHO1 protein.

II.1.2 Culture media, buffers and solutions

All culture media, buffers and solutions used in these studies were described in the Appendix VII.1.

II.1.3 Culture and growth conditions

The *C. graminicola* WT, RNAi (*GLS1*) and Δ*rho1* strains were cultivated on oat meal agar (OMA; appendix VII.1.2, **M1**); Werner *et al.*, 2007; Koneman *et al*, 1997), complete medium (CM; **M2**; Leach *et al*., 1982), potato-dextrose (PD; **M3**; Difco Laboratories, Sparks, MD, USA.), regeneration medium, or (RM; **M4**) synthetic minimal medium (SMM; **M5**). To grow RNAi strains, the medium chosen was supplemented with 0.15 M KCl, 1 M sorbitol, or 1 M sucrose.

In liquid media, strains were grown in an incubation shaker (Unitron, Infors, Bottmingen, Switzerland) at 110 rpm and 23°C. On solidified media, containing 1.5% (w/v) agar-agar (Difco Laboratories, Sparks, MD, USA), strains were grown at 23°C under

continuous fluorescent light (Climas Control CIR, UniEquip, Martiensried, Germany; Werner *et al*., 2007).

The *Saccharomyces cerevisiae* Δ*fks1* mutant Y05251 (BY4741; Mat a; his3D1; leu2D0; met15D0; ura3D0; YLR342w::kanMX4) and the reference strain Y00000 (parental S288C) (Mat a, his3D1, leu2D0, met15D0, ura3D0) (Euroscarf, Frankfurt, Germany) were grown at 30°C and 150 rpm in liquid YPD (**M6**) (Difco, Sparks, MD, USA.) or yeast synthetic complete medium (YSCM) (**M7**) (Difco, Sparks, MD, USA) lacking uracil. Solidified media contained 1.5% (w/v) agar agar (Difco, Sparks, MD, USA). Strain Y05251 was grown in the presence of 1 M sorbitol.

II.4 *In vitro* differentiation of infection structures and infection assays

Infection structure differentiation of the *C. graminicola* WT and RNAi strains was induced on polyester, glass or onion epidermis as described (Horbach *et al*., 2009), with 0.15 M KCl added as an osmolyte.

Twelve days old whole maize (*Zea maize* cv Nathan) plants, 7 cm long leaf segments of the 2nd and 3rd leaf, depending on the experiment, or epidermal cell layers from onion (*Allium cepa* cv Grano) bulbs were used to assess virulence of the WT and different strains generated in this study. Maize leaf segments and onion epidermal layers were inoculated with four 10 µL droplets containing 10^4 conidia in 0.01% (v/v) Tween 20. For wound inoculation, single pricks were inserted immediately prior to inoculation as described (Werner *et al*., 2009). Alternatively, 0.5 g mycelium of 10 days old liquid CM cultures (containing 0.15 M KCl) was washed with distilled water twice and fragmented in 15 mL sterile 0.01% (v/v) Tween 20 using an Ultra Turrax (IKA Labortechnik). Maize plants were inoculated with four 10 µL droplets mycelial fragment suspensions. Leaf segments were incubated in sealed Petri dishes (Ø 9 cm) in a BOD 400 incubator (Uni Equip, Planegg, Germany) in darkness at 25°C. For each treatment and time point analyzed, 12 leave segments were evaluated. Mock inoculation was performed with 0.01% (v/v) Tween 20. Symptoms were photographed 6 DAI. For quantitative PCR, inoculation was done with 10 detached 2nd or 3rd leaves each repetition, treatment and time-point, in which each leaf received a single 10 µL droplet containing

1 x 10^4 conidia. For qRT-PCR, whole maize plants were spray-inoculated with 10 mL of a spore suspension containing 10^6 spores / mL. For each treatment and time point, the 2nd and 3rd leaf of nine plants were used per repetition. Mock inoculation was performed using sterile 0.01% (v/v) Tween 20. Plants were incubated in a Percival AR-75L (CLF Laborgeräte, Emersaker, Germany) growth chamber (12 h light; 200 mE; 70% relative humidity; 25°C).

All experiments were performed in triplicate and with four repetitions.

II.2 Oligonucleotide sequences used in this study

All oligonucleotides used in these studies were listed in the table A1 (Appendix VII.2; Table A1).

II.3 Complementation of the *S. cerevisiae* Δ*fks1* mutant with the *C. graminicola GLS1* cDNA

In order to synthesize cDNA of *GLS1*, total RNA was extracted (Chirgwin *et al*., 1979) from vegetative mycelium of *C. graminicola*. mRNA was isolated using the Nucleotrap mRNA Purification Kit, and cDNA synthesis was performed using the Creator SMART cDNA Library Construction Kit (BD Biosciences Clontech, Heidelberg, Germany). The primers CgGLS1NotI-Fw and CgGLS1NotI-Rv have been used to amplify the *GLS1* cDNA, which was cloned into the *Not*I site of the yeast cDNA expression vector pAG300 (www.addgene.org; Horbach *et al*., 2009). These primers, and others mentioned here, are listed in Table 1. Correct orientation of the DNA was confirmed by sequencing, using the primer pAG300-Fw. The empty vector (pAG300) and the vector containing the *GLS1* cDNA were transformed into *S. cerevisiae* strain Y05251 using the lithium acetate procedure (Becker *et al*., 2001) to yield the complemented yeast strains $T_{Cg\mathit{GLS1}}1$. As control, strain Y05251 was also transformed with empty pAG300, yielding T_{pAG300}. Yeast cells were grown on YSCM agar lacking uracil.

II.4 Preparation of fungal genomic DNA and total RNA

II.4.1 Mini preparation of fungal genomic DNA

For small-scales of DNA, about 400 mg of mycelia was dried with paper towel, crushed in 800 µL of DNA lysis buffer (**B1**) in a 2 mL safe lock tube with one steel ball in a Retsch mill (Qiagen, Hilden, Germany; 30 rotations/s for 30 seconds). A single extraction of the aqueous phase was performed with 800 µL of Phenol-Chloroform-Isoamylalcohol 25:24:1, Rotiphenol, (PCI). The centrifugation was performed in an Eppendorf centrifuge 5403 (Eppendorf, Hamburg, Germany) (23000 x *g* for 20 min at 4°C). The supernatant was transfered to a fresh 2 mL safelock tube. The DNA was precipitated by adding one volume of isopropanol and centrifuged (6800 x *g* at 4°C for 20 min). The resulting pellet was washed twice with 70% (v/v) ethanol. For final resolubilisation, the DNA pellet was resuspended in 50 L of 1x TE buffer (see appendix VII.1.3; **B2**) or bidistilled water and stored at -20°C.

II.4.2 Maxi preparation of fungal genomic DNA

Genomic DNA was isolated from 500 mg of vegetative mycelium of *C. graminicola* (Döbbeling *et al*., 1997). The fungal mycelium was dried with paper towel and crunched to a frozen powder with liquid nitrogen in a mortar. The powder was transferred into a 2 ml safelock tube and 800 µL of extraction buffer I (**B3**). Afterward, this solution was mixed vigorously on a vortexer, incubated on ice for 10 min and mixed again. The tubes were centrifugate in an Eppendorf centrifuge 5403 (Eppendorf, Hamburg, Germany) (23,000 x *g* at 4°C for 20 min). The supernatant was transferred to a fresh 2 mL safelock tube and extracted with chloroform. The DNA was precipitated by addition of one volume of isopropanol centrifuged (23000 x *g* at 4°C for 20 min), resuspended in 700 µL of extraction buffer II (**B4**), and subsequent treated by RNase A and Proteinase K. RNase A treatment (Roche Diagnostics, Mannheim, Germany) was performed at 37°C for 30 min as described in the manufacturer protocol. Proteinase K treatment (Roche Diagnostics, Mannheim, Germany) was performed at 60°C for 60 min as described in the manufacturer protocol. As for the Mini preparation of fungal genomic DNA, the supernatant was transferred to a fresh 2 ml

safelock tube. The DNA was precipitated by one volume of isopropanol and centrifuged (6800 x *g* at 4°C for 20 min). The resulting sediment was washed twice with 70% ethanol. The DNA pellet was resuspended in 50 µL of 1x TE buffer (**B2**) or steril bidistilled water and stored at -20°C.

II.4.3 Preparation of fungal total RNA

Total RNA was extracted from melanized mycelium (Chirgwin *et al*., 1979); mRNA was isolated using the Nucleotrap mRNA Purification Kit (BD Biosciences Clontech, San Jose, California, USA.), as indicated by the manufacturer.

II.4.4 Determination of DNA and RNA concentration

Quantity and quality controls of DNA preparations were performed with a NanoDrop ND-1000 (Thermo Scientific, Wilmington, USA) and visually controlled after gel electrophoresis. Quantity and quality controls of RNA preparations included UV spectrophotometry were performed with a 2100 Bioanalyzer (Agilent, California, USA).

II.5 Gel electrophoretic separation of nucleic acids

II.5.1 DNA electrophoresis

DNA and PCR-products were separated according to their size using agarose gels of 0.6% (w/v) to 2.5% (w/v) agarose (Seakem LE-Agarose; Biozym Scientific GmbH, Hess. Oldendorf, Germany). Agarose powder was boiled in 1 x TAE buffer (**B5**) in a microwave. The agarose solution was mixed on a magnetic stirrer until the temperature was less than 60°C and poured into a gel tray. DNA samples containing $1/6^{th}$ of their volumes of loading buffer (**B6**) were separated in a constant electrical field of 2 to 12 V/cm in a gel electrophoresis chamber filled with 1 x TAE. Gels were stained with ethidium bromide (1

µg/mL for 30 min, washed for 5 min with distilled water and were digitally recorded under UV irradiation (320 nm) with an AlphaImager (Biozym Scientific GmbH, Hess. Oldendorf, Germany). The DNA fragments of defined length (GeneRuler DNA Ladder Mix, Fermentas, St. Leon-Rot, Germany) were used as size standard.

II.5.2 RNA electrophoresis

The quality of the RNA was mensured with a 2100 Bioanalyzer (Agilent, California, USA). The integrity of the ribosomal 18 S and 28 S RNA bands were visualize using UV-light at a wavelength of 320 nm.

Agarose was dissolved at a concentration of 1% (w/v) in RNA running buffer (**B7**) in the microwave. RNA samples were diluted in containing 1/2th of their volumes of loading buffer (**B8**). The separation of RNA fragments was carried out at constant voltage (1V per cm). Subsequently the gel was incubated in an ethidium bromide solution (1µg/ml in bidistilled H_2O) for 60 min.

II.6 Southern hybridization

II.6.1 Preparation of probes

Hybridisation probes were generated by standard PCR (see II.7.1), except that the regular dNTP mix was replaced by the Roche DIG (digoxygenin)-labeling Kit (2 mM dATP; dCTP; dGTP; 0.19 mM dTTP and 0.1 mM DIG-11-dUTP, alkali labile). Labeling was verified by agarose gel electrophoresis (see II.5.1). DIG labeled fragments appeared larger in gel electrophoresis compared to non-labeled reference fragments, due to the incorporated Digoxygenin.

II.6.2 Digestion of genomic DNA

DNA samples of 10 µg were digested over night with the appropriate restriction enzymes and buffers in a total volume of 25 µL and incubated at 37°C for 16 hours. Reactions were set up on ice and the enzymes were denatured by incubation at 80°C for 30 min in a heating block (Eppendorf, Hamburg, Germany). A mini test gel was prepared to confirm complete digestion.

II.6.3 Capillary transfer of digested DNA

Digested DNA was precipitated with 100 µl of isopropanol, centrifuged (23000 x *g* at 4°C for 20 min), washed twice with 70% (v/v) ethanol, dried under a sterile bench and resuspended in 20 µL of sterile, distiled water. Digested DNA was separated on a 0.7% (w/v) agarose TAE gel, and digestion was visually confirmed after ethidium bromide staining. The gel was destainied with distiled water for 30 min, depurined with 0.25 M HCl and denatured with 0.4 M NaOH, each at RT for 30 min. DNA fragments were transferred by the upward capillary method to a positively charged nitrocellulose membrane (Hybond-N+, Amersham Pharmacia Biotech, Freiburg, Germany) 12 to 16 h.

The gel-sized transfer membrane was drained in 20 x SSC (**B9**) and placed on a stack of paper towels and two 20 x SSC drained filters paper squares (Whatman 3MM, Whatman GmbH, Dassel, Germany) (gel-sized). The destained gel was placed tightly on the membrane and covered with three 20 x SSC-drained Whatman filters (gel-sized). Two glass petri dishes with diameters of 12 cm were placed besides the blot and filled with 20 x SSC. A long rectangular strip of Whatman filter paper was folded, drained in 20 x SSC and placed from the left petri dish to the right one, touching the upper filter paper of the blot tightly, thereby supplying buffer reservoir for blotting. Finally a glass plate containing a 250 mL bottle of water was placed upon the blot construction. After transfer, DNA fragments were cross-linked to the filter for 2 h at 80°C in a hybridisation oven (Biozym Scientific GmbH, Hess. Oldendorf, Germany).

II.6.4 Hybridization

After cross-linking, the membrane was placed in a glass tube and incubated at 65°C for 2 h with 20 mL of prehybridisation buffer (**B10**) in an hybridisation oven (Biozym Scientific GmbH, Hess. Oldendorf, Germany). For hybridisation, the whole DIG-UTP-labeled probe was denaturated at 100°C for 2 min in a heating block, cooled briefly on ice and was added to the prehybridisation buffer in the tube. Hybridisation was performed at 65°C for 3 h.

II.6.5 Detection of bound probe

The membrane was washed stepwise in the tube for 5 min with 2 x SSC (**B11**), 15 min with 0.5 x SSC (**B12**), 15 min with 0.25 x SSC (**B13**), and was equilibrated for 1 min with buffer M. Afterwards, the filter was incubated for 2 h with 1x blocking solution (**B14**) and subsequently with anti-DIG antibody-coupled alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) for 1 h in a sealed plastic bag (Carl Roth GmbH, Karlsruhe, Germany). The filter was then incubated with detection buffer (**B15**) in another sealed plastic bag. Emitted chemiluminiscence was documented with an X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech, Freiburg, Germany) that was developed with an Optimax TR device (MS Laborgeräte, Heidelberg, Germany).

Membranes were kept wet in 20 x SSC, sealed in a plastic bag and stored at 4°C. For removal of the alkali-labile DIG chemiluminophores the membrane was washed twice in dist. water and incubated with stripping solution (**B16**) for 15 min. The membrane was washed twice with 2 x SSC for 5 min and exposed with an X-ray film for 18 h to verify unlabeling efficiency. Therefore, the membrane could be hybridised with a different probe subsequently using the previous protocol of probe detection.

II.7 Polymerase chain reaction PCR

II.7.1 Standard test PCR

All PCR reactions were performed with a PTC-150 thermal cyclers (MJ Research, Waltham, USA) or Tpersonal thermal cycler (Biometra, Göttingen, Germany). The enzymes and reaction buffers were obtained from NEB (Frankfurt / Main, Germany). Standard PCR was applied to examine insertion events in fungal transformants using Taq (*Thermus aquaticus*) polymerase (Fermentas native Taq Polymerase / Frankfurt / Main, Germany) in 20 µL reactions.

Reaction mixture had the following composition:

2 µl PCR-Puffer 10x

1 µl dNTPs 10 mM

1 µl Primer 1 (2 µM)

1 µl Primer 2 (2 µM)

0.2 µl Taq-Polymerase 5 units/ml

DNA (1-50 ng)

ad bidistilled H_2O to final volume 20 µL

The PCR program used to amplify DNA with Taq-Polymerase was the following (Table 2):

Table 2: PCR reactions to test for insertion events in fungal strains

N°	Step	Temperature	Time
1	Denaturation	94°C	1 min
2	Denaturation	94°C	30 sec
3	Annealing	Tm*	30 sec
4	Elongation	72°C	1 min/kb
	Repeat steps 2-4 for 34 times		
5	Elongation	72°C	5 min
6	Pause	10°C	∞

* $TM = 69.5 \pm C + (0.41x\%GC) - 650/\text{bp}$

Approximately 30-40 cycles proved for the majority of PCR applications as adequate.

The duration of the initial denaturation was dependent of the type of template DNA and DNA polymerase. As the annealing temperature (Tm), the lowest melting temperature of the primers was selected. The elongation time depended on the size of the amplified fragment (~ 1 kb / min). The most effective conditions were empirically determined by variation of the individual parameters determined.

II.7.2 DJ-PCR

Constructs used for gene deletion were generated by double-joint PCR (Yu *et al*., 2004). In a first 50 µL reaction, the 5' and 3' flanks of the target gene and the resistance cassette were indenpendently amplified. Phusion High Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) was used and reactions were mixed to a final total of 50 µl. This polymerase was chosen because of its 3'-5' exonuclease activity, which reduces the error rate compared to Taq polymerase and its higher synthesis rate of 4 kb/min. Therefore, the

standard PCR protocol was modified to reduced elongation times according to the sizes of templates and a shorter denaturation step at higher temperature (Table 3).

Reaction mixture (50 µL) had the following composition:

10 µl HF-Buffer 5x
1 µl dNTPs (10 mM)
5 µl Primer 1 (2 µM)
5 µl Primer 2 (2 µM)
0.5 µl Phusion Polymerase 2 units/ml
DNA (1-20 ng)
ad bidistilled H_2O

The PCR program used to amplify 1 kb of flanking regions of target gene and the resistance cassette was the following:

Table 3: PCR program to amplify 1 kb of flanking regions of target genes

N°	Step	Temperature	Time
1	Denaturation	98°C	1 min
2	Denaturation	98°C	30 sec
3	Annealing	Tm	30 sec
4	Elongation	72°C	15-30 sec/kb*
	Repeat steps 2-4 for 34 times		
5	Elongation	72°C	5 min
6	Pause	10°C	∞

* Synthesis time depended on the length of the amplicon. For 1 kb, 20 s were chosen.

The products of the first PCR were then used in a second PCR mixture. In this reaction, about 50 ng DNA of the each flank reagion and 100 ng of amplified resistance cassette were used as a template.The 5’ flank reverse primer and 3’ flank forward primer contain

overhands with the sequence of the correspondent resistance cassette primes. Thus, these complementary overhangs care a primer function in this second round of the DJ-PCR.

In a second PCR step, DNA fragments purified as described in the SureClean protocol (Bioline, Luckenwalde) were fused, using different annealing temperatures in order to optimise the obtation of PCR fusion product (see Table 4, and the follows reactions mixtures).

Reaction mixture (50 µL) and PCR conditions were as follows:

10 µl HF Puffer 5x
1 µl dNTPs 10 mM
0.5 µl Phusion Polymerase 2 Units/ml
DNA: (ratio 1:3:1, 5'-flank - nat cassette - 3'-flank)*
ad bidistilled H_2O (to complete total volume for 50 µL/reaction)
*Usually 20:60:20 ng for 5'-flank (1Kb): nat cassette (2Kb): 3'-flank (1Kb)

Table 4: Two-step DJ-PCR program to fuse 5' and 3' flanking regions of target genes and nourseothricin resistance cassette

N°	Step	Temperature	Time
1	Denaturation	98°C	1 min
2	Denaturation	98°C	30 sec
3	Annealing	Tm	30 sec
4	Elongation	72°C	1 min
	Repeat steps 2-4 for 10 times		
5	Denaturation	98°C	30 sec
6	Elongation	72°C	1:30 min
	Repeat steps 2-4 for 24 times		
7	Elongation	72°C	5 min
8	Pause	10°C	∞

Purified DJ-PCR products were used as templates for nested PCR in a third step (see Table 5 and the follows reactions mixtures).

Reaction mixture (50 µL) and PCR conditions were as follows:

10 µl HF Puffer 5x
1 µl dNTPs 10 mM
5 µl nested-Primer1 2 µM
5 µl nested-Primer2 2 µM
0.5 µl Phusion Polymerase 2 Units/ml
1 µl fusions fragment
26.5 µl bidistilled H_2O

Table 5: **Nested PCR program to amplify 4-5 kb of deletion cassettes or constructs**

N°	Step	Temperature	Time
1	Denaturation	98°C	1 min
2	Denaturation	98°C	30 sec
3	Annealing	Tm	30 sec
4	Elongation	72°C	15-30 sec/kb*
	Repeat steps 2-4 for 34 times		
5	Elongation	72°C	5 min
6	Pause	10°C	∞

* Synthesis time depended on the length of the amplicon. For 1 kb, 20 s were chosen.

The KO construct was amplified and cleaned by using the SureClean protocol (Bioline, Luckenwalde), as described by the manufacturer (Qiagen, Hilden, Germany). 0.25-5 µg of the purified KO construct was used for fungal protoplasts transformation.

II.8 Targeted deletion of *GLS1*, construction of RNAi strains and generation of *GLS1*:*eGFP* replacement strains of *C. graminicola*

For targeted deletion of the 5940-bp *GLS1* gene, the NatR cassette was PCR-amplified from pNR1 (Malonek *et al*., 2004), using primers Nourse1pNR1-Fw, and Nourse1pNR1-Rv. The 1009-bp 5’ and the 1003-bp and 3’ flanking regions of the *GLS1* gene were amplified from genomic DNA using primers CgPGLS1-fw, CgP1GLS15'-flank-rv and CgTGLS13'-

flank-fw and CgTGLS1-rv, respectively. The products were fused by double-joint-PCR (Yu *et al*., 2004), and nested primers CgPGLS1nest-fw and CgTGLS1nest-rv were used to amplify the 4210-bp KO construct, which was transformed into conidial protoplasts as described (Werner *et al*., 2007). Tests for homologous integration of the KO construct was done with primers CgPGLS1test-fw and CgTGLS1test-rv.

The RNAi cassette from plasmid pRedi (Janus *et al*., 2007) was used to generate an RNAi construct targeting *GLS1* transcripts of *C. graminicola*. The 475-bp *GLS1* sense and antisense fragments were amplified from genomic DNA, using the primers RNAi(*GLS1*)-fw and RNAi(*GLS1*)-Rv, and RNAi(*GLS1*)i-fw and RNAi(*GLS1*)i-Rv, respectively. The sense and antisense fragments were integrated into the *Xho*I-*SnaB*I and *Bgl*II-*Apa*I sites of pRedi, and were thus separated by 135-bp of the intron of the *M. oryzae Cut2* gene (NCBI: XM_365241.1), existing in pRedi as a linker (Janus *et al*., 2007). The resulting 6.24-kb RNAi construct was excised from pRedi by *Dra*I digestion, purified by gel elution, and transformed into conidial protoplasts of *C. graminicola*. Single spore isolates were generated as described (Werner *et al*., 2007).

To study cell-specificity of expression of *GLS1* and localization of the protein, a *GLS1*:*eGFP* replacement construct consisting of the 3'-1 kb end of the coding region of the *GLS1* gene fused in frame to the *eGFP* gene, followed by the Hyg^R resistance cassette and the 1kb 5'-non-coding *GLS1* sequence containing the terminator was transformed into the *C. graminicola* WT strain. The 3'-coding region of *GLS1* was amplified with the primers CgGLS1GFP-Fw and CgGLS1GFP5'-flank-Rv, using genomic DNA of *C. graminicola* as the template. The *eGFP* gene and the Hyg^R cassette were amplified using primers EGFP-Fw and HygR-Rv, with plasmid pSH1.6EGFP as template. Using genomic DNA as template the 3'- flank of *GLS1* was amplified with primers CgGLS1GFP3'-flank-Fw and CgTGLS1GFP-Rv. The *GLS1*:*eGFP* construct was fused by double-joint-PCR (Yu *et al.*, 2004), and the complete 6.2-kb fragment was amplified with nested primers CgGLS1:GFP.nest-fw and CgGLS1:GFP.nest-rv. Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) was used in all PCR reactions. The *GLS1*:*eGFP* construct was transformed into conidial protoplasts (Werner *et al*., 2007), and single spores isolates were tested for site-specific integration and replacement of the WT *GLS1* gene by Southern hybridization.

II.9 Targeted deletion of *C. graminicola RHO1*

For targeted deletion of the 1081-bp *C. graminicola GLS1* gene, the NatR cassette was PCR-amplified from pNR1 (Malonek *et al*., 2004), using primers Nourse1pNR1-Fw, and Nourse1pNR1-Rv. The 1009-bp 5' and the 1003-bp and 3' flanking regions of the *RHO1* gene were amplified from genomic DNA using primers CgP*Rho1*-fw, CgP1Rho*1*5'-flank-rv and CgTRho13'-flank-fw and CgT*Rho1*-rv, respectively. The products were fused by double-joint-PCR (Yu *et al*., 2004), and nested primers CgPRho1nest-fw and CgTRho1nest-rv were used to amplify the 4210-bp KO construct, which was transformed into conidial protoplasts as described (Werner, 2001; Werner *et al*., 2007). Tests for homologous integration of the KO construct was done with primers CgPRho1test-fw and CgTRho1test-rv. The isolates were tested for site-specific integration by Southern hybridization.

II.10 Targeted promoter exchange and overexpression of *C. graminicola GLS1*

For overexpression of *GLS1*, the trpC promoter of *A. nidulans* was amplified from pSM1 (Pöggeler *et al.*, 2003), using primers PtrpC-Sac1-Fw and PtrpC-Sac1-Rv. The toxB promoter of *Pyrenophora tritici-repentis* was amplified from pCM29 (Andrie *et al*., 2005), using primers PtoxB-Sac1-Fw and PtoxB-Sac1-Rv. The PCRs products were digested by *Sac*I, purified, and integrated into *SacI*-digested pNR1. The complete *GLS1* gene was amplified with the primers CgGLS1NotI-Fw and CgTGLS1NotI-Rv. The 7050-bp PCR product was *Not*I-digested, purified and ligated into pNR1. The resulting 10032-bp (P_{trpC}:*GLS1*:*NatR*) and 10,058-bp (P_{toxB}:*GLS1*:*NatR*) constructs were amplified using primes NatOvExp.nest-Fw and NourspNR1-Rv, and transformed into conidial protoplasts (Werner *et al.*, 2007). Single spore isolates were tested for numbers of the *GLS1* overexpression constructs integrates by Southern hybridization.

For targeted promoter exchange, P_{trpC} and P_{toxB} were amplified as decribed above; the NatR cassette was PCR-amplified from pNR1, as described above. The 1009-bp 5'- and the 1003-bp and 3'-flanking regions of the *GLS1* promoter and the 5'-part of *GLS1* gene coding sequence were amplified from genomic DNA using primers P-TrpCGLS5'flank-Fw, P-TrpCGLS5'flank-Rv, CgGLS3'flank-Fw and P-TrpCGLS3'flank-Rv, respectively. The

products were fused by double-joint-PCR (Yu *et al.*, 2004), and nested primers P-TrpCGLS1nest-Fw and P-TrpCGLS1nest-Rv were used to amplify the 4210-bp promoter replacement construct, which was transformed into conidial protoplasts (Werner *et al.*, 2007). Homokariotic strains were tested for site-specific integration by Southern hybridization.

II.11 Analysis of genomic DNA gel blot

To analyze transformants for the number of integrations of the RNAi construct, genomic Southern blot analyses were performed with 10 mg of *Xho*I-digested DNA. The 511-bp alkali-labile DIG-dUTP-labeled probe (Roche Diagnostics, Mannheim, Germany) specific for the nourseothricin acetyl transferase gene (*NatI*) was amplified from plasmid pNRl, using primers NatR probe-Fw and NatR probe-Rv. To analyze transformants for the correct integration of a single copy of the *GLS1*:*eGFP* cassette into genomic DNA of WT and RNAi strains of *C. graminicola*, 10 µg of *Sac*I-digested genomic DNA was Southern-blotted and hybridized with a 500-bp DIG-dUTP-labeled hygromycin phosphotransferase (Hyg^R)-specific probe amplified from the *GLS1*:*eGFP* construct as template with primers HygR probe-Fw and HygR probe-Rv. To analyze transformants for the correct integration of a single copy of the *RHOI*-KO cassette genomic DNA of transformants of *C. graminicola* were Southern-blotted and hybridized with a 500-bp DIG-dUTP-labeled *RHO1*-specific probe amplified from genomic DNA of the WT strain as template with primers RhoI probe-Fw and RhoI probe-Rv.

Hybridization and probe detection followed the recommended protocol (Roche Diagnostics, Mannheim, Germany). The membrane was exposed to Hyperfilm ECL X-ray film (Amersham Pharmacia Biotech, Piscataway, USA).

II.12 Quantitative RT-PCR and PCR

Quantitative RT-PCR (qRT-PCR) was performed with one-hundred ng (*GLS1*) or 50 ng of total RNA (*actin* and *histone 3*) pretreated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) in 10 µL reactions (One Step RT-PCR Kit; Qiagen, Valencia, California USA). cDNA synthesis was performed at 50°C for 30 min. Cycling conditions consisted of an initial denaturation step (95°C, 3 min), followed by 40 cycles of denaturation (95°C, 30 s), annealing (60°C, 20 s), and extension (72°C, 50 s). The absence of contaminating genomic DNA was confirmed by a control PCR with RNA not reverse transcribed. Primers used were CgGLS1qRT-Fw and CgGLS1qRT-Rv for *GLS1*, CgACTqRT-F1 and CgACTqRT-R1 for the α-actin and CgH3-qRT.F1 and CgH3-qRT.R1 for the histone-3 reference gene. Melting curve and agarose gel analyses indicated amplification of a single product. Transcript abundance was calculated and normalized as described (Guimil et al., 2005; Gutjahr et al., 2008). CT values of three independent replicates were used to calculate mean values and standard deviations. qRT-PCR was performed using a MyiQ-Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, California, USA) equipped with iQ5 standard edition software (version 2.0.148.60623).

To quantify fungal development, DNA was isolated as described above, with an additional phenol-chloroform extraction and precipitation step (SureClean; Bioline, Luckenwalde, Germany). qPCR reactions contained 100 ng template DNA, 100 nM of each primer (CgITS2-q1, CgITS2-q2), and 10 µL iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a final volume of 20 µL. Cycling conditions consisted of an initial denaturation (95°C, 3 min), followed by 40 cycles of denaturation (95°C, 30 s), annealing (63°C, 20 s), and extension (72°C, 15 s). Melting curve and agarose gel analyses indicated amplification of a single product. PCR efficiency was calculated using the LinRegPCR software version 7.2 (Ramakers *et al*., 2003). CT values of three independent replicates were used to calculate mean values and standard deviations. All values are standardized to the average threshold cycle value obtained with DNA extracted from non-wounded leaves inoculated with the *C. graminicola* wild-type strain at 0 HAI.

II.13 Expression of *eGFP* under control of biotrophy- and necrotrophy-specific promoters in *C. gramminicola* infection structures

To identify biotrophy-specific promoters of *C. graminicola*, 1.2 to 1.3 kb of the 5' non-translated flanks of homologs of biotrophy-specific genes of *Colletotrichum higginsianum* encoding a NmrA-like family protein, a saccharopine dehydrogenase, a pentafunctional AROM protein, an argininosuccinate lyase, an argininosuccinate synthase, and of the non-annotated unigenes 3, 125 and 143 (Takahara *et al*., 2009), were amplified using primers containing *Eco*47III restriction sites in the forward primer and *Eco*47III sites in the reverse primers for unidirectional cloning into plasmid pSH1.6EGFP, 5' of *eGFP* (Figure 22A). The promoter:*eGFP* fusions, together with the *HygR* gene controlled by the *GPDA* promoter of *Aspergillus nidulans*, were PCR-amplified from pSH1.6EGFP using the primers PSH1.6-fw and Hyg.treminator-rev, transformed into *C. graminicola*, and microscopically screened for specific expression in infection vesicles and primary hyphae. Of the putative biotrophy-specific promoters tested, the 1.2 kb 5'-non-translated region of the gene encoding a saccharopine dehydrogenase (SDH) and the 1.3 kb-5'-non-translated region of a gene encoding an NmrA-like family protein mediated biotrophy-specific expression of eGFP. To identify necrotrophy-specific promoters of *C. graminicola*, putative promoters of genes that had previously shown necrotrophy-specific expression in qRT-PCR experiments (Krijger *et al*., 2008), i.e. 5' non-coding regions of gene encoding a secreted peptidase and of an extracellular serine-rich protein, were tested as described above, and the 1.2 kb 5' non-translated region of the secreted peptidase gene mediated expression specifically in secondary hyphae. *eGFP* fusions with the promoters of the genes encoding the NmrA-like family protein, the saccharopine dehydrogenase, and the secreted peptidase were further used and transformed into the *C. graminicola* WT and class I RNAi strains. Primers used to produce promoter:*eGFP* fusions for these genes were CgPrNmrA-Fw and CgPrNmrA-Rv (NmrA like family protein), CgPrSacDh-Fw and CgPrSacDh-Rv (saccharopine dehydrogenase), and CgPrSecPep-Fw and CgPrSecPep-Rv (secreted peptidase).

To visualize fluorescence of eGFP driven by biotrophy- and necrotrophy-specific promoters in infection structures of WT and class I RNAi strains, 10^4 conidia were

inoculated onto intact or wounded epidermal cell layers from bulbs of the alternative host onion (*Allium cepa* cv Grano). Fluorescence was evaluated at the time 0, 12, 24, 48, 72 HAI.

II.14 Analysis of β-1,3-glucan in cell walls of infections structures at different phases of pathogenesis

To analyze β-1,3-glucan contents of cell walls of pre-penetration and *in planta* differentiated infection structures, infected maize leaves were harvested at 0, 12, 24, 48, and 72 HAI and stained with Aniline Blue Fluorochrome (Biosupplies Australia Pty Ltd, Parkville Victoria, Australia) as described (Vogel *et al*., 2000).

II.15 Microscopy and imaging

Fluorescence microscopy was performed with a Nikon Eclipse 90i confocal laser scanning microscope (Nikon, Düsseldorf, Germany) with the following settings: excitation wavelength, 488 nm; laser light transmittance, 25% (ND4 in, ND8 out); pinhole diameter, 30 mm; lens, Plan Apo 60/1.4 oil lens.

Bright-field and differential interference contrast (DIC) microscopy were performed with a Nikon Eclipse E600 microscope (Nikon, Düsseldorf, Germany) as described (Werner *et al.*, 2007).

Fluorescence levels of *GLS1*:*eGFP* expressing transformants of *C. graminicola* were evaluated at 0, 6, 12, 24, and 72 HAI, using a Observer.Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with a AxioCam HRm camera (Carl Zeiss, Oberkochen, Germany). Image acquisition and analysis were performed using Zeiss AxioVision 4.8.2 (06-2010) software with the Physiology module. Video rate FRET imaging was done using a Observer.Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany), using a UApo 40 1.35 numerical aperture (NA) oil-immersion objective (Plan Apochromat 63x/1.40 oil DIC M26) equipped with an AxioCam HRm camera (Carl Zeiss, Oberkochen,

Germany). Image acquisition and analysis were performed by using Zeiss AxioVision 4.8.2 (06-2010) software with the Physiology module.

Wide-field fluorescence observations were made with an Observer.Z1 inverted microscope (Carl Zeiss, Oberkochen, Germany), using a UApo 40 1.35 numerical aperture (NA) oil-immersion objective (Plan Apochromat 63x/1.40 oil DIC M26). Epi-illumination was used at an excitation cutoff limit of 365 nm with barrier filter 49 DAPI (Carl Zeiss, Oberkochen, Germany) for Aniline Blue Fluorocrome and an excitation wavelength, 488 nm with barrier filter 38 HE eGFP (Carl Zeiss, Oberkochen, Germany) for eGFP. Laser light transmittance was fixed to 25% (ND4 in, ND8 out) (pinhole diameter, 30 mm).

II.16 Other methods

Appressorial turgor pressure was measured as incipient cytorrhizis, using polyethylene glycol 6000 (PEG 6000) as described (Howard *et al*., 1991b).

Fluorescein diacetate FDA staining was performed as described (Söderström *et al*., 1977).

To measure conidiation, Petri dishes (Ø 9 cm) with 14-d-old fungal cultures grown on OMA were washed on a rotary shaker with 10 mL of Tween 20 for 10 min to yield homogenous conidial suspensions. Conidia were counted in a Thoma chamber.

Penetration assays were performed as described (Brush and Money, 1999).

II.17 Statistics

Calculations (t-test, analysis of variance) were performed with the software XLSTAT version 2009.4.02 (Addinsoft, Paris, France).

II.18 Accession Numbers

Sequence data for genes of *C. graminicola* used in this thesis can be found in the GenBank/EMBL database under the following accession numbers: *GLS1*, EFQ30502; saccharopine dehydrogenase (*SDH*), EFQ35895; NmrA-like family protein (*NmrA*), GLRG_07923; secreted peptidase (*SPEP*), EFQ25677; developmentally regulated MAPK-interacting protein, EFQ32584; *RHO1*, EFQ35184.

II.19 Vectors

All vectors used in these studies are listed in the table A2 (Appendix VII.3; Table A2).

III Results

III.1 *GLS1* of *C. graminicola* is a single-copy gene encoding a membrane-integral β-1,3-glucan synthase

BLASTX searches performed with GLS proteins of the yeast *S. cerevisiae* (FKS1), and the filamentous Ascomycota *Magnaporthe oryzae*, *Aspergillus nidulans*, *Paracoccidioides brasiliensis*, *Neurospora crassa*, and *Fusarium solani* (FKS1) suggested that, like in most other filamentous fungi (Latgé, 2007), only a single GLS gene exists in the annotated genome of *C. graminicola* (http://www.broadinstitute.org/annotation/genome/colletotrichum_group/FeatureSearch.html; O'Cornel *et al*., 2012). BLASTX searches performed with more distantly related GLS proteins, i.e. GLSs of *U. maydis*, *Pichia pastoris*, *S. cerevisiae* (FKS3) and *F. solani* (FKS2), did not identify further homologs in the proteome of *C. graminicola*. We designated *GLS1* to the single *GLS* gene of *C. graminicola*. The phylogenetic tree calculated on the basis of the derived amino acid sequences shows that the proteins of filamentous ascomycetes form a single clade, distinct from the GLSs of yeasts and basidiomcetes (Figure 8A). On the amino acid level, GLS1 of *C. graminicola* shares 77.7%, 75.5% and 78.9% sequence identity with the GLSs of *N. crassa*, *M. oryzae* and *Fusarium solani* (FKS1), respectively, but only 59.3%, 59.9%, and 45.0% identity with FKS1, FKS2, and FKS3 of *S. cerevisiae*. However, plants are also capable of synthesizing a β-1,3-linked glucans polymer called callose. Callose synthase-like proteins of plants, e.g. GLS5 and GLS7 of Arabidopsis and CSLF3 of maize, share only 32, 30 and 17% identity with GLS1 of *C. graminicola*.

The size of the predicted fungal GLS proteins ranges from 1729 (BGS1 of *Schizosaccharomyces pombe*) to 1955 amino acids (GLS1 of *N. crassa* and *FKS1* of *C. neoformans*), with GLS1 of *C. graminicola* containing 1940 amino acids. The vast majority of GLSs, including GLS1 of *C. graminicola*, have 16 predicted transmembrane domains flanking the glucan synthase domain (Figure 9). *GLS* genes of filamentous ascomycetes contain two introns at conserved sites at their 5'- and 3'-ends, respectively.

Gene sizes range from 5184 (*Bgs1* of *S. pombe*) to 5865 bp (*GLS1* of *N. crassa* and *FKS1* of *C. neoformans*). *GLS1* of *C. graminicola* consists of 5820 bp (Figure 9).

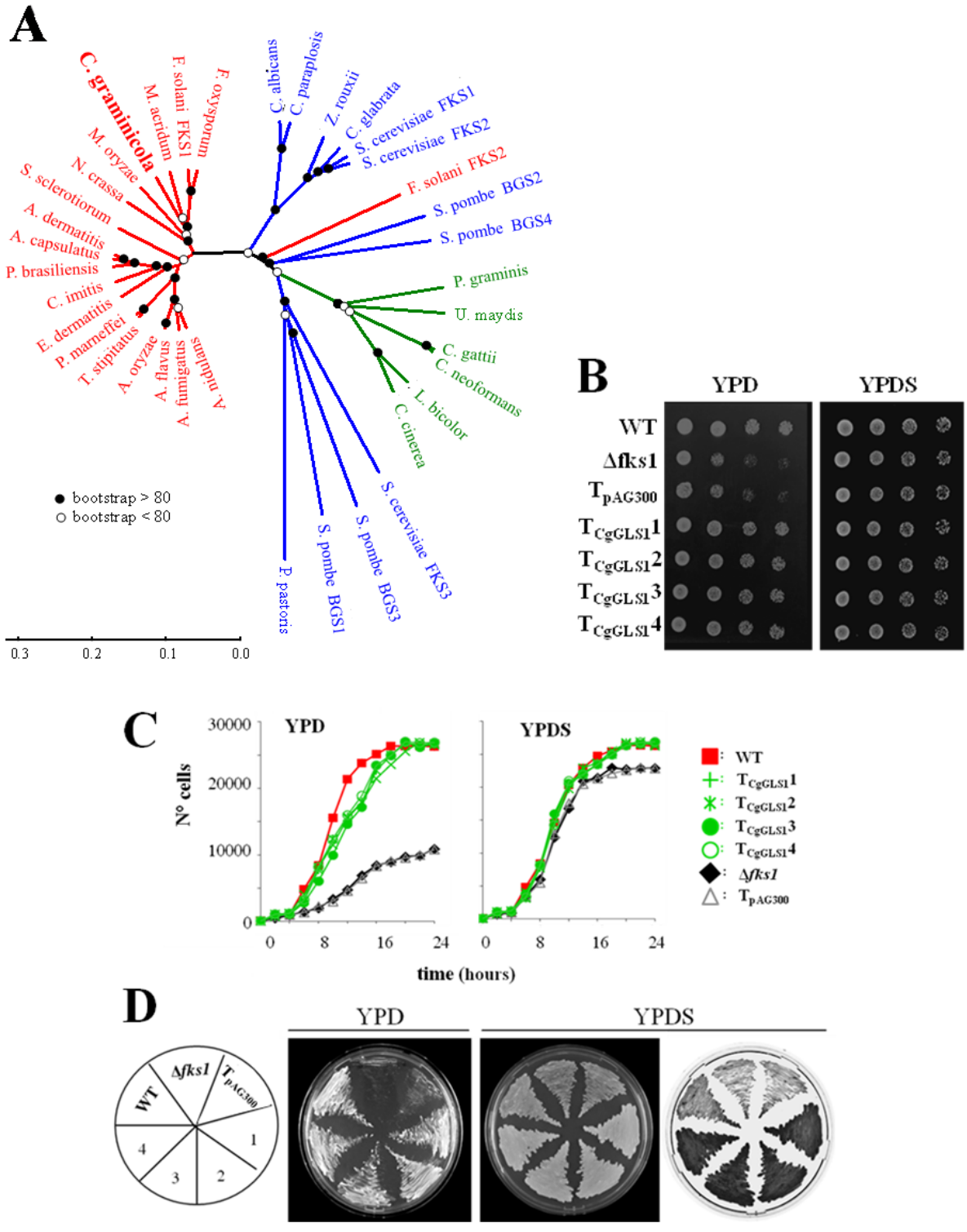

Fig. 8: *GLS1* of *C. graminicola* encodes a functional β-1,3-glucan synthase

(A) The phylogenetic tree indicates close relatedness of *GLS1* of *C. graminicola* with other β-1,3-glucan synthases of filamentous fungi. *A. capsulatus*, *Ajellomyces capsulatus*, (XP001539822.1); *A. dermattidis*, *Ajellomyces dermatitidis* (XP002629106.1); *A. flavus*, *Aspergillus flavus FKS1* (XP002383406.1); *A. oryzae*, *Aspergillus oryzae FKS1* (XP_001816673.1); *A. flavus*, *Aspergillus flavus* (XP_002383406.1); *A. fumigatus*, *Aspergillus fumigatuss* (XP751118.1); *A. nidulans*, *Aspergillus nidulans* (XP661333.1); *C. albicans*, *Candida albicans* (XP721429.1); *C. glabrata*, *Candida glabrata* (XP446406.1); *C. paraplosis*, *Candida parapsilosis* (ABX80511.1); *C. gattii*, *Cryptococcus gattii* (XP003197612.1); *C. imitis*, *Coccidioides immitis* (XP_001247982.1); *C. posadasii*, *Coccidioides posadasii* (XP003065511.1); *C. graminicola*, *Colletotrichum graminicola GLS1* (EFQ30502); *C. cinerea*, *Coprinopsis cinerea okayama* (XP001837755.2); *C. neformans* , *Cryptococcus neformans*_var. *neoformans* (XP771791.1); *E. dermatitis*, *Exophiala dermatitidis* (ABL63820.1); *F. solani* (FKS1), *Fusarium solani* (ABC59463); *F.solani* (FKS2), *Fusarium solani* (XP003040299.1); *L. bicolor*, *Laccaria bicolor* (XP001878782.1); *M. oryzeae*, *Magnaporthe oryzae Magnaporthe oryzae* (XP368379.2); *M. acridum*, *Metarhizium acridum* (EFY92417.1); *N. crassa*, *Neurospora crassa* (XP957980.1); *P. brasiliensis*, *Paracoccidioides brasiliensis* (XP002792935.1); *P.marneffei*, *Penicillium marneffei* (XP002147495.1); *P. pastoris*, *Pichia pastoris* (XP002491155.1); *P. graminis*, *Puccinia graminis* f. sp. *tritici* (XP003307175.1); *S .cereviseae* (FKS1), *Saccharomyces cerevisiae* (NP013446.1); *S.cereviseae* (FKS2), *Saccharomyces cerevisiae* (NP011546.1); S.cereviseae (FKS3), *Saccharomyces cerevisiae* (NP014036.1); *S. pombae* (Bgs1), *Schizosaccharomyces pombe* (NP595971.1); *S. pombae*, *Schizosaccharomyces pombe* (Bgs2) (NP594032.1); *S. pombae* (Bgs3), *Schizosaccharomyces pombe* (NP_594766.1); *S. pombae* (Bgs4), *Schizosaccharomyces pombe* (CAA20125.1); *S. sclerotiorum*, *Sclerotinia sclerotiorum* (XP001586992.1); *T. stipitatus*, *Talaromyces stipitatus* (XP002481640.1); *U. maydis*, *Ustilago maydis* (XP757786.1); *Z. rouxii*, *Zygosaccharomyces rouxii* (XP002494904.1).

(B) Complementation of *S. cerevisiae* Δfks1 by *GLS1* cDNA of *C. graminicola* suggests that *GLS1* encodes an active β-1,3-glucan synthase. WT, *S. cerevisiae* reference strain Y00000; Δfks1, *FKS1*-deficient *S. cerevisiae* strain Y05251; T_{pAG300}, Δfks1 transformant harboring the empty binary vector pAG300, T_{CgGLS1} 1 - 4, independent Δfks1 transformants expressing the *GLS1* cDNA of *C. graminicola*; YPD, YPD medium; YPDS, YPD medium supplemented with 1M sorbitol. Yeast cell numbers inoculated were (left to right) $5x10^4$, $5x10^3$, $5x10^2$, and 5x10.

(C) The reference yeast strain Y00000 (wild-type) and FKS1 mutants expressing *GLS1* cDNAs from *C. graminicola*, respectively, were able to grow on medium lacking osmotic stabilization (YPD). By contrast, Δfks1 or the Δfks1 transformant containing the empty expression vector (T_{pAG300}) show non satisfatory growth in YPD medium.

(D) All yeast strains show satifatory growth on solid medium amended with sorbitol (YPDS). By contrast, under the expossion of light, the four transformants expressing *GLS1* cDNA of *C. graminicola* reduced the light penetration.1-4, T_{CgGLS1} 1 – 4.

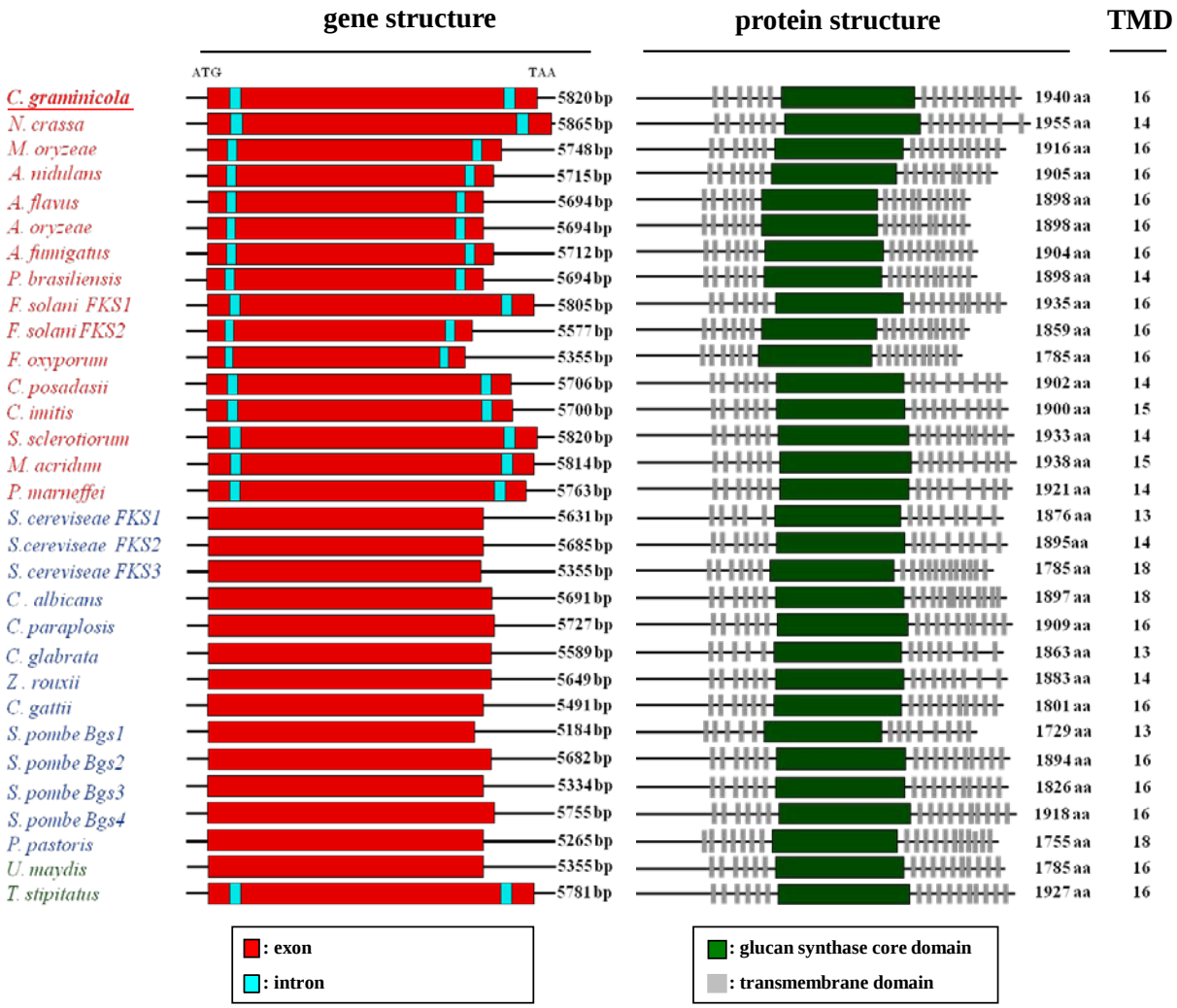

Fig. 9: Gene (left) and protein structure (right) of β-1,3-glucan synthases of different Asco- and Basidiomycota

Exons are shown as red bars, introns are blue bars. Names of species belonging to filamentous Ascomycota are in red, names of yeasts are in blue, and names of Basidiomycota are in green. *GLS* genes of filamentous Ascomycota show highly conserved sequences and intron positions. In yeasts and in the dimorphic Basidiomycete *U. maydis*, introns are absent. The positions of introns in the *GLS1* gene of *Talaromyces stipitatus* are similar to those of filamentous Ascomycota. bp, base pairs.

Domain organization of the GLS proteins of different filamentous Ascosmycota, yeasts, and Basidiomycota. The predicted proteins show a central GLS core region (green bars) and several predicted transmembrane domains (gray bars). aa, amino acids; TMD, transmembrane domains.

The function of *GLS1* of *C. graminicola* was confirmed by complementation of yeast strain Y05251, carrying a deletion of the *GLS* gene *FKS1*. Due to the presence of three *GLS* genes, deletion of *FKS1* is not lethal in *S. cerevisiae* but results in severe growth defects on osmotically non-stabilized medium (Figure 8B,C,D, YPD). Growth defects of the Δ*fks1* strain were fully rescued by osmotically stabilizing the YPD medium with 1M sorbitol (Figure 8B, YPDS). Approx. one million yeast transformants carrying the *C. graminicola GLS1* cDNA were generated, four of which (T_{CgGLS}1-4) were randomly chosen and used in growth assays. All yeast transformants expressing the *C. graminicola GLS1* cDNA showed growth rates comparable to those of the reference strain Y00000, irrespective of osmotic support. The Δ*fks1* strain transformed with the empty expression vector pAG300 (T_{pAG300}) was indistinguishable from the Δ*fks1* strain and showed poor growth on non-stabilized YPD plates (Figure 8B,C,D, compare Δ*fks1* and T_{pAG300}).

Collectively, the yeast complementation experiments show that *GLS1* of *C. graminicola* represents a functional *GLS* gene.

Programs such as TMHMM Server v. 2.0 predict that GLS1 of *C. graminicola* is a plasma membrane-integral protein. To study localization of *GLS1*, four independent *C. graminicola* replacement strains with single *GLS1*:*eGFP* integrations were randomly chosen, expressing the *GLS1*:*eGFP* fusion under the control of the native *GLS1* promoter (P_{GLS1}) (Figures 10A and B). The *GLS1*:*eGFP* fusion was fully functional in the replacement strain, as i) osmotic stabilization was not required for vegetative growth, ii) growth and iii) sporulation rates did not differ from those of the WT strain CgM2 (Figure 10C-E), and iv) cell wall defects did not occur (see below). The *GLS1*:*eGFP* replacement strains revealed strong eGFP fluorescence associated with the apical plasma membrane (Figure 11A, insert, left panel, arrowhead), slightly extending into sub-apical regions (Figure 11A, insert, left panel, short arrow). Fluorescence of GLS1:eGFP co-localized with that of the red-fluorescing membrane marker dye FM-464 only at the apical and sub-apical plasma membrane, indicating a tip-oriented gradient of GLS1 (Figure 11A, central and right panels, arrows). To further show that the GLS1:eGFP fusion protein is a plasma membrane-integral protein, protoplasts were generated, using fungal cell wall lysing enzymes from *Trichoderma harzianum* (Figure 11B, DIC)(Werner *et al*., 2007). Clearly, these protoplasts showed eGFP-fluorescence in their plasma membranes (Figure 11B, GLS1:eGFP, arrows).

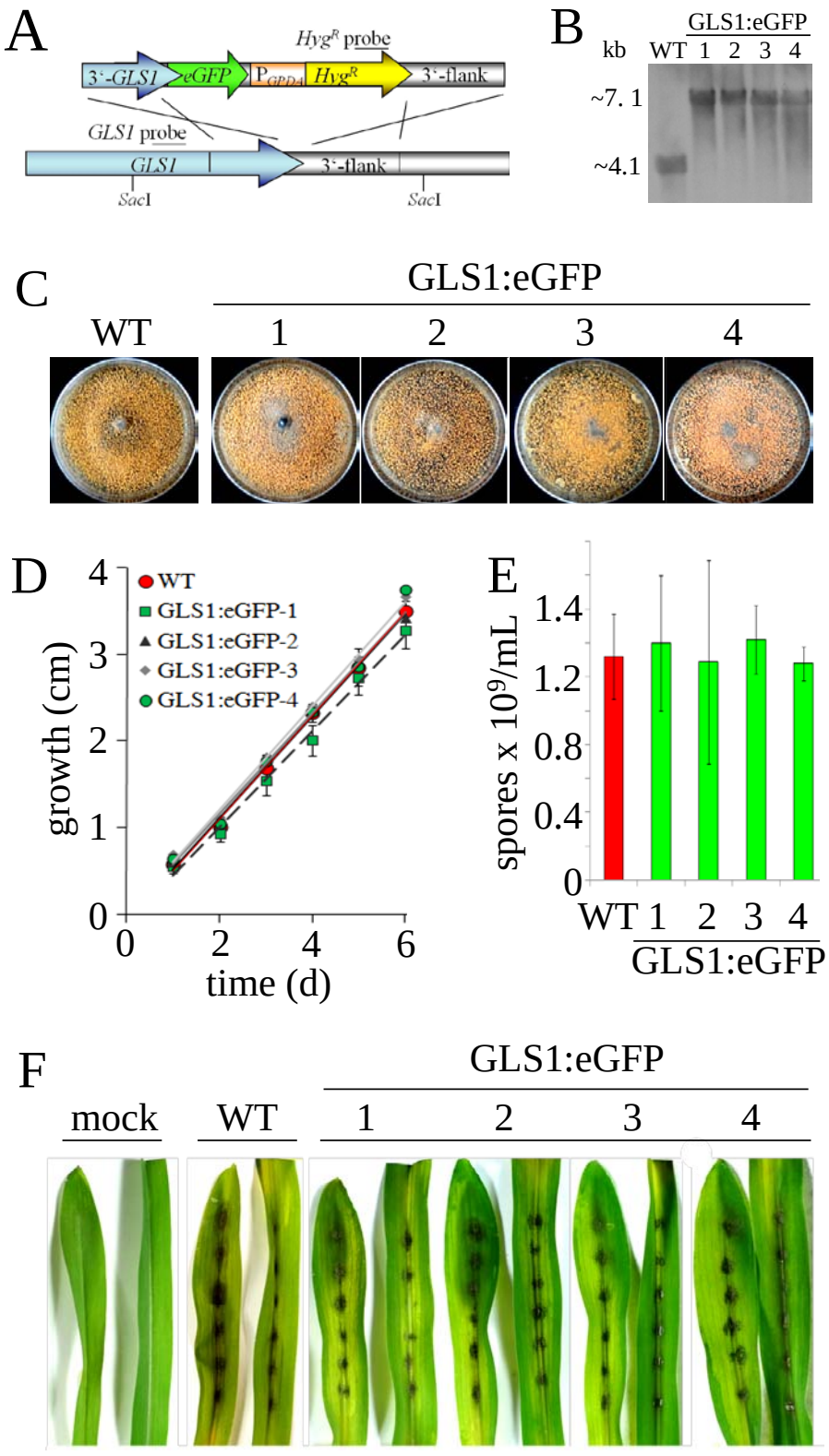

Fig.10: Generation of *GLS1:eGFP* replacement strains and comparison of the WT and the replacement strains

(A) Strategy of *eGFP*-tagging of the *GLS1* gene by homologous recombination. The 3'-end of the *GLS1* gene was fused to *eGFP*. The hygromycin phosphotransferase gene (Hyg^R) of *E. coli* is controlled by the *GPDA* promoter (P_{GPDA}) of *A. nidulans*. The 3'-non-coding region served as the 3'-flank. Bars indicate probes used in Southern blot experiments.

(B) Southern blot of *Sac*I-digested DNA isolated from *C. graminicola* wild-type (WT) and four independent transformants (1 – 4) with homologous integration of the *GLS1*:*eGFP* construct. Fragment sizes are given in kb.

(C) Morphology of colonies of *C. graminicola* wild-type (WT) and four independent *GLS1*:*eGFP* replacement strains, growing on oat meal agar plates.

(D) Radial growth rates of *C. graminicola* wild-type (WT, red circles) and four independent *GLS1*:*eGFP* replacement strains (green symbols).

(E) Sporulation of *C. graminicola* wild-type (WT, red bar) and four independent *GLS1*:*eGFP* replacement strains (GLS1:eGFP 1 – 4, green bars). Experiments were performed on oat-meal agar plates and evaluated 14 days after inoculation.

(F) Infection assays of *C. graminicola* wild-type (WT) and four independent *GLS1*:*eGFP* replacement strains (GLS1:eGFP 1 – 4). Non-wounded second (left) and third (right) leaves of 14 days old maize (*Z. mays* cv. Nathan) plants were inoculated with a suspension containing 10^6 spores/mL and evaluated 5 days after inoculation. Mock inoculation (mock) was done with 0.01% (v/v) Tween 20.

Bars in D and E represent ± standard deviations.

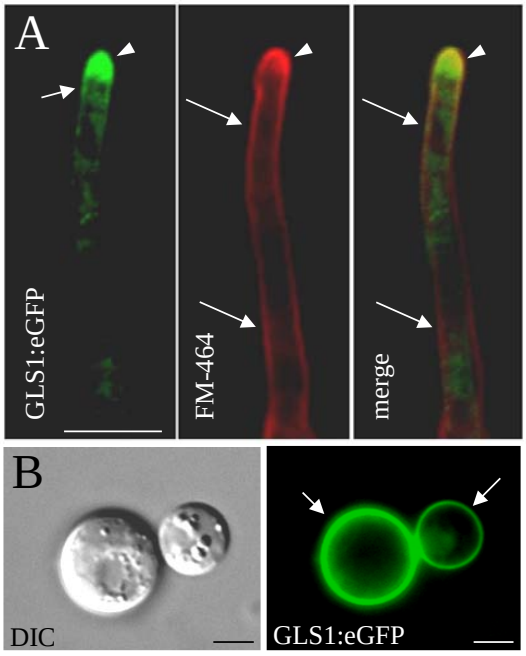

Fig. 11: GLS1 of *C. graminicola* is plasma membrane-localized.

(A) GLS1:eGFP exhibits a tip-oriented gradient. Significant eGFP fluorescence is localized at the hyphal apex (GLS1:eGFP, arrowhead), in the sub-apical plasma membrane (GLS1:eGFP, short arrow), and in macro-vesicles (GLS1:eGFP, long arrow). Staining of hyphae with the membrane-specific fluorescent dye FM-464 visualizes the hyphal plasma membrane (FM-464 and merge, arrowhead).

(B) Protoplast produced from a *GLS1*:*eGFP* expressing hypha show membrane-localization of GLS1. Arrow indicates eGFP fluorescence.

Bars are 10 µm.

III.2 RNAi-mediated reduction of *GLS1* expression causes severe cell wall defects and hyper-melanization of vegetative hyphae

Like in experiments performed with other filamentous fungi (Latgé, 2007), attempts to delete the *GLS* gene of *C. graminicola* failed (Figure 12A). PCR screens performed with more than 100 single-spore isolates of transformants always led to amplification of a 1 kb fragment of the *GLS1* gene (Figure 12B), suggesting ectopic integration of the deletion cassette. As average rates of homologous integration in this fungus rarely are below 30%, these results suggest that deletion of the *GLS1* gene of *C. graminicola* is lethal.

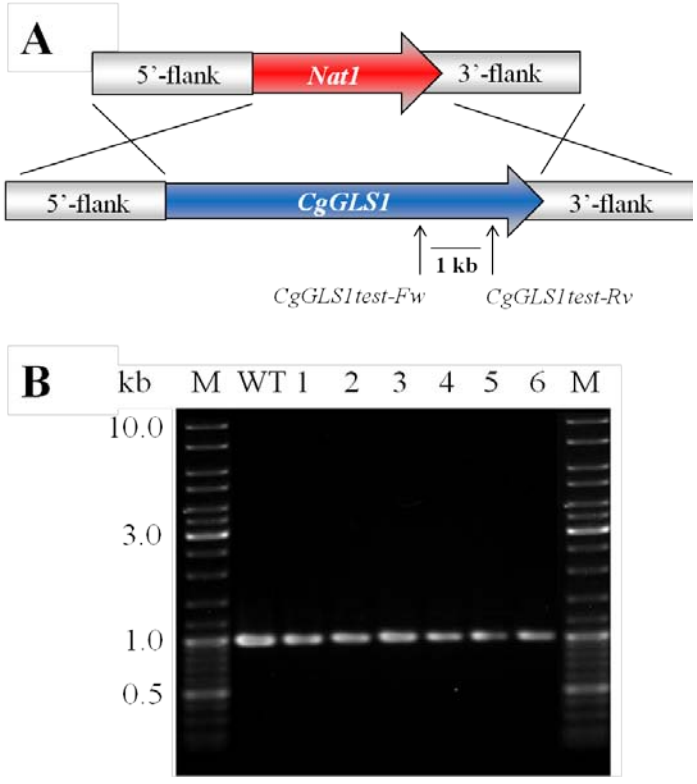

Fig.12: Strategy for targeted deletion of *GLS1* of *C. graminicola* and screen for transformants with homologous integration of the deletion cassette

(A) The deletion cassette consisted of the Nourseothricin acetyltransferase gene (*Nat-1*) from *S. noursei*, controlled by the *oliC* promoter from *A. nidulans*, flanked by the 5'- and 3'-flanks of the *GLS1* gene of *C. graminicola*. Arrows indicated binding sites of primers CgGLS1test-Fw and CgGLS1test-Rv; bar indicates the predicted ~1 kb-PCR band in the wild-type (WT) strain and transformants with ectopically integrated deletion cassette. Not to scale.

(B) PCR screen for homologous integration of the deletion construct. PCR primers (CgGLS1test-Fw and CgGLS1test-Rv) have binding sites in the coding sequence of GLS1, so that the 1 kb band can only be amplified from the wild-type (WT) strain and strains harboring an ectopically integrated deletion cassette. Six independent transformants (1 – 6) of a total of 200 are shown. PCR analyses showed a ~1 kb band for all transformants tested, indicating ectopic integration of the deletion cassette and the indispensability of *GLS1*. M, DNA size marker.

To circumvent the lethal phenotype of a *GLS1* deletion, an RNAi-based *knock down* strategy was adopted (Kück and Hoff, 2010) (Figure 13). The RNAi vector consisted of the *trpC* promoter, a 475 bp sense and antisense fragment of the second exon of the *GLS1* gene of *C. graminicola*, separated by 135 bp of the second intron of the *Cut2* gene from *M. oryzae*, followed by the *trpC* terminator of *A. nidulans*. The Nourseothricin resistance gene from *Streptomyces noursei* (Malonek *et al.*, 2004) was used as the selection marker (Figure 13A).

*Xho*I-digested genomic DNA of the WT strain and of nine RNAi strains was analyzed by Southern hybridization. Different numbers of RNAi constructs had integrated into the genome of different transformants (Figure 13B), and the copy number of the RNAi construct correlated with the reduction of *GLS1* transcript abundance, as indicated by qRT-PCR (compare Figures 13B and C). According to different degrees of reduction of *GLS1* transcript abundance, the nine RNAi strains were grouped into three classes. As compared with the WT strain, class I (strains 1-3) showed transcript abundances between 55 and 67%, class II (strains 4-6) between 33 and 37%, and class III (strains 7-9) below 25% (Figure 13C). All transformants exhibited severely reduced growth rates (Figures 13D-F).

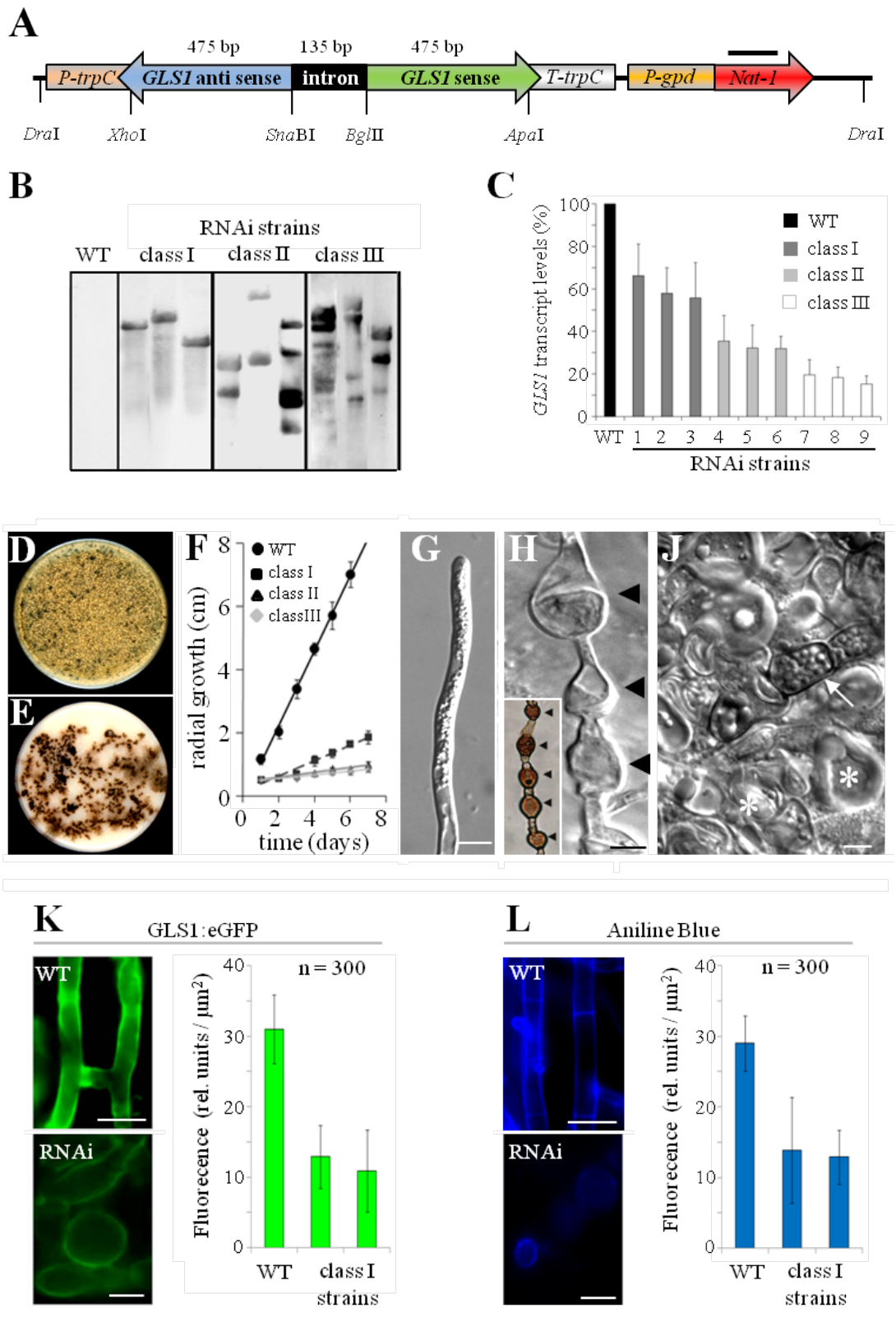

Fig. 13: RNAi-mediated reduction of *GLS1* transcript abundance causes reduction of β-1,3-glucan contents of cell walls, hyphal distortion, and retarded growth

(A) RNAi construct transformed into *C. graminicola*. Bar indicates probe used for Southern blots. P_{trpC}, *trpC* promoter; T_{trpC}, *trpC* terminator, *Nat-1*, Nourseothricin acetyl transferase gene. Not to scale.

(B) Southern blot of *Xho*I-digested genomic DNA of *C. graminicola* WT and RNAi strains. A digoxygenin-labelled fragment of the *Nat-1* gene served as the probe (bar in Figure 3A).

(C) *GLS1* transcript abundance measured by qRT-PCR.

(D, E) Morphology of colonies of WT **(D)** and a class I RNAi strain **(E)** on OMA supplemented with 0.15 M KCl.

(F) Radial growth of WT, class I, class II and class III RNAi strains on oat meal agar (OMA) supplemented with 0.15 M KCl. Bars represent ± standard deviation.

(G) Vegetative hypha of the WT strain.

(H) Vegetative hypha of a class I RNAi strain showing hyphal swellings (arrowheads). Insert shows pigmentation of swellings.

(J) Severely distorted vegetative hyphae of a class II RNAi strain. Even on OMA supplemented with 0.15 M KCl the mycelium consists of swellings (asterisks), some of which are strongly melanized (arrow). Bars in G – J are 5 µm.

(K) eGFP fluorescence and quantification of fluorescence intensities in the WT and a class I RNAi strain carrying a *GLS1*:*eGFP* replacement construct.

(L) β-1,3-glucan distribution and quantification of fluorescence in a WT and a class I RNAi strain carrying a *GLS1*:*eGFP* replacement construct. Hyphae were stained by Aniline Blue Fluorochrome. Bars in micrographs in K and L are 10 µm, bars on columns represent ± standard deviation.

As compared with the WT strain (Figure 13D), all RNAi strains showed a strongly altered colony phenotype and formed compact hyper-melanized colonies (Figure 13E). While the WT strain developed normal filaments (Figure 13G), vegetative hyphae of class I RNAi strains exhibited severe hyphal swellings (Figure 13H, arrowheads), which often were strongly pigmented (Figure 13H, insert, arrowheads). Class II RNAi strains showed even more pronounced cell wall distortions (Figure 13J, asterisks), and many of the swellings were strongly melanized (Figure 13J, arrow).

In order to visualize that reduction of transcript abundance led to reduced GLS1 protein contents, the *GLS1* gene of the WT strain and of the class I RNAi strain 1 was replaced by

the *GLS1*:*eGFP* construct (see Figures 10 and 14). Two representative transformants and the *GLS1*:*eGFP* replacement strain lacking the RNAi construct were comparatively analyzed by quantitative fluorescence microscopy (Figures 13K and L). Vegetative hyphae of the two class I RNAi strains showed a reduction of eGFP fluorescence by 66.4±18.9% and 70.4±29.4% (Figure 13K), and a reduction of Aniline Blue fluorescence by 56.7±23.4 % and 60.1±13.2% (Figure 13L), clearly indicating down-regulation of *GLS1*:*eGFP* expression and, accordingly, synthesis of β-1,3-glucan by RNAi.

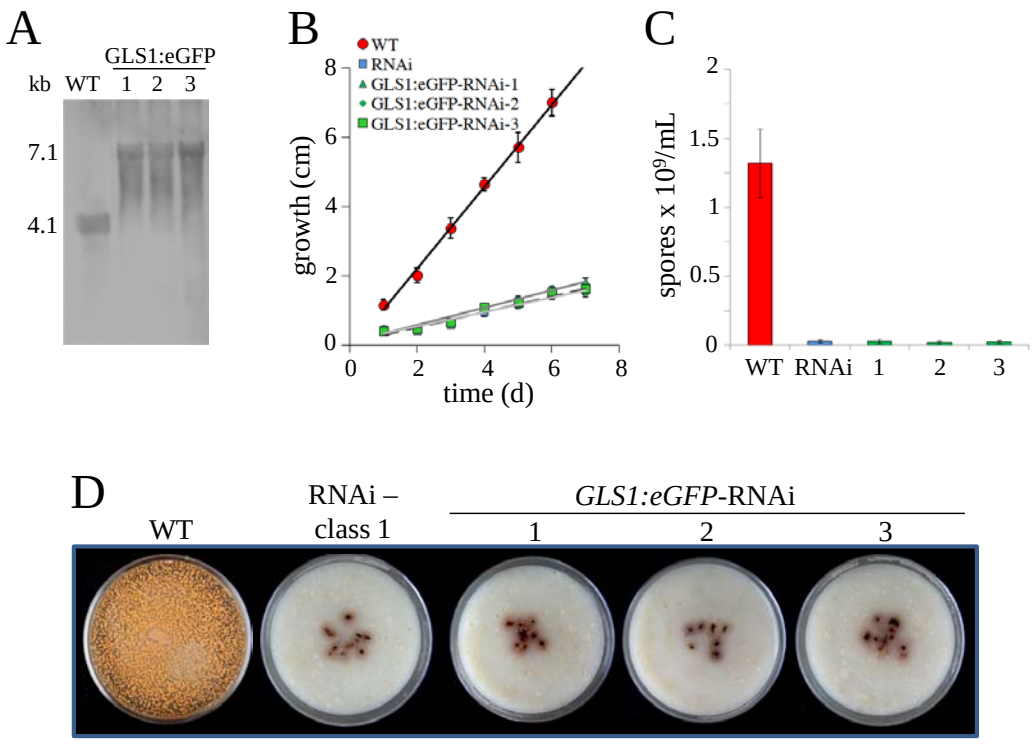

Fig. 14: Replacement of *GLS1* by an *GLS1*:*eGFP* fusion in three RNAi strains, and comparison of the WT and the replacement strains

(A) Southern blot of DNA isolated from the *C. graminicola* wild-type (WT) strain and from three independent transformants with the homologously integrated *GLS1*:*eGFP* construct. The construct and strategy used for replacement of *GLS1* by *GLS1*:*eGFP* is shown in Figure 10; DNA was *Sac*I-digested and hybridized with the *GLSI* probe as indicated in Figure 10.

(B) Radial growth rates of the *C. graminicola* wild-type (WT, red circles) strain, class I RNAi strain 1 (RNAi, blue circles), and of three independent transformants with the homologously integrated *GLS1*:*eGFP* construct (GLS1:eGFP-RNAi 1 – 3, green symbols).

(C) Sporulation of the *C. graminicola* wild-type (WT, red bar) strain, class I RNAi strain 1 (RNAi, blue bar), and of three independent transformants with the homologously integrated *GLS1*:*eGFP* construct (GLS1:eGFP-RNAi 1 – 3, green bars). Strains were allowed to grow on oat-meal agar plates, spores were counted 14 days after inoculation. Black bars indicate ± standard deviation. Four plates were counted for the WT and each mutant strain. Bars in B and C represent ± standard deviations.

(D) Morphology of colonies of *C. graminicola* wild-type (WT), class I RNAi strain 1 (RNAi-class 1), and of three independent transformants with the homologously integrated *GLS1*:*eGFP* construct (GLS1:eGFP-RNAi 1 – 3).

These data show for the first time that RNAi allows characterizing essential genes in the maize anthracnose fungus *C. graminicola*.

III.3 *GLS1* expression and cell wall β-1,3-glucan contents are drastically down-regulated in biotrophic hyphae

GLS genes are thought to be constitutively expressed during vegetative hyphal growth (Mouyna *et al*., 2004; Ha *et al*., 2006). During pathogenesis, infection structure-specific regulation of *GLS* gene may be required to support the function of the specialized pathogenic cells or hyphae. Expression of *GLS* genes and synthesis of β-1,3-glucan during the fungal infection process has so far not been analyzed. Unfortunately, as infection structure differentiation of *C. graminicola* on maize leaves does not occur in a synchronized fashion, neither qRT-PCR-based quantification of infection structure-specific *GLS1* transcript abundance nor chemical quantification of β-1,3-glucan is feasible. We therefore used the *GLS1*:*eGFP* replacement strains of *C. graminicola* (Figure 13) to quantify *GLS1* expression and β-1,3-glucan contents in infection structures of *C. graminicola* by measuring eGFP signals (Figure 15A, C) and β-1,3-glucan-specific fluorescence after Aniline Blue Fluorochrome staining (Figures 15B, D). Virulence of the *GLS1*:*eGFP* replacement strains did not differ from that of the WT strain (Figure 10F). Strong eGFP fluorescence was observed in non-germinated conidia, appressoria, and necrotrophic secondary hyphae (Figure

15A, 0 HAI, co; 12 HAI, ap; 72 HAI, sh; Figure 15C). Melanization of appressoria only slightly reduced eGFP fluorescence (Figure 15C). Unexpectedly, biotrophic primary hyphae showed only background eGFP fluorescence (Figures 15A, 24 HAI, ph; Figure 15C). Note that the fluorescence signal marked with an arrowhead was emitted by the appressorium on the cuticle, not by biotrophic hyphae (Figure 15A, 24 HAI). Accordingly, non-germinated conidia, non-melanized immature appressoria and necrotrophic secondary hyphae showed strong Aniline Blue fluorescence (Figure 15B, 0 HAI, co; 12 HAI, ap; 72 HAI, sh; Figure 15D). Mature appressoria emitted low levels of fluorescence (Figure 15D), likely due to the fluorescence-scavenging activity of melanin (Henson *et al*., 1999). Importantly, biotrophic infection vesicles and primary hyphae exhibited strongly reduced fluorescence intensities (Figure 15B, 24 HAI, ph; Figure 15D). To exclude the possibility that the lack of Aniline Blue labeling in biotrophic hyphae was due to apposition of α-1,3-glucan, as suggested for *M. oryzae* (Fujikawa *et al*., 2009), specimens were treated with alkaline (0.1 N NaOH, 20 min at 60° or 121°C) (Sietsma *et al*., 1985) (Figure 16). Alkaline treatment did not restore Aniline Blue-labeling, indicating that differential expression of *GLS1*, and not masking of β-1,3-glucan by α-1,3-glucan apposition caused lack of Aniline Blue fluorescence in biotrophic structures.

In conclusion, quantitative fluorescence microscopy revealed that *GLS1* expression and β-1,3-glucan contents of cell walls are prominent in conidia, appressoria and necrotrophic hyphae but are lacking in biotrophic infection structures of *C. graminicola*.

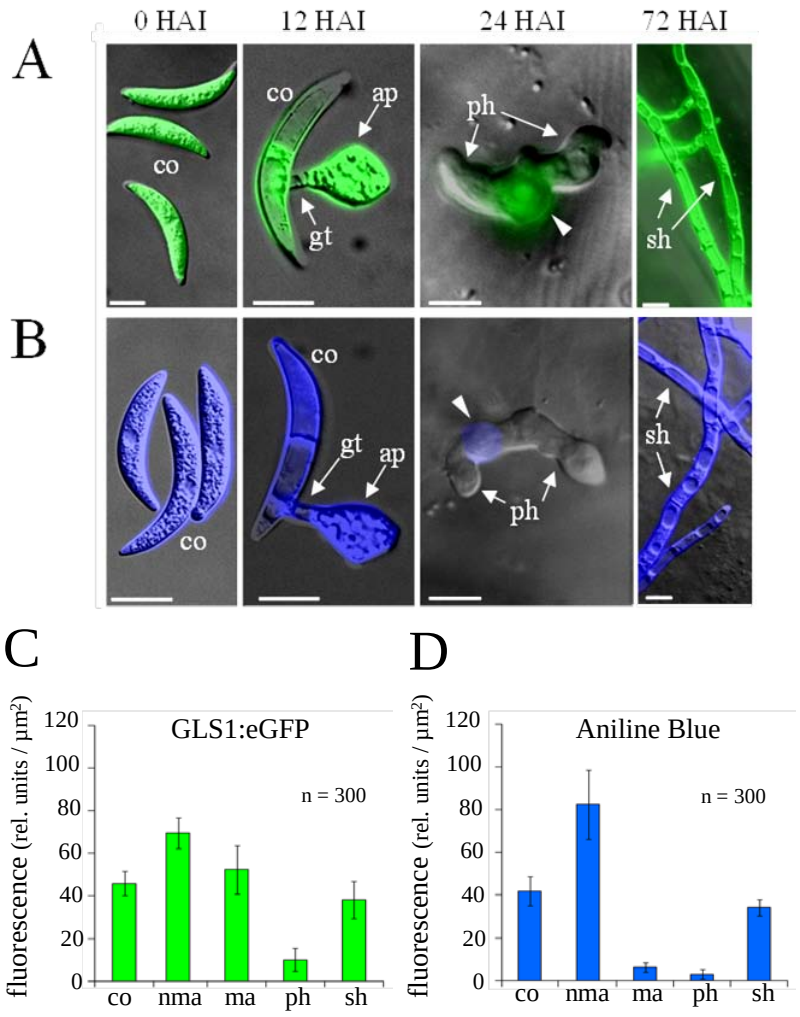

Fig. 15: Infection structure-specific expression of *GLS1*:*eGFP* and synthesis of β-1,3-glucan in *C. graminicola*

(A) *GLS1*:*eGFP* is strongly expressed in conidia (co; 0 HAI), appressorium (ap; 12 HAI), and necrotrophic secondary hyphae (sh; 72 HAI), but not in biotrophic primary hyphae (ph; 24 HAI).

(B) Staining of infection structures with β-1,3-glucan-specific Aniline Blue Fluorochrome shows β-1,3-glucan in conida (co; 0 HAI), appressorium (ap; 12 HAI), and necrotrophic secondary hyphae (sh; 72 HAI), but not in biotrophic primary hyphae (ph; 24 HAI). Bars in A and B are 10 µm.

(C) Quantification of eGFP fluorescence in infection structures. co, condia; gt, germ tubes; nma, non-melanized appressoria; ma, melanized appressoria; ph, biotrophic primary hyphae; sh, necrotrophic secondary hyphae. 200 infection structures were measured.

(D) Quantification of β-1,3-glucan in infection structures using Aniline Blue Fluorochrome. co, condia; gt, germ tubes; nma, non-melanized appressoria; ma, melanized appressoria; ph, biotrophic primary hyphae; sh, necrotrophic secondary hyphae. 200 infection structures were measured; bars in C and D represent ± standard deviation.

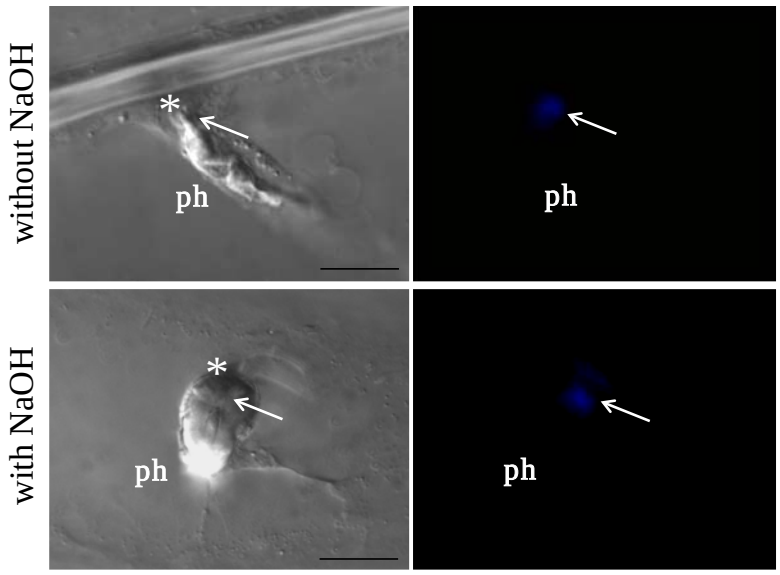

Fig. 16: β-1,3-glucan is not masked by alkali-soluble cell wall appositions

Alkali-treatment (0.1 N NaOH, 60°C, 20 min) before staining with Aniline Blue Fluorochrome did not alter the fluorescence pattern. Asterisks indicate the position of the appressorium, arrow points to the penetration site, ph indicates biotrophic primary hypha. Note that appressoria can not be seen, due to different focal plane. Bars are 10 µm.

III.4 *GLS1* of *C. graminicola* is required for asexual sporulation, adhesion and differentiation of functional appressoria

Intense fluorescence of conidia in transformants expressing a *GLS1*:*eGFP* fusion and after Aniline Blue Fluorochrome staining (Figure 15) suggested that *GLS1* expression and β-1,3-glucan synthesis are required for formation of asexual spores. Indeed, reduction of *GLS1* transcript levels by RNAi strongly affected asexual sporulation rates and the conidial shape (Figure 17).

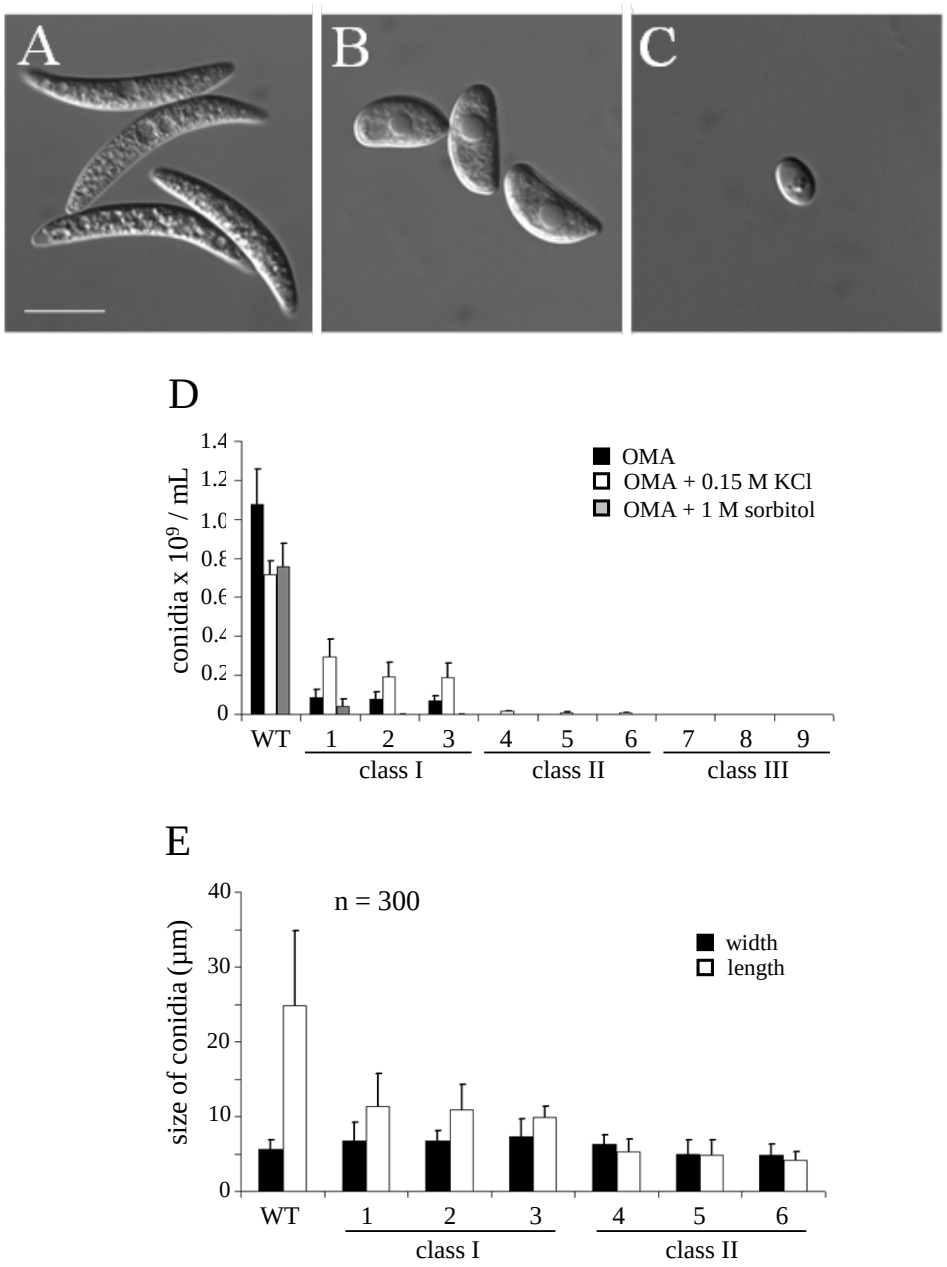

Fig. 17: Formation of asexual spores by the *C. graminicola* WT and independent class I and II RNAi strains

(A-C) Shape of conidia of the WT (A) and class I (B) and II (C) RNAi strains. Bar is 10 µm.

(D) Sporulation of the WT strain and of independent class I, II and III RNAi strains on oat meal agar in the presence / absence of different osmolytes (0.15 M KCl or 1 M sorbitol). In all RNAi strains, irrespective of the osmolyte added, sporulation rates were strongly reduced.

(E) Size of conidia of the WT strain and of independent class I and II RNAi strains. Conidial length of the WT strain differed significantly from that of all RNAi strains. Means of three biologic repeats, with 300 spores each measured, are shown.

Bars in D and E are standard deviations.

Osmotically stabilized conidia of the WT strain and the class I RNAi strains (Figures 18A and B, asterisk) germinated and differentiated appressoria on artificial substrata (Figures 18A and B, arrow). Intriguingly, while appressoria of the WT strain subsequently remained unaltered, approx. 15% of all appressoria formed by class I RNAi strains exploded (Figure 18B, arrows; Figure 18F) and released cell components such as lipid droplets (Figure 18B, arrowheads). Interestingly, several appressoria of class I RNAi strains that did not explode (Figure 18C, white arrow; Figure 18F) developed hyphae reminiscent of biotrophic primary hyphae (Figure 18C, black arrow). Hyphae developing from appressoria of class I RNAi strains on the surface of epidermal cells of onion had a diameter of 7.8 ± 3.2 µm, which is in good agreement with and statistically not different ($p > 0.05$) from diameters of 8.1 ± 3.2 µm measured for biotrophic primary hyphae formed *in planta* (Politis and Wheeler, 1973; Horbach *et al.*, 2009). As tight adhesion of appressoria is required for directed growth of the penetration peg into the plant cell wall, failure in penetration and development of primary hyphae on the cuticle is likely due to compromised adhesion. Indeed, as compared with the WT strain, adhesion of infection structures of the class I RNAi strains tested was 36.7-; 34.5-; and 26.9-fold reduced on onion epidermal cells, polyester and glass (Figure 18G).

While appressoria of the WT strain were strongly melanized (Figure 18A, arrow), melanization was clearly reduced in the appressoria of class I RNAi strains (Figures 18B and 18C, insert). Interestingly, melanin often formed a ring on the underlying polyester and onion epidermis, surrounding the appressorium (Figure 18C, insert, arrowheads), suggesting that an intact β-1,3-glucan network is required for incorporation of melanin into the cell wall. Melanization appeared to be severely de-regulated in class II RNAi strains. Conidia of these strains were able to germinate (Figure 18D, arrow), but failed to form appressoria (Figure 18F). While germlings of WT conidia never melanize, those of class II RNAi strains produced massive amounts of pigment that was secreted into the medium and precipitated on the substratum surrounding the hypha (Figure 18E, class II RNAi strains).

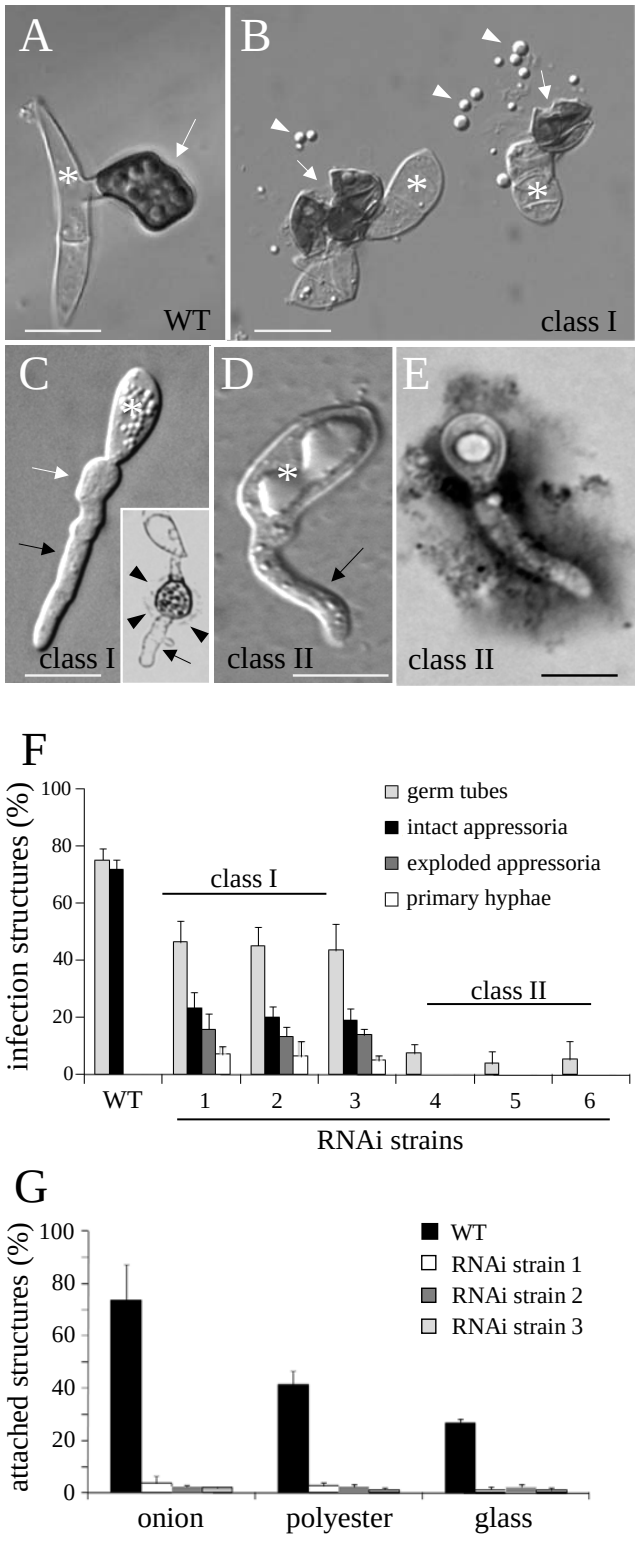

Fig. 18: Appressoria of RNAi strains of *C. graminicola* lack cell wall rigidity, have melanization defects, and are non-adhesive

(A) On polyester, conidia (asterisk) the WT strain germinate and form melanized appressoria (arrow).

(B) Conidia (asterisk) of class I RNAi strains germinate and form appressoria, many of which explode (arrow) and release lipid bodies (arrowhead).

(C) Appressorium (white arrow) of a class I RNAi strain with a voluminous hypha (black arrow) reminiscent of a primary hypha. Insert shows an irregularly melanized appressorium surrounded by a ring of melanin (arrowheads). A hypha with large diameter reminiscent of a primary hypha is marked by a black arrow.

(D) Conidia (asterisk) of class II RNAi strains are able to form germ tubes (arrow) but fail to differentiate appressoria.

(E) Germling of a class II strain surrounded by a melanin precipitate.

Bars in A – E are 10 µm.

(E) Quantification of infection structure differentiation on polyester. In each of the three independent experiments performed, 100 infection structures have been counted.

(G) Adhesion of infection structures formed by WT and three independent class I RNAi strains on onion epidermis or artificial surfaces 24 HAI. In each of the three independent experiments performed, 100 infection structures have been counted. Bars in E and G represent standard deviations.

When class I RNAi strains were allowed to form appressoria in sterile distilled water, these cells ruptured (Figures 18B and F). Those appressoria that did not explode allowed measuring appressorial diameters (Figures 19A and B). In distilled water, class I RNAi strains showed significantly increasing appressorial diameters. Interestingly, addition of the osmolyte PEG6000 (400 mg/mL) caused shrinking of the appressoria of the class I RNAi strains by approx. 50% to the WT level, indicating that appressorial cell walls of the class I RNAi strains were highly elastic. The appressorial diameter of the WT strain was unaffected by addition of the osmolyte (Figures 19A and B). Increased appressorial cell wall elasticity was expected to affect the appressorial turgor pressure. Indeed, WT appressoria showed incipient cytorrhizis at a PEG6000 concentration of more than 400 mg/mL, whereas approx. 190 mg/mL were sufficient in the class I RNAi strains (Figure 19C), indicating significantly reduced appressorial turgor pressure of class I RNAi strains, as compared with the WT.

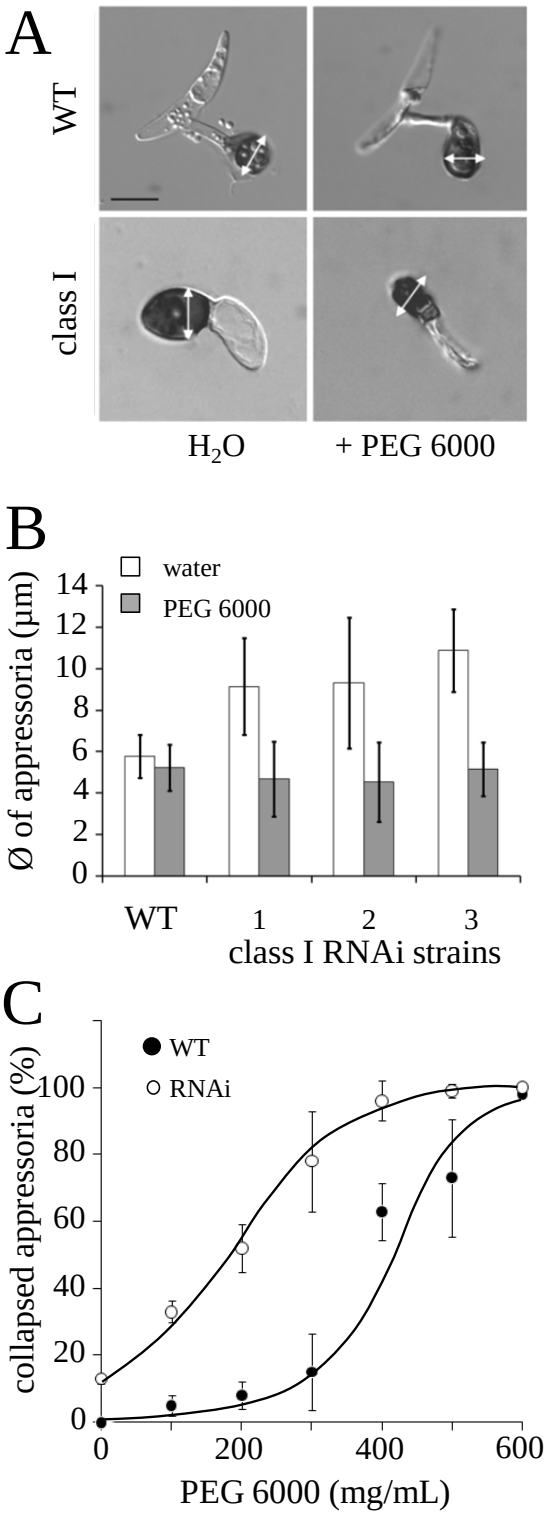

Fig. 19: Down-regulation of appressorial β-1,3-glucan contents increases cell wall elasticity and reduces turgor pressure

(A) Appressoria of the WT strain show comparable sizes in water and in the osmolyte PEG 6000 (400 mg/mL), as indicated by the double arrow. Appressoria of class I RNAi strains had larger diameters in water than in PEG 6000. Bar is 10 µm.

(B) Quantification of appressorial diameters of the WT and three class I RNAi strains in water and after addition of PEG 6000 (400 mg/mL). Three times 100 appressoria were measured.

(C) Incipient cytorrhizis indicates that the appressorial turgor pressure of class I RNAi strains is considerably lower than that of the WT strain. Three times 100 appressoria were counted. Bars in B and C represent ± standard deviations.

These data show that synthesis of β-1,3-glucan is indispensable for appressorial adhesion, cell wall rigidity and melanization, and for generation of turgor pressure.

III.5 *GLS1* is indispensable for appressorial penetration, development of necrotrophic hyphae and anthracnose disease symptoms in maize

In order to investigate the role of *GLS1* of *C. graminicola* in plant infection, conidia of the WT strain and of class I and class II RNAi strains were inoculated onto intact and wounded segments of the youngest fully expanded leaf of 2- to 3-week-old maize plants, and virulence was evaluated 7 days after inoculation (DAI). The WT strain caused disease symptoms on both intact and wounded leaves. In contrast, the RNAi strains were unable to invade intact leaves. On wounded leaves, however, the RNAi strains caused minor necroses at the margins of the wounds (Figure 20A). Fungal development assessed by quantitative PCR (qPCR), using internal transcribed spacer two (ITS2) primers (Behr *et al*., 2010), fully confirmed macroscopically observed disease development (Figure 20B). Thus, fungal virulence correlated with *GLS1* transcript abundance (compare Figures 13C and 20B).

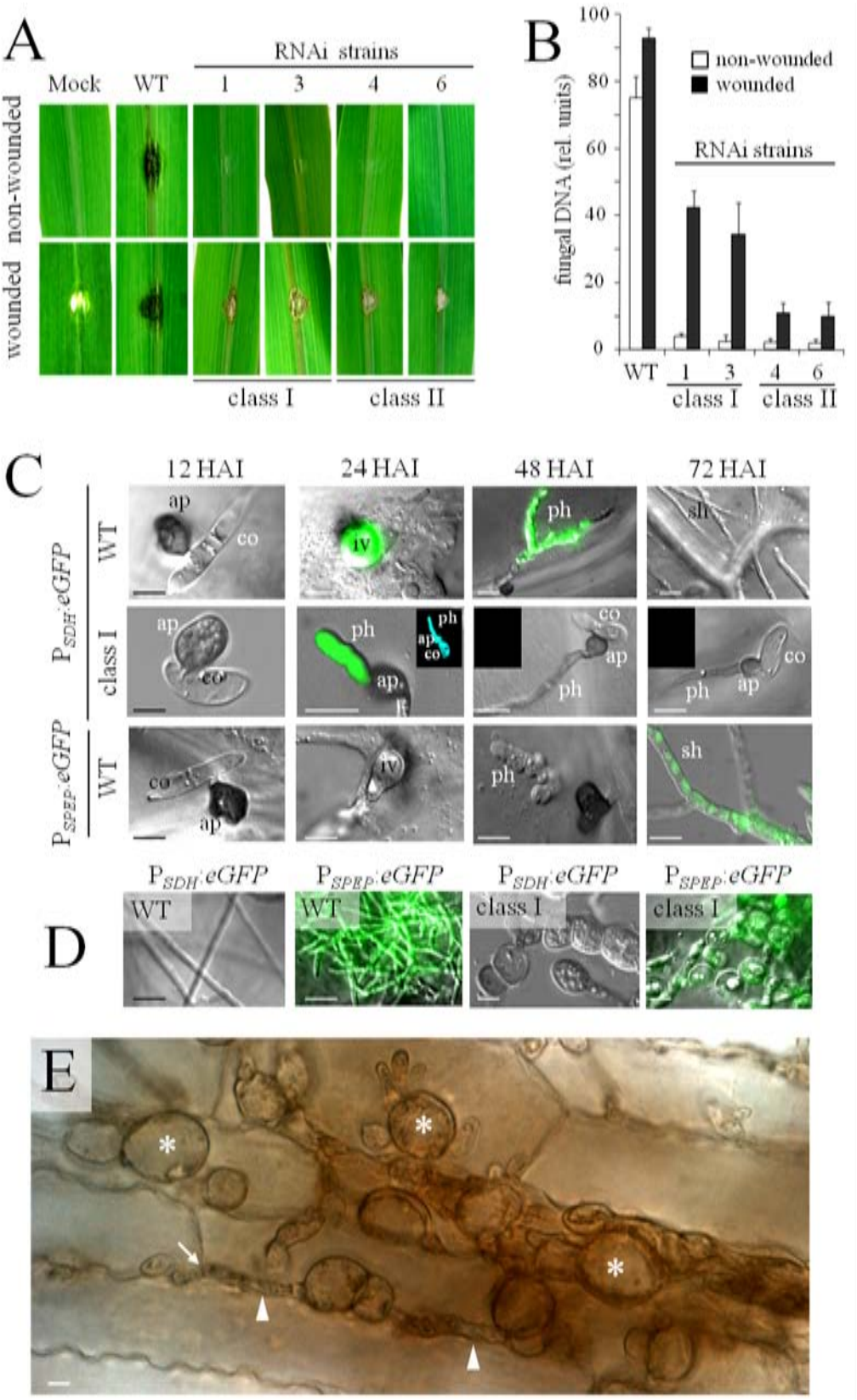

Fig. 20: RNAi strains of *C. graminicola* form severely distorted necrotrophic hyphae and are non-pathogenic on maize

(A) Disease symptoms on wounded and non-wounded maize leaves after inoculation with the WT and independent class I (1 and 3) and class II RNAi strains (4 and 6). Mock inoculated leaves were treated with 0.01% (v/v) Tween 20.

(B) Quantification of fungal development on intact or wounded maize leaves after inoculation with the strains used in (A). Three biological repeats, with two technical repeats each, were analyzed; bars represent standard deviations.

(C) Development of infection structures of WT and a class I RNAi strain expressing the *eGFP* gene under the control of the biotrophy-specific *SDH* promoter (P_{SDH}), and of the WT strain expressing the *eGFP* gene under the control of the necrotrophy-specific *SPEP* promoter (P_{SPEP}) on intact maize leaves. ap, appressorium; co, conidium; ph, primary hypha; sh, secondary hypha. Inserts in class I, 24 – 72 HAI show viability staining with fluorescein diacetate.

(D) Development of infection structures of the WT and a class I RNAi strain on wounded maize leaves. *eGFP* gene expression is under control of the biotrophy-specific *SDH* (P_{SDH}) or the necrotrophy-specific *SPEP* promoter (P_{SPEP}).

(E) Necrotrophic hyphae of a class I RNAi strain in a maize leaf after wound inoculation. Hyphae show severe swellings (asterisks), connected by narrow hyphae (arrowheads). Arrow indicates penetration point in an anticlinal plant cell wall. Bar in C – E are 10 µm.

In order to understand the role of GLS in the infection process of the hemibiotroph *C. graminicola*, differential interference contrast (DIC), in combination with eGFP-based fluorescence microscopy, was employed. Discrimination of biotrophic and necrotrophic infection structures *in planta* is usually based on morphology, but as morphology of infection hyphae may be severely compromised in RNAi strains exhibiting cell wall defects, additional morphology-independent criteria were needed.

Therefore, to construct biotrophy reporter strains, we took advantage of biotrophy-specific genes identified in a cDNA library prepared from intracellular biotrophic hyphae of the Arabidopsis pathogen *Colletotrichum higginsianum* (Takahara *et al.*, 2009). Based on the expression level in *C. higginsianum*, we selected nine homologous genes of *C. graminicola*, fused approx. 1.5 kb of their 5'-upstream regions, likely harboring their promoters, to the *eGFP* gene, transformed these constructs into the *C. graminicola* WT strain and analyzed the transformants for biotrophy-specific *eGFP* expression. Based on fluorescence intensities and biotrophy-specificity of eGFP expression, we selected the promoters of a saccharopine dehydrogenase gene (accession number EFQ35895) and of a gene encoding a NmrA-like

family protein (GLRG07923). A necrotrophy-specific gene encoding a secreted peptidase of the class of subtilases (*SPEP*; EFQ25677) had previously been identified in a YSST library of *C. graminicola* (Krijger *et al.*, 2008), and the promoter of this gene was used for necrotrophy-specific eGFP expression (Figure 21).

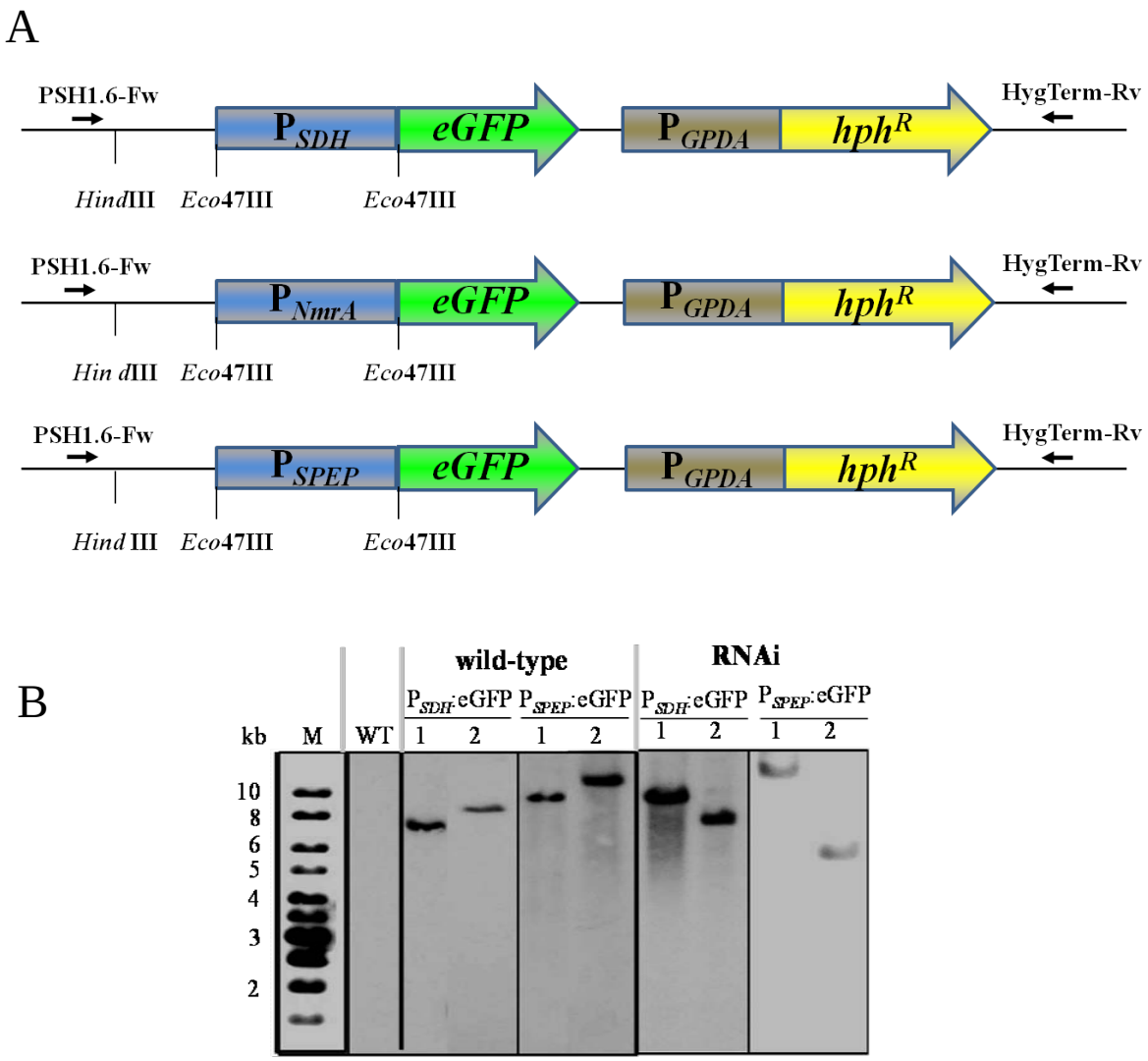

Fig. 21: Construction of biotrophy- and necrotrophy-specific promoter:*eGFP* fusions and confirmation of the integration of a single copy of the different constructs into the genome

(A) The biotrophy-specific promoters of the saccharopine dehydrogenase gene *SDH* (P_{SDH}) and of a gene encoding a NmrA-like family protein (P_{NmrA}), and of the necrotrophy-specific promoter of a gene encoding a secreted peptidase (P_{SPEP}) were fused with the *eGFP* gene. All constructs contained the hygromycin resistance gene (*hph*) driven by the glyceraldehydes-3-phosphate dehydrogenase promoter (P_{GPDA}) of *A. nidulans*. Arrows indicate primers used to amplify the constructs transformed into *C. graminicola* WT and class I RNAi strains.

(B) Southern blot of *Hind*III-digested DNA isolated from *C. graminicola* WT and independent class I RNAi strains harboring the biotrophy- or necrotrophy-specific promoter:eGFP constructs. Note that single copies of these constructs had integrated into the strains used in further experiments. Bars on Hygromycin resistance cassette (A) indicate probes used in Southern blot experiments. M, DNA ladder (marker); wild-type and RNAi, WT and RNAi strains harboring constructs as indicated; WT, non-transformed wild-type strain.

The promoter-*eGFP* fusions chosen were transformed into the WT and all class I RNAi strains, and morphology of infection structures and eGFP fluorescence were compared after inoculation onto non-wounded and wounded maize leaves (Figures 20C and D). On intact leaves, the WT strain had formed appressoria on the cuticle after 12 HAI, penetrated the epidermal cell wall by 24 HAI, and brightly fluorescing biotrophic infection vesicles had formed in the epidermal host cell, due to *eGFP* expression under control of the biotrophy-specific saccharopine dehydrogenase promoter (P_{SDH}) (Figure 20C, P_{SDH}:*eGFP*, WT, 24 HAI). Also primary hyphae fluoresced intensively (Figure 20C, P_{SDH}:*eGFP*, WT, 48 HAI). In necrotrophic secondary hyphae, no fluorescence was detected, and fluorescence in primary hyphae had ceased when necrotrophic hyphae had formed (Figure 20C, P_{SDH}:*eGFP*, WT, 72 HAI). In comparison, under control of the necrotrophy-specific promoter of the gene encoding a secreted peptidase (P_{SPEP}), eGFP fluorescence was only observed in fast growing necrotrophic hyphae (Figure 20C, P_{SPEP}:*eGFP*, WT, 72 HAI). Thus, using these promoters allowed discriminating biotrophic and necrotrophic hyphae on a morphology-independent basis.

Class I RNAi strains harboring the P_{SDH}:*eGFP* construct were allowed to form appressoria on intact leaves (Figure 20C, P_{SDH}:*eGFP*, class I, 12 HAI). As observed on artificial surfaces (Figure 18C), several appressoria of these strains developed voluminous hyphae on the cuticle of the host plant, reminiscent of biotrophic primary hyphae

(Figure 20C, P_{SDH}:*eGFP*, class I, 24 HAI). These hyphae fluoresced brightly, indicating that they indeed represent biotrophic hyphae. Viability staining with fluorescein diacetate (FDA) showed that on the cuticle biotrophic hyphae were viable at 24 HAI, but not at later time points after inoculation (Figure 20C, P_{SDH}:*eGFP*, class I, 24 – 72 HAI, inserts). These observations were confirmed by experiments using *eGFP* fusions with the second biotrophy-specific promoter, controlling the expression of an NmrA-like gene (Figure 22).

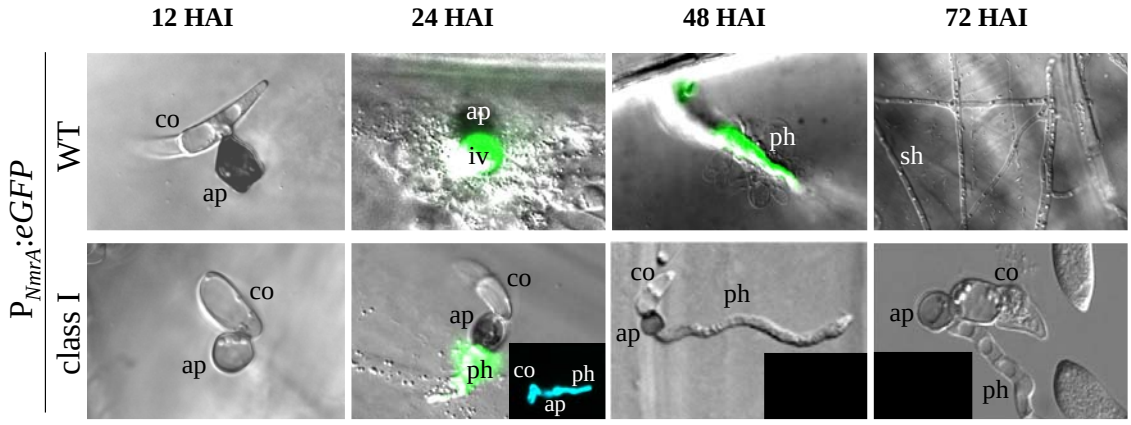

Fig. 22: Expression of the biotrophy-specific P_{NmrA}:*eGFP* reporter construct in the WT and in a class I RNAi strain of *C. graminicola*. ap, appressorium; co, conidium; HAI, hours after inoculation; iv, infection vesicle; ph, primary hypha; sh, secondary hypha. Insert at 24, 48, and 72 HAI show fluorescein diacetate-stained infection structures. Bars are 10 µm.

When the WT strain was inoculated onto wounded leaves, biotrophic hyphae were not observed, and fast growing thin necrotrophic hyphae formed immediately after wound inoculation (Figure 20D, WT). In agreement with hyphal morphology of the WT strain, fluorescence was observed in strains expressing eGFP under the control of the necrotrophy-specific (Figure 20D, P_{SPEP}:eGFP, WT), but not under the control of the biotrophy-specific promoter (Figure 20D, P_{SDH}:eGFP, WT). When class I RNAi strains were inoculated onto wounded leaves, hyphae with large swellings formed (Figure 20E, asterisks). These hyphae, in contrast to necrotrophic hyphae of the WT strain (Figure 20C, WT, 72 HAI), were strongly melanized and grew slowly, but were able to penetrate the anticlinal maize cell walls

(Figure 20E, arrow). The hyphal swellings observed in host cells were connected by thin hyphae (Figure 20E, arrowheads), suggesting that these hyphae are necrotrophic hyphae with severe cell wall distortions. Indeed, the distorted hyphae showed eGFP fluorescence under the control of the necrotrophy-specific promoter (Figure 20D, P_{SPEP}:*eGFP*, class I), but not of the biotrophy-specific *SDH* promoter (Figure 20D, P_{SDH}:eGFP, class I). Formation of necrotrophic hyphae by class I RNAi strains is in agreement with the necroses observed after inoculation of these strains onto wounded leaves (see Figure 20A, wounded, class I RNAi strains). Importantly, formation of necrotrophic hyphae is significantly affected if *GLS1* transcript abundance is reduced by only ~ 40% (see Figure 13C).

III.6 Forced expression of *GLS1* during biotrophic development triggers defense responses in maize and reduced virulence

The profiles of *GLS1* expression and synthesis of β-1,3-glucan (Figure 15) suggest that exposition of β-1,3-glucan must be avoided during biotrophic growth of *C. graminicola* in order to evade β-1,3-glucan-triggered immunity. To test this hypothesis, two different approaches were taken. First, the *GLS1* promoter (P_{GLS1}) was replaced by either of the two constitutively active promoters, i.e. the *trpC* promoter (P_{trpC}) of *Aspergillus nidulans* (Pöggeler *et al.*, 2003) or the *toxB* promoter (P_{toxB}) of the wheat pathogen *Pyrenophora tritici-repentis* (Andrie *et al.*, 2005). Second, the *GLS1* gene fused to P_{trpC} or P_{toxB} was ectopically integrated into the genome of *C. graminicola* (Figure 23A-C). Infection assays performed with both, the promoter exchange strains and the strains harboring an additional ectopic copy of *GLS1*, showed that these strains were severely impaired in virulence (Figure 24A). As both promoter exchange strains showed lower vegetative growth rates than the WT strain (Figure 23D), likely due to the fact that the promoter activity of P_{GLS1} is stronger than that of P_{trpC} and P_{toxB}, the infection process was studied in detail with the strains harboring an ectopic *GLS1* copy controlled by P_{trpC}. All strains used for infection assays also harbored the P_{SDH}:*eGFP* biotrophy reporter construct, allowing to visualize biotrophic development and to demonstrate that β-1,3-glucan is indeed synthesized in biotrophic infection structures. Interestingly, the diameters of biotrophic infection structures

formed by two independent transformants carrying the ectopically integrated P_{trpC}:*GLS1* construct were significantly thinner (P_{trpC}:*GLS1*-1: 5.1±1.9 and P_{trpC}:*GLS1*-2: 4.2±2.4 µm) than those of the WT strain (8.4±3.3 µm) (Figure 24B, WT and P_{trpC}:*GLS1*, P_{SDH}:*eGFP*, arrowheads). Necrotrophic hyphae of the WT strain had a diameter of 3.8±2.3 µm, which is statistically not different ($p < 0.05$) from the diameter of the biotrophic hyphae of the P_{trpC}:*GLS1* strains. Aniline Blue Fluorochrome staining clearly showed that β-1,3-glucan was exposed on the surface of biotrophic hyphae of the P_{trpC}:*GLS1* strains, but not on those of the WT strain (Figure 24B, biotrophy, WT and P_{trpC}:*GLS1*, Aniline blue, arrowheads; Figure 24C, WT and P_{trpC}:*GLS1* light blue bars). Also, necrotrophic hyphae of the P_{trpC}:*GLS1* strains showed stronger Aniline blue fluorescence than necrotrophic hyphae of the WT strain (Figure 24B, necrotrophy, WT and P_{trpC}:*GLS1*, Aniline blue; Figure 24C, WT and P_{trpC}:*GLS1* dark blue bars). Importantly, host cells attacked by appressoria of the *C. graminicola* WT strain only rarely exhibited defense responses, indicating effective evasion of recognition. In contrast, P_{trpC}:*GLS1* strains triggered whole cell and/or cell wall fluorescence in 30 – 40% of the host cells decorated by a single appressorium, as compared with only ~2% of those attacked by single WT appressoria (Figures 24D and E, compare WT and P_{trpC}:*GLS1*). Furthermore, while hyphae of the WT strain invaded living host cells without elicitation of formation of infection-related brown vesicles in the plant (Vargas *et al.*, 2012), massive formation of vesicles was observed in cells colonized by the P_{trpC}:*GLS1* strains (Figure 24F, compare WT and P_{trpC}:*GLS1*). Vesicles formed in the cytoplasm were initially colorless (Figure 24F, P_{trpC}:*GLS1*, arrowhead), but turned dark brown (Figure 24F, P_{trpC}:*GLS1*, insert) and eventually decorated the hypha completely (Figure 24F, P_{trpC}:*GLS1*, arrow).

In conclusion, down-regulation of *GLS1* expression and avoidance of surface exposure of β-1,3-glucan in *C. graminicola* WT leads to reduction of cell wall rigidity and thus formation of voluminous biotrophic hyphae, but is required to evade β-1,3-glucan-triggered immunity in maize.

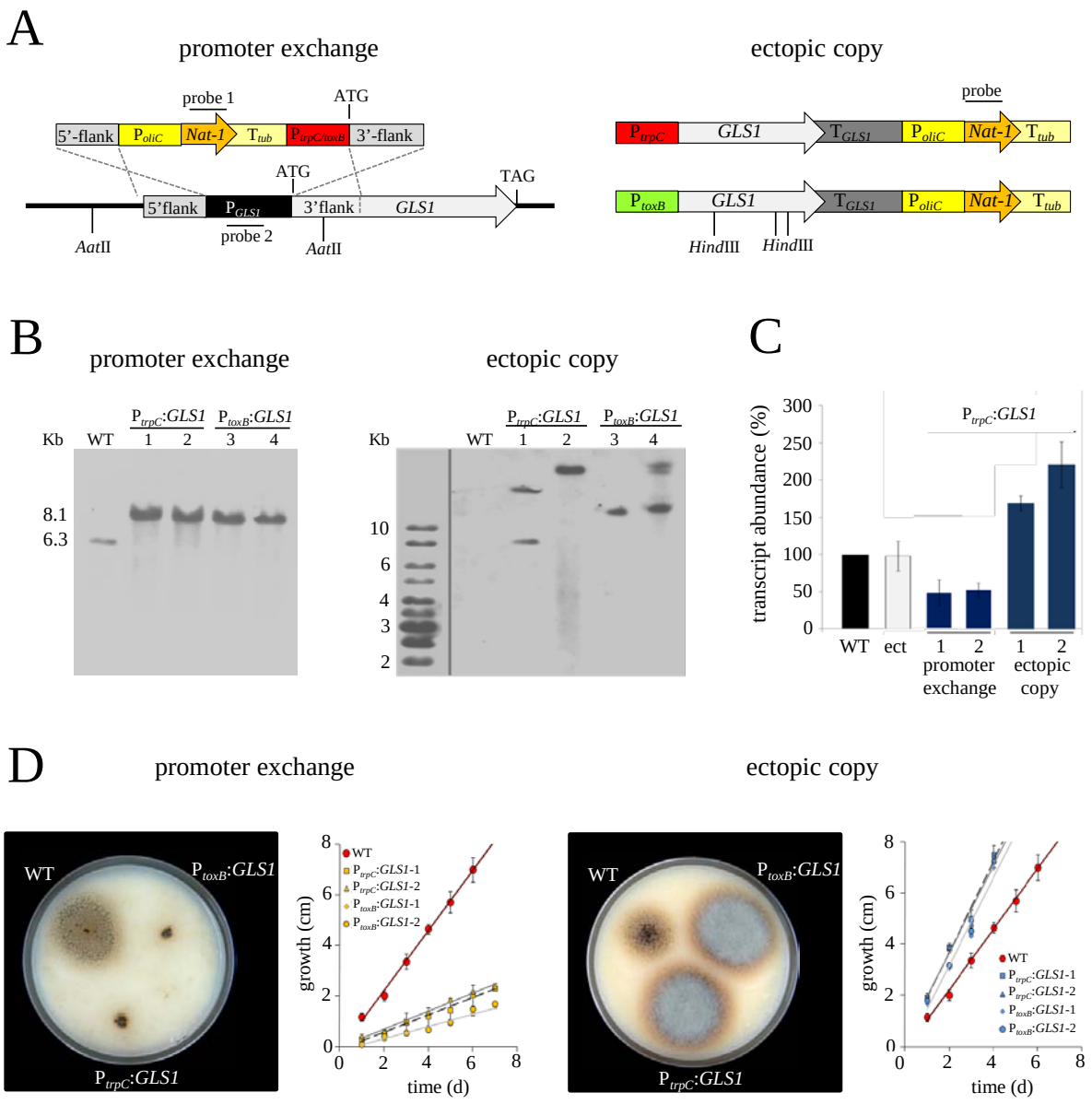

Fig. 23: Forced expression of *GLS1* by promoter exchange and ectopic introduction of an additional copy of *GLS1* controlled by constitutive promoters, and comparison of vegetative development of the WT strain and transformants

(A) Strategies for promoter exchange and introduction of an additional copy of *GLS1* controlled by constitutive promoters. Promoter exchange constructs consisted of the Nourseothricin resistance gene *Nat-1* driven by the *oliC* promoter of *A. nidulans*, and either the constitutive *trpC* or *toxB* promoter of *A. nidulans* or *P. tritici-repentis*, respectively. The construct was flanked by the 5'-non-coding region (5'-flank) and the 5'-coding region (3'-flank) of the *GLS1* gene. The entire construct was PCR-amplified and transformed into the WT strain of *C. graminicola*. Alternatively, P_{trpC}:*GLS1* or P_{toxB}:*GLS1* fusions ligated to the *Nat-1* gene, lacking homologous flanks, were transformed into the *C. graminicola* WT strain to yield strains with an ectopic *GLS1*

copy. Bars indicate probes used in Southern blot experiments. P_{toxB}, toxB promoter; P_{trpC}, trpC promoter; P_{GLS1}, *GLS1* promoter from *Colletotrichum graminicola.*

(B) Southern blots of the *C. graminicola* WT strain and transformants with homologous integration of the *GLS1* promoter exchange construct (promoter exchange) and transformants carrying ectopic integrations of the *GLS1* overexpression construct (ectopic copy). DNA of promoter exchange strains was digested with *AatII*, DNA of strains carrying an ectopic copy of *GLS1*were *Hind*III-digested. The blot was hybridized with both probes indicated in Supplemental Figure 9A.

(C) *GLS1* transcript abundance in vegetative hyphae of the *C. graminicola* WT strain, a strain carrying an ectopic copy of the promoter exchange construct, and of two independent transformants, carrying either a homologously integrated promoter exchange construct (promoter exchange) or an ectopic copy of *GLS1*. The promoter exchange strains and strains harboring an ectopic copy of *GLS1* used in transcript abundance measurement expressed *GLS1* under the control of P_{trpC}. In all qRT-PCR experiments, three biological replicates and two technical repeats were used.

(D) Morphology of colonies and radial growth rates of *C. graminicola* WT strain and of P_{toxB}:*GLS1* and P_{trpC}:*GLS1* promoter exchange strains, and of strains harboring an ectopically integrated copy of strain and of P_{toxB}:*GLS1* and P_{trpC}:*GLS1*. Growth assays were performed on oat-meal agar plates. Red circles, growth rates of the WT strain; yellow symbols, growth rates of two independent P_{toxB}:*GLS1* and P_{trpC}:*GLS1* promoter exchange strains; blue symbols, growth rates of strains harboring ectopically integrated P_{toxB}:*GLS1* and P_{trpC}:*GLS1* constructs.

Bars in C and D represent ± standard deviations.

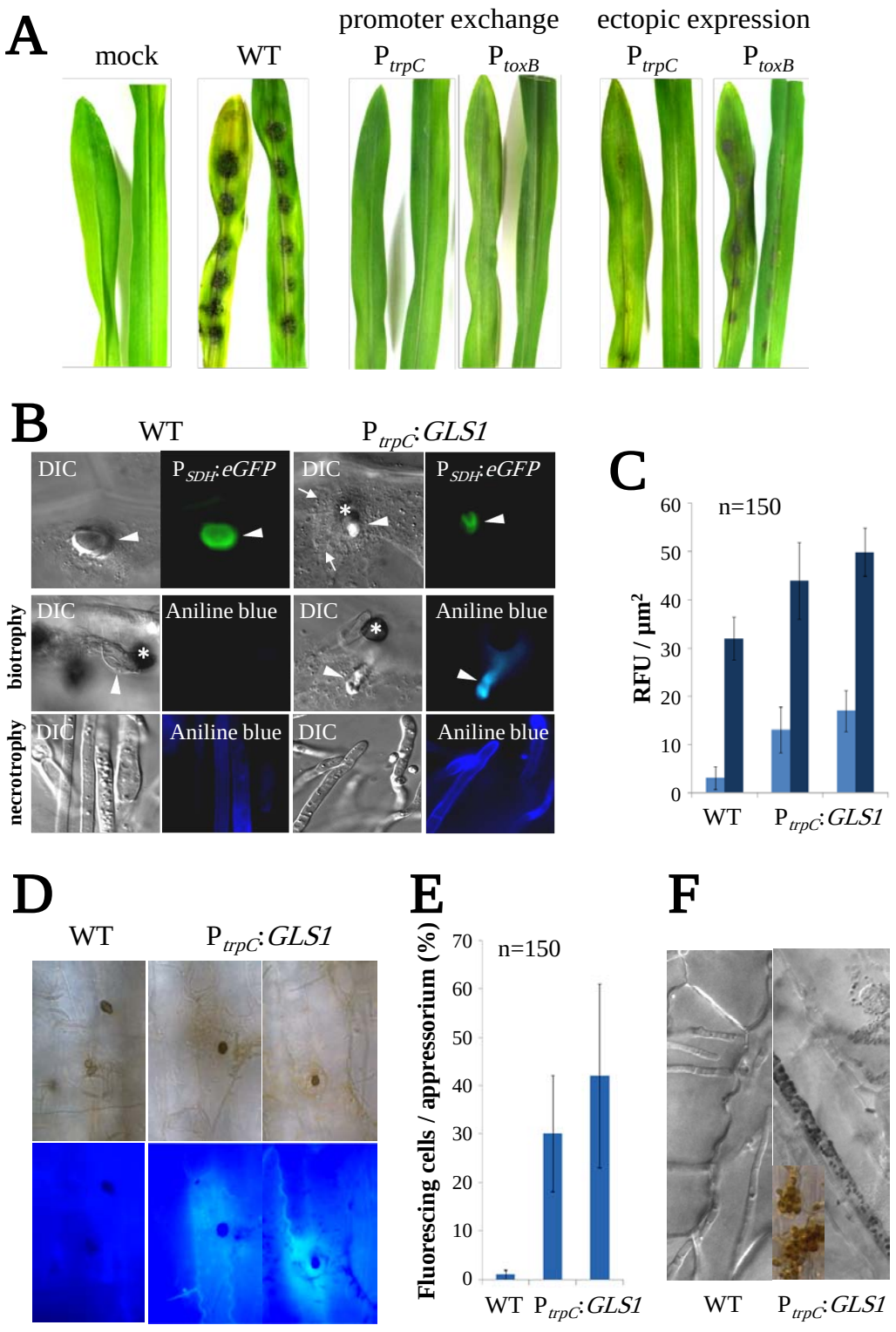

Fig. 24: Forced expression of *GLS1* in biotrophic hyphae of *C. graminicola* induces defense responses in maize and causes reduced fungal virulence

(A) Disease symptoms on non-wounded maize leaves after inoculation with the WT, strains with the *GLS1* promoter exchanged by the *trpC* or *toxB* promoter (P_{trpC}; P_{toxB}) of *A. nidulans* or *P. tritici-repentis*, respectively, and strains harboring a single ectopically integrated extra copy of *GLS1* controlled by P_{trpC} or P_{toxB}. Mock inoculated leaves were treated with 0.01% (v/v) Tween 20.

(B) Comparison of biotrophic infection vesicles (top panel, arrowhead) formed by the WT strain and strains harboring an ectopically integrated *GLS1* copy controlled by P_{trpC} indicated that forced expression of *GLS1* led to reduction of hyphal diameters. Both, WT and P_{trpC}:*GLS1* strains carried a P_{SDH}:*eGFP* construct confirming biotrophic lifestyle of fluorescing structures. While aniline blue fluorescence indicated that β-1,3-glucan was present in biotrophic hyphae of the P_{trpC}:*GLS1* strain (biotrophy; aniline blue; P_{trpC}:*GLS1*; arrowhead), no fluorescence was visible in the WT strain (biotrophy; aniline blue; WT; arrowhead). Necrotrophic hyphae of both, WT and P_{trpC}:*GLS1* strain showed aniline blue fluorescence (necrotrophy; aniline blue; WT and P_{trpC}:*GLS1*; arrowhead). DIC, differential interference contrast micrographs. Bars are 10 µm.

(C) Quantification of aniline blue fluorescence in biotrophic (light blue) and necrotrophic hyphae (dark blue) of the WT and two independent P_{trpC}:*GLS1* strains. Three times 100 measurements have been performed with each strain; bars are ± standard deviations.

(D) Both WT and P_{trpC}:*GLS1* strains differentiated melanized appressoria on maize intact leaves (top panel; arrowheads), but only the P_{trpC}:*GLS1* strains caused whole cell (white arrows) or cell wall fluorescence (white arrowheads) in maize under UV light, indicative of defense responses.

(E) Quantification of fluorescing maize cells decorated with single appressoria of the WT or P_{trpC}:*GLS1* strains. Bars represent ± standard deviations.

(F) Maize cells infected by hyphae of P_{trpC}:*GLS1* strains formed vesicles (arrowhead) that turned dark brown (insert), and densely decorated the invading hyphae (arrow). Maize cells infected by WT hyphae rarely showed vesicles.

III.7 Deletion of the *RHO1* gene of *C. graminicola* mimics the knock-down of the *GLS1* gene with respect to hyper-melanization, hyphal cell wall integrity, growth rates and sporulation

To functionally characterize the *RHO1* gene, encoding a regulatory protein of β-1,3-glucan synthase (GLS1), mutants were generated in which the entire *RHO1* gene was replaced by the nourseotrycin acetyltransferase gene (*Nat-1*^{R}) from *Streptomyces noursei*. The *RHO1* deletion vector consisted of 980 bp of the *RHO1* 5'-flank, 913 bp of the *RHO1* 3'-flank and 2164 bp of the *Nat-1* cassette (Figure 25A). The construct was transformed into the *C. graminicola* WT strain M2.

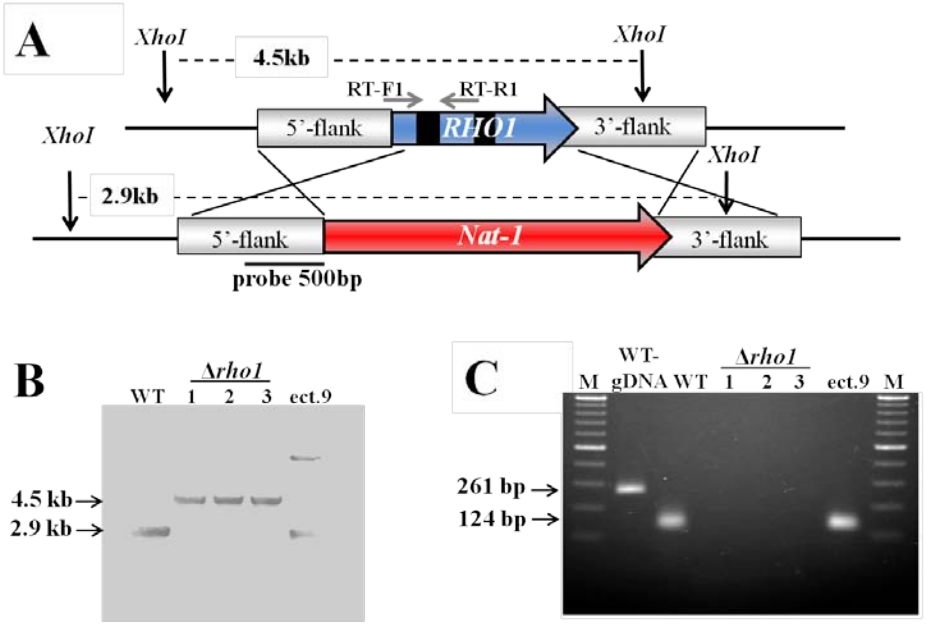

Fig. 25: Deletion of *RHO1* and discrimination between transformants with homologous and ectopic integration of the deletion cassette

(**A)** Deletion strategy of *RHO1* of *C. graminicola*. Introns are drawn in black. Black arrows indicate *Xho*I restriction sites, small grey arrows indicate the RT-PCR primers, bar indicates probe used in the Souther blot analysis. RT-F1, CgRho1RT-PCR-Fw; RT-R1, CgRho1RT-PCR-Rv. Not to scale.

(**B)** Southern blot analysis showing homologous or ectopic integration of the deletion construct.

(**C)** RT-PCR confirming the absence of *RHO1* transcripts in the Δ*rho1* mutants.

M, DNA ladder (marker), **WT**, wild-type; **1-3**, three independent Δ*rho1* deletion mutants; **ect.9**, transformant with ectopic integration of the deletion cassette.

Southern blot analyses were performed with *Xho*I-digested DNA from the wild-type CgM2 and different independent transformants (Figure 25A and B). A 500-bp probe corresponding to the 5'-flank was generated using the primers CgRhoIprobe-Fw and CgRhoIprobe-Rv. The Southern blot indicated three transformants with a single homologous integration of the deletion cassette into the genome (Figure 25B). The absence of the *RHO1* gene in the Δ*rho1* mutants was also confirmed by RT-PCR (Figure 25C).

As compared with the WT strain, the Δ*rho1* strains showed a strongly altered colony phenotype and formed small compact colonies with increased pigmentation (Figure 26A). Ectopic transformants were comparable with the WT (Figure 26A).

In order to investigate the role of *RHO1* of *C. graminicola* in the vegetative growth, growth assays were performed with the WT, the ectopic transformant and idependent Δ*rho1* strains on PDA (Figure 26B). In the absence of osmotical stabilization, all three Δ*rho1* strains exhibited poor growth rates (Figure 26B).

Fungal growth rates and the ability to invade solid substrata are usually tightly correlated. Penetration assay were performed on PDA containing 0.15 M KCl with different concentrations of agar (Figure 26C). In all agar concentrations the Δ*rho1* strains exhibited penetration rates close to zero. In contrast, the WT strain and the ectopic strain showed best penetration rates at agar concentrations of 1 and 2% (w/v), and were even able to invade PDA containing 4% (w/v) agar (Figure 26C).

Microscopical analyses of vegetative hyphae were in PD media containing osmotic stabilization (0.15 M KCl). While the WT strain developed normal filaments (Figure 26D), the three independent Δ*rho1* strains exhibited vegetative hyphae with severe hyphal swellings (Figure 26E, arrowheads) and pronounced cell wall distortions (Figure 26F asterisk). Many of these swellings of Δ*rho1* hyphae were melanized (Figure 26E, insert arrowheads, and F, arrow). These observations clearly indicate that the Δ*rho1* deletion strains and the RNAi (*GLS1*) strains were comparable with respect to cell wall defects and melanization (see Figure 13 D-E; G-J). Thus, the hyphal defects of Δ*rho1* mutants are likely due to reduced β-1,3-glucan synthase activity.

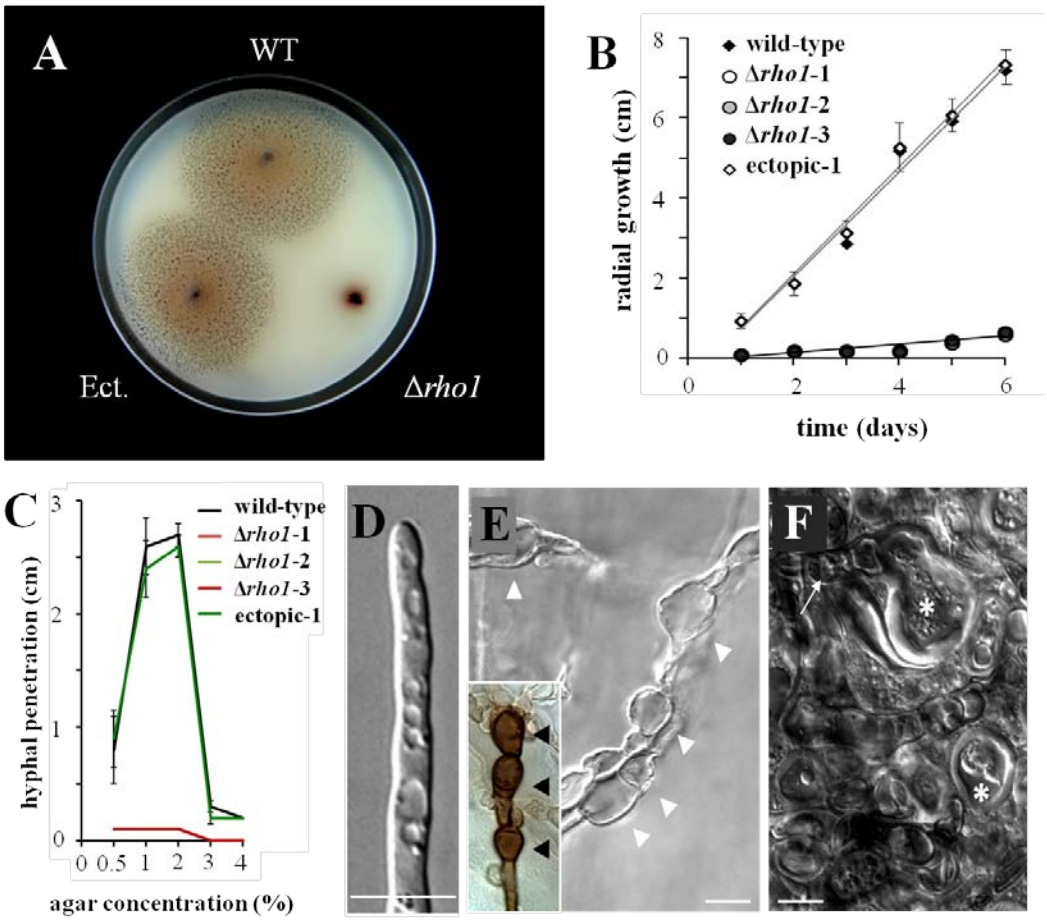

Fig. 26: Morphology of colonies, growth rates, agar penetration and phenotype of vegetative hyphae of *C. graminicola* WT, ectopic and Δ*rho1* strains

(A) Morphology of colonies of WT, ectopic and a *ΔrhoI* strain on OMA supplemented with 0.15 M KCl. Picture was taken 12 DAI.

(B) Radial growth of *C. graminicola* WT, ectopic and three Δ*rhoI* strains on Potato-Dextrose-Agar (PDA).

(C) Penetration of *C. graminicola* WT, ectopic and three Δ*rho1* strains in PDA medium suplemented with different concentrations of agar-agar.

Bars in B – C are ± standard deviations.

(D) Vegetative hyphae of the WT strain.

(E) Vegetative hypha of a Δ*rho1* strains showing hyphal swellings (arrowheads). Insert shows pigmentation of swellings (arrowheads).

(F) Severely distorted vegetative hyphae of a Δ*rho1* strain. The mycelium consists of swellings (asterisks), some of which are strongly melanized (arrow).

Bars in D – F are 10 µm.

In order to confirm reduced β-1,3-glucan contents in hyphae of the Δ*rho1* strains, aniline blue fluorocrome staining was performed (Figure 27). In comparison with the reference strain CgM2, the three Δ*rho1* strains showed a drastic reduction of fluorecence intensity of the cell wall.

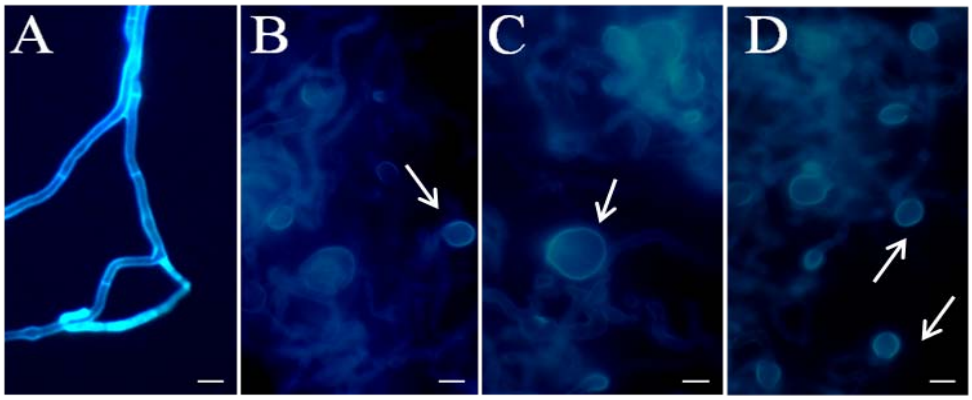

Fig. 27: The β-1,3-glucan contents are reduced in hyphae of Δ*rho1* strains of *C. graminicola*

β-1,3-glucan contents, as indicated by fluorescence, in vegetative hyphae of the WT (A) and three idependent Δ*rho1* strains (B-D). Hyphae of Δ*rho1* strains shoed severe swellings (arrows). Hyphae were stained by Aniline Blue Fluorochrome. Bars are 10 µm.

In order to investigate the role of *RHO1* of *C. graminicola* in the formation of asexual spores, sporulation assays were performed with the WT strain, an ectopic strain and three idependent Δ*rho1* strains in different osmotically stabilized OMA or CM medium. All Δ*rho1* strains were unable to sporulate, independently of osmotic stabilization (Fig.29A-B). Moreover, the sporulation defects of Δ*rho1* strains were quantitatively comparable with those of class III RNAi (*GLS1*) strains, in which the the *GLS1* transcript levels were reduce to approximately 17-25% (Figure 13C).

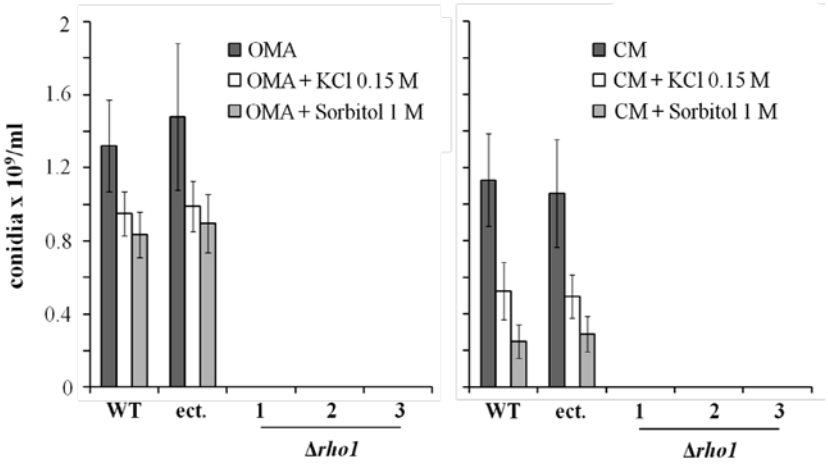

Fig. 28: Δ*rho1* strains are unable to produce asexual spores

(A-B) Sporulation on OMA **(A)** and liquid CM medium **(B)** with or without osmotic stabilization (0.15 M KCl; 1 M sorbitol). The three independent Δ*rho1* strains were unable to form conidia, indepent of osmotical stabilization. The WT strain and the ectopic strain showed reduction of sporulation levels under osmotic stabilization. WT, wild-type; ect., ectopic strain; Δ*rho1* 1-3, three independent Δ*rho1* strains.

Bars in A-B represent standard deviations.

In order to investigate the role of *RHOI* of *C. graminicola* in maize infection, mycelia of the WT strain M2, the ectopic strain and three independent Δ*rhoI* strains were inoculated onto intact and wounded segments of the 2nd and 3rd youngest fully expanded leaf of 2- to 3-week-old maize plants, and virulence was evaluated 7 DAI. The WT strain and the ectopic strain caused anthracnose symptoms on both intact and wounded leaves (Figure 30A). In contrast, the three Δ*rhoI* strains were non-pathogenic in both wounded and non-wounded leaves (Figure 30A).

Microscopy of the infection sites was done 7 DAI. The WT strain showed formation of tubular destructive hyphae, which penetrated anticlinal cell walls (Figure 30B). On wounded and non-wounded leaves, hyphae of Δ*rho1* strains were unable to colonize the host tissue and only the mycelia used for inoculation has been observed on the leaf surface (Figure 30C). Large hyphal swellings ussually occurred along hyphae at irregular intervals (Figure 30C). In contrast with the RNAi (*GLS1*) strains (Figure 20E), the hyphae were restricted to the external part of the leaves (Figure 30C).

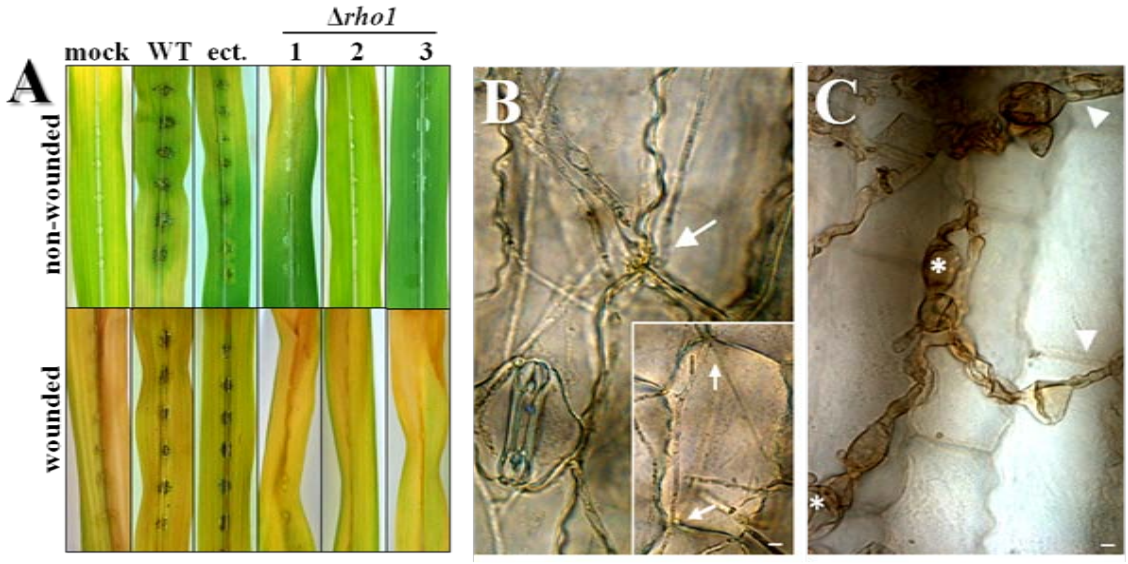

Fig. 29: Δ*rho1* strains are nonpathogenic on wounded and non-wounded leaves

(A) Disease symptoms on wounded and non-wounded maize leaves after inoculation with the wild-type strain M2, the ectopic strain and three independent Δ*rho1* strains. Mock, mock-treated control; WT, wild-type; ect, ectopic transformant; Δ*rho1* 1-3, three independent Δ*rho1* strains.

(B) The WT strain shows thin tubular hyphae penetrating anticlinal plant cell walls (arrows).

(C) Growth of hyphae of Δ*rho1* strains on a maize leaf after wound inoculation. Hyphae show severe swellings (asterisks), connected by narrow hyphae (arrowheads).

Bars in B-C are 10 µm.

IV Discussion

In filamentous fungi, β-linked polysaccharides represent the most prominent fraction of polymers, with glucose polymers being most abundant and widely distributed. Different β-linked polyglucans, i.e. linear β-1,3-glucans, β-1,3-glucans with β-1,6- and β-1,4-linkages, and β-1,6-linked glucans have been identified (Ruiz-Herrera, 1991). Indeed, chemical analysis of the *C. graminicola* WT strain M2 revealed that the cell wall contains 59.7% (w/w) glucose (Werner *et al*., 2002), suggesting that β-linked glucans polymers play an important structural role in vegetative hyphae. β-1,3-glucan chains form covalent β-1,4-bonds with chitin chains, leading to a three-dimensional network, which is not only responsible for the structural integrity, but also for the shape of the cell. Other polymers, i.e. homopolymeric α-1,3-glucan and glycosylated proteins are attached to this β-linked-glucan-chitin core, modifying the physico-chemical properties of the cell wall (Bowman *et al*., 2006; Latgé, 2007; Walker *et al*, 2008; Kollár *et al*., 1995). During hyphal growth a delicate balance between sub-apical rigidity and apical plasticity is mediated by tight regulation of enzymes involved in cell wall biogenesis (Sietsma *et al*., 1985; Walker *et al*., 2008; Cabib *et al*., 1998; Drgonová *et al*., 1996 ; Wessels, 1993).

Due to the essential nature of single copy GLS genes, targeted deletion is not feasible in filamentous fungi harboring a haploid genome (Figures 8A and 12).

We have taken advantage of RNAi interference in combination with *GLS*:*eGFP* fusion and β-1,3-glucan-specific staining to evaluate the role of GLS of the maize pathogen *C. graminicola* in vegetative development and, for the first time, analyzed the importance of this enzyme during differentiation of infection structures on the plant surface and *in planta*.

IV.1 Role of *GLS1* in vegetative growth

Genetic evidence underlining the importance of GLS has been presented in some fungi. In *N. crassa*, an antisense construct has been used to reduce GLS formation, resulting in reduced growth rates and swellings of the hyphal tips (Tentler *et al*., 1997). In *A. fumigatus*, an air-born fungus infecting immunocompromised patients, the *fks1* gene was disrupted in a diploid by homologous recombination. Subsequent haploidization did not yield transformants

with a disrupted *fks1* gene, suggesting that *fks1* is an essential gene in *A. fumigatus* (Firon *et al*., 2002). These findings have been confirmed by in an elegant study utilizing an RNAi approach (Mouyna *et al*., 2004). Likewise, in *F. solani*, a plant pathogen that can also infect immunocompromised humans, RNAi approaches have shown that GLS is indispensable for hyphal growth (Ha *et al*., 2006).

The genome of the maize pathogen *C. graminicola*, like that of the vast majority of filamentous fungi, contains a single *GLS* gene denominated *GLS1* (Figure 8A). We established RNAi in *C. graminicola* and obtained nine transformants, falling into three classes, according to their levels of *GLS1* transcript abundance (Figure 13C). Stepwise reduced *GLS1* transcript abundance correlated with the severity of cell wall and growth defects in vegetative hyphae. Interestingly, hyphae of class I strains formed strongly pigmented swellings (Figure 13H), and lysis has been observed in rare cases, comparable to that observed in hyphae of *F. solani* expressing an RNAi construct (Ha *et al*., 2006). These data are in agreement with those reported for other filamentous fungi and indicate that *GLS1* of *C. graminicola* is indispensable for vegetative development. Interestingly, melanization is massively de-regulated in vegetative hyphae of RNAi strains of *C. graminicola*. In contrast to germ tubes and vegetative hyphae of the WT strain, germinating spores of class II RNAi strains produced large amounts of melanin and secreted it into the growth medium (Figure 18E), and hyphal swellings of class I RNAi strains were melanized as well (Figure 13H). The hyper-melanization of hyphal swellings and the formation of brown colonies on solid medium were also observe in Δ*rho1* strains (Figures 26E), which confirmed the *GLS1* knockdown phenotype. Increased melanization has also been observed after deletion of genes encoding α-1,3-glucan and chitin synthase in the human pathogens *A. fumigatus* and *W. dermatitidis* (Liu *et al*., 2004; Maubon *et al.*, 2006). In the human pathogen *W. dermatitidis* melanization of cell walls indeed increases cell wall rigidity, as indicated by stab inoculation experiments testing for competence of invasive growth (Brush and Money, 1999). The loss of melanin from the cell wall of RNAi strains of *C. graminicola* reported here suggests that an intact β-1,3-glucan polymer network is essential for incorporation and linking melanin to the cell wall.

IV.2 *GLS1* is indispensable for adhesion of infection structures, appressorial cell wall rigidity and for invasion of intact host leaves

Adhesion of infection cells formed on the plant surface is essential for penetration, and this is particularly true for fungi that form appressoria to forcefully penetrate their host (Nicholson and Epstein, 1991). In *Colletotrichum* species, glycoproteins required for adhesion have been identified in the appressorial mucilage (Hutchison *et al*., 2002). Interestingly, conidia of class I RNAi strains were able to form appressoria on hard artificial surfaces such as glass or polyester and on the cuticle of the alternative host onion, but in contrast to the WT strain, these appressoria were unable to adhere (Figure 18G). Through an ester bond between a deaminated glutamine residue in internal repeats, some fungal cell wall proteins are covalently linked to a hydroxyl group of a β-1,3-glucan chain (De Groot *et al*., 2005; Ecker *et al*., 2006), and β-1,3-glucan may similarly be linked to adhesive glycoproteins. As breaching of cuticle and cell wall and directed growth into the epidermal cell wall requires tight sealing of the infection cell (Mercure *et al*., 1994; Mercure *et al*., 1995; Howard *et al*., 1991a,b; Howard *et al*., 1996), compromised adhesion may lead to lifting of the appressorium from the substratum and growth of the primary hypha on the surface of the underlying substratum. Indeed, on both polyester and onion epidermis (Figures 18G) appressoria of class I strains were unable to penetrate the host, likely due to lack of adhesion, and primary hyphae formed on the surface of the substratum. Failure of directed invasive growth into the host tissue, due to compromised adhesion, is fully sufficient to explain non-pathogenicity of class I RNAi strains on intact leaves (Figures 13, 18, 19 and 20).

Appressoria of class I RNAi strains swell in media that were not osmotically stabilized (Figures 19A and B) and exploded (Figure 18B). Unexpectedly, when adding an osmolyte (PEG 6000) to those enlarged appressoria that had not exploded, their diameter shrunk to approx. half of the size, clearly showing the enormous elasticity of appressorial cell walls of class I RNAi strains. Appressoria of class I RNAi strains were unable to integrate melanin adequately into their cell walls, and often melanin formed a ring surrounding the appressorium of class I RNAi strains or a massive precipitate on the underlying substratum in the vicinity of germ tubes of class II RNAi strains (see Figure 18C, insert and Figure 18E). Insufficient integration and cross-linking of melanin in the appressorial wall is unlikely to

affect lack of rigidity, as melanin-deficient mutants of *C. graminicola* (Horbach *et al*., 2009), *C. lagenarium* (Kubo *et al*., 1991) and *M. oryzae* (Chida and Sisler, 1987) and did not show increased elasticity. Also for chitin synthase deficient mutants increased elasticity of appressorial cell walls has not been reported (Madrid *et al*., 2003; Weber *et al*., 2006; Werner *et al*., 2007). However, in Δ*chsV* mutants of *C. graminicola*, appressorial initials developed normally, but cell walls lysed when appressoria matured, and hyphopodia showed increased sizes (Werner *et al*., 2007). The data shown here clearly demonstrate that the *GLS* gene of *C. graminicola* is indispensable for differentiation of rigid and functional appressoria. As these infection cells generate enormous turgor pressure of approx. 5.5 MPa (55 bar) (Bastmeyer *et al*., 2002; Bechinger *et al*., 1999) it is not surprising that β-1,3-glucan is a prominent polymer in appressorial cell walls, as shown by quantification of *GLS1*:*eGFP* expression and aniline blue staining (Figures 15).

IV.3 Differential roles of *GLS1* in biotrophic and necrotrophic infection structures

Integrity and composition of the cell wall must constantly be monitored during growth and morphogenesis (Bowman and Free, 2006; Latgé, 2007; Walker *et al*., 2008), and this is especially true in plant pathogenic fungi, which differentiate highly specialized infection structures before and after penetration into the plant cell. Indeed, staining experiments with polymer-specific dyes, lectin- and immunocytochemistry have shown changes in cell wall composition in *in planta* differentiated infection structures of several plant pathogenic fungi (Freytag and Mendgen, 1991; Fujikawa *et al*, 2009; Mendgen and Deising, 1993; O'Connell *et al*., 1996), and the cell wall modifications occurring after appressorial penetration may represent a mechanism of adaptation to the new environment in the plant. In *C. graminicola*, lectin- and immunocytochemistry revealed that chitin is converted to chitosan on the surface of invading biotrophic hyphae (El Gueddari *et al*., 2002), and similar results were reported in biotrophic rust and smut fungi, in the rice blast fungus *M. oryzae*, and in the human pathogen *C. neoformans* (Baker *et al*., 2007; El Gueddari *et al*., 2002; Fujikawa *et al*., 2009; Treitschke *et al*., 2010). Interestingly, in the rice blast fungus chitin and β-1,3-glucan appear to be masked by α-1,3-glucan appositions. At 24 HAI, when infectious hyphae had developed in rice sheath cells, transcript levels of the *GLS* gene, *FKS1*, increased massively and decrease

during further *in planta* development of the pathogen (Fujikawa *et al*., 2009). The study by Fujikawa *et al*. (2009) clearly demonstrates that cell wall composition is morphogenetically regulated, but unfortunately biotrophic and necrotrophic infection structures differentiated *in planta* have not been distinguished. In contrast to the rice blast fungus, β-1,3-glucan synthesis is clearly reduced in biotrophic infection structures of *C. graminicola*, as indicated by (i) integrity of biotrophic primary hyphae in the absence of osmotic stabilization, (ii) low levels of a *GLS1*:*eGFP* fusion protein expressed under the control of the native *GLS1* promoter, and (iii) weak staining by the fluorescence-labeled low molecular weight β-1,3-glucan-specific dye aniline blue. Studies suggest that β-1,3-glucan may serve as MAMPs and trigger innate immune responses in the plant (Daxberger *et al*., 2007). Therefore, avoiding the exposition of β-1,3-glucan, either my masking or/and by infection structure-specific reduction of synthesis of this polymer, may be essential for hemibiotrophs at early stages of pathogenesis.

In necrotrophic secondary hyphae of *C. graminicola* β-1,3-glucan appears to be a major cell wall component, as deduced from high levels of the GLS1:eGFP fusion protein, and intensive aniline blue staining (Figure 15). Furthermore, we have shown by expression of *eGFP* under the control of biotrophy- and necrotrophy-specific promoters that necrotrophic, but not bitotrohic, hyphae of class I RNAi strains exhibit severe cell wall defects and, as a consequence, grow slowly (Figures 20C-E). In addition to necrotrophy-specific eGFP-fluorescence, narrow hyphal diameters between the large swellings provide supporting morphological evidence that these distorted hyphae are necrotrophic secondary hyphae. Cell wall defects occurring in necrotrophic hyphae with reduced *GLS1* transcript levels have not been observed in biotrophic hyphae formed on the plant surface (Figures 20C-D).

Taken together, these data indicate that β-1,3-glucan synthesis and thus *GLS1* of *C. graminicola* are of significant importance during efficient colonization and spreading necrotrophic growth of the fungus in the host plant tissue.

IV.4 Forced expression of β-1,3-glucan in biotrophic hyphae of *C. graminicola* triggers immunity in maize

Down-regulation of *GLS1* expression and surface exposition of β-1,3-glucan during formation of biotrophic hyphae of *C. graminicola* suggests that evasion of PAMP recognition may be fundamental for escaping PAMP-triggered immunity. Fragments of structural cell wall polymers, including linear and/or branched β-1,3-glucans, function as PAMPs in plants and mammals, and trigger innate immune responses in both host backgrounds (Nürnberger *et al*., 2004). In humans, the membrane-bound non-classical C-type lectin receptor Dectin-1 recognizes either linear β-1,3-glucan or β-1,3–1,4-glucan polymers, or linear β-1,3-glucan polymers with short linear β-1,6-glucose side chains (Latgé J.P., 2010, and references therein). In plants, β-glucan recognition receptors have as yet not been functionally characterized. However, structurally different β-glucans are differentially recognized (Yamaguchi *et al*., 2000; Klarzynski *et al*., 2000). A putative high-affinity β-glucan receptor of soybean exhibiting an endo-cleaving β-1,3-glucanase domain has biochemically been characterized in detail (Fliegmann *et al*., 2004). This protein has β-1,3-glucanase, but no β-1,6-glucanase activity, and specific disintegration of β-1,3-glucan chains may thus amplify elicitor-active β-glucan elicitor molecules with a structure specifically recognized by a putative β-glucan receptor complex. Intriguingly, genes encoding proteins related to the β-glucan receptor from soybean exist in presumably all plant species (Fliegmann *et al*., 2004).

The majority of pattern recognition receptors (PRRs) are plasma membrane-located (Postel and Kemmerling, 2009), and exposure of PAMPs may therefore be particularly critical during biotrophic development, i.e. when pathogenic hyphae develop in the interfacial matrix in close vicinity of the host membrane. As enzymatic modification and/or high-affinity binding of β-1,3-glucan has not been reported, masking or down-regulation of β-1,3-glucan synthesis during critical stages of the infection process, or a combination thereof, may be employed to evade recognition of this PAMP.

In the human pathogen *A. fumigatus*, coating by the surface hydrophobin RodA and melanin apposition renders conidia immunologically inert, although several immunogenic molecules are localized beneath the surface (Aimanianda *et al*., 2009; Chai *et al*., 2010).

Whether or not surface appositions, e.g. apposition of α-1,3-glucan, as suggested by Fujikawa *et al.* (2009), contribute to masking of PAMPs in plant pathogenic fungi is currently unclear.

For the first time, the data reported here provide evidence that biotrophy-associated down-regulation of *GLS1* expression and synthesis and exposition of β-1,3-glucan is required for evading β-glucan-triggered immunity. In biotrophic hyphae of *C. graminicola*, β-1,3-glucan is not detectable, and in WT-inoculated maize (*Z. mays* cv Nathan) leaves, less than 5% of epidermal cells attacked by a single appressorium exhibit defense responses, as indicated by whole cell or cell wall fluorescence (Figures 24D). In contrast, strains over-expressing *GLS1* during biotrophic growth, expose significant amounts of β-1,3-glucan on the hyphal surface (Figures 24B and C) and evoke dramatic defense responses, i.e. whole cell or cell wall fluorescence in 30-40% of the epidermal cells attacked (Figures 24D). Furthermore, in maize cells attacked by hyphae exposing β-1,3-glucan, but not containing WT hyphae, formation of large numbers of brown vesicles possibly containing antifungal compounds such as the recently identified diterpenoid phytoalexins (Schmelz *et al*., 2011) occurs. Although the content of these vesicles is unknown at present, formation of such vesicles has recently been reported as a defense response of maize against *C. graminicola* (Vargas *et al*., 2012).

Taken together, our data strongly suggest that biotrophy-specific down-regulation of β-1,3-glucan synthase is required for the establishment of a compatible interaction between *C. graminicola* and maize.

V Conclusions

Due to the uniqueness of the fungal cell wall with respect to its polymer composition and the enzymes involved in its biogenesis, several approaches have been taken to use the fungal cell wall as a target in medicine. In particular, GLS inhibitors such as echinocandins and related compounds have been used to combat invasive mycoses caused by *A. fumigatus*, *C. neoformans, C. albicans*, and others (Georgopapadakou *et al*., 2001). In addition to drugs targeting cell wall biogenesis, antibodies directed against β-1,3-glucan exhibited a direct antimicrobial effect *in vitro* and provided protection against infection by *Aspergillus* and *Candida* (Torosantucci *et al*., 2005).

Live-cell imaging and Aniline Blue Fluorochrome-staining indicated that the cell wall composition of the maize pathogen *C. graminicola* is highly dynamic during infection structure development and required for host infection and compatibility (Figures 24). Massive β-1,3-glucan and chitin synthesis, providing the cell wall rigidity required for controlling turgor pressure, occurs in appressoria (Figures 15, 18, 19 and 31), and polymeric β-1,3-glucan is likely to carry β-1,6-glucan side chains. Infection vesicles and primary hyphae encased by the plant plasma membrane do not contain significant amounts of β-1,3-glucan and develop into voluminous hyphae, probably due to insufficient cell wall rigidity. Furthermore, these hyphae secrete chitin deacetylases into the interfacial matrix layer yielding chitosan, which is both a poor substrate for plant chitinases and a poor elicitor (El Gueddari *et al*., 2002, and references therein). Thus, at this delicate stage of pathogenesis defense responses are circumvented, and neither elicitor-active β-1,3-glucan nor chitin is exposed on the surface of biotrophic hyphae. When necrotrophic development is initiated, *GLS1* is massively expressed and β-1,3-glucan is a prominent cell wall polymers in secondary hyphae (Figure 31). At this stage of pathogenesis, although host defense may be activated to some extent (Vargas *et al*., 2012), defense responses are overcome, possibly due to secretion of toxic fungal metabolites by secondary hyphae (Horbach *et al.*, 2009; Horbach *et al*., 2011).

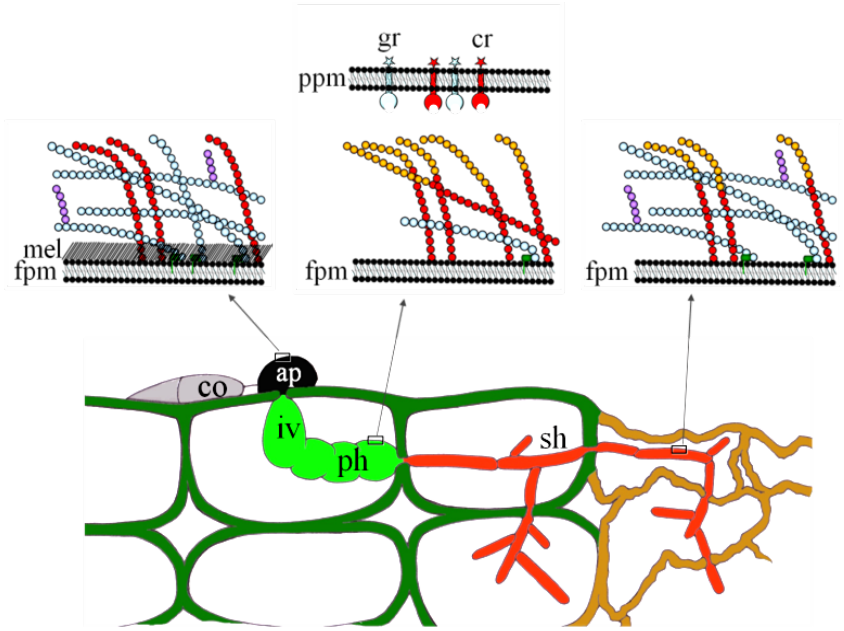

Fig. 30: Model showing cell wall modifications in infection hyphae of *C. graminicola*

In appressoria (ap) cell walls consist of significant amounts of chitin (red circles), β-1,3-glucan (light blue circles) and β-1,6-glucan (violet), forming a rigid scaffold, with melanin (mel) forming a layer in the vicinity of the fungal plasma membrane (fpm). Biotrophic infection vesicles (iv) and primary hyphae (ph) (green) are located within the interfacial matrix and surrounded by the plant plasma membrane (ppm), with integrated β-glucan (gr) and chitin receptors (cr). The walls of these voluminous hyphae show no or very low amounts of β-1,3-glucan, and surface-localized chitin is deacetylated to yield chitosan (brown circles). Cell walls of thin secondary hyphae (sh; orange) exhibit chitin (red circles), chitosan (brown circles), and β-1,3- (light blue circles) and β-1,6-glucan (violet). These hyphae are highly destructive and cause necrosis of host cells.

Also in plants the fungal cell wall represents a promising target for strategies to improve resistance. While initial attempts in overexpression of genes encoding individual cell wall-degrading enzymes, e.g. chitinases or β-1,3-glucanases, or combinations thereof, did not lead to results allowing implementing these plants in practical agriculture, a novel strategy called host-induced gene silencing (HIGS) (Nowara *et al.*, 2010) may represent a methodological break-through. Short interfering RNAs (siRNAs) corresponding to essential genes of a fungal pathogen are produced from RNAi constructs in transgenic plants and taken up by the fungus during the initial infection process, and the RNA-induced silencing complex

in the fungus will subsequently degrade its own essential mRNA, and fungal pathogenic development will be terminated (Nowara *et al*., 2010).

Our results have shown that *GLS1* of the leaf anthracnose and stem rot fungus of maize, *C. graminicola*, is an excellent HIGS target, as it is a viability factor and required during destructive necrotrophic *in planta* development. However, although fungi and plants belong to different kingdoms and are thus taxonomically remote, fungal and plant glucan synthases display high sequence similarities, as indicated by *E*-value of $6e^{-80}$ between the *A. thaliana* gene (accession number: At_1g05570) and the *S. pombe* (accession number: SPAC_19B12.03) gene (Latgé, 2007). Comparing the *C. graminicola GLS1* gene with the most closely related callose synthase-like gene of maize, *CSLF3* (accession number: NP_001147926.1), shows even lower similarity. These lower similarities may be taken as an indication of the low risk of off-target effects to occur in plants expressing HIGS constructs. For targeted RNA degradation mediated by the RNA-induced silencing complex (RISC), 21-bp of perfect matches are required allowing clear predictions. Over the entire *GLS1* segment of *C. graminicola* used in the RNAi construct, the largest segment of perfect matches is 5 bp (Appendix VII.4), making siRNA-mediated silencing of cellulose synthase-like proteins in maize, the host plant of *C. graminicola*, unlikely. Interestingly, Tinoco *et al*. (2010) used transgenic tobacco expressing a GUS-RNAi construct to study trans-species gene silencing of the GUS gene in *Fusarium verticillioides*. Ten fungal colonies were isolated from GUS-RNAi and from non-transgenic control plant lines each, and two colonies isolated from GUS-RNAi plants exhibited a reduction of GUS activity by 62 and 96%, respectively (Tinoco *et al*., 2010). In *C. graminicola* a reduction of *GLS1* transcript abundance by only approx. 40% was sufficient to cause non-pathogenicity (Figure 20A-B). If the efficiency of HIGS in the maize – *C. graminicola* interaction would be comparable to that reported for the interaction between tobacco and *F. verticillioides*, employment of a HIGS construct targeting *GLS1* may significantly increase the resistance against *C. graminicola*. Future work will show whether or not HIGS targeting fungal *GLS* genes, either alone or in combination with other fungal cell wall biogenesis genes, is suited as a novel and effective method to durably control pathogen ingress. These studies are underway.

VI References

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VII Appendix

VII.1 Culture media, buffers and solutions

VII.1.1 Culture media

M1: Oat Meal Agar, **OMA** (Werner *et al.*, 2007; Koneman *et al.*, 1997).

The medium contains 50 g oatmeal homogenized in a blender (Waring Blender BB 90 E, Waring, Torrington, USA). The powder oatmeal and agar-agar 2.4% were mixed in bidistilled H_2O. The total volume was adjusted to 1000 ml, and the medium was autoclaved for 40 min.

M2: Complete medium, **CM** (modified according to Leach *et al.*, 1982).

The medium contains 10 ml of each Stock solution A and B (VII.2.2; **S1** and **S2**), together with 10 g glucose, 1 g yeast extract and 1 g of casein (Difco Laboratories, Augsburg). The total volume was adjusted to 1000 ml with bidistilled H_2O and autoclaved.

M3: Potato dextrose agar, **PDA**

24 g of PDA (BD, Heidelberg, Germany) and 2.4% agar-agar were dissolved in bidistilled H_2O. The total volume was adjusted 1000 ml and autoclaved. Hygromycin B (II.2.2; **S3**) or nourseothricin (VII.2.2; **S4**) was added im the media (cooled to about 45°C) at a final concentration of 100 µg / ml.

M4: Regeneration medium, **RM**

In a total volume of 1000 ml of bidistilled H_2O; 342.3 g sucrose; 1 g yeast extract; 1 g casein and 6 g of agar-agar were added and autoclaved.

M5: Synthetic minimal medium, **SMM**

10 g glucose; 1 g $Ca(NO_3)_2$; 0.2 g, KH_2PO_3; 0.25 g $MgSO_4$; 0.054 g NaCl and 15 g of agarose were dissolved in bidistilled H_2O. The total volume of the media was completed to 900 ml with bidistilled H_2O and autoclaved. 100 ml of 10x Aminoacids mix (**S5**) was added after cooling (45°C). The final concentrations of the amino-acids were 100 µg / ml.

M6: Yeast Pepton Dextrose, **YPD**

In a total volume of 1000 ml of bidistilled H_2O; 10 g glucose; 2.5 g yeast extract and 5 g pepton were added and autoclaved.

M7: Yeast synthetic complete medium, Y**SCM**

In a total volume of 900 ml, 20 g glucose; 1.7 g YNB without amino acids (BD, Heidelberg, Germany); 5 g $(NH_4)\ 2SO_4$ and 15 g of agarose were dissolved and autoclaved. 100 ml of 10x Aminoacids mix (**S5**) was added after cooling (45°C). The final concentrations of the amino-acids were 100 µg / ml.

VII.1.2 Solutions

S1: Stock solution A

50 g $Ca\ (NO_3)_2$

ad 500 ml of bidistilled H_2O

autoclaved

S2: Stock solution B

10 g KH_2PO_3

12.5 g $MgSO_4$ x $7H_2O$

2.7 g NaCl

ad 500 ml of bidistilled H_2O

autoclaved

S3: Hygromycin- Stock solution

100 mg/ml Hygromycin B (InvivoGen, Toulouse, France) in bidistilled H_2O, steril filtrated.

S4: Nourseothricin- Stock solution

100 mg/ml Nourseothricin (Werner BioAgents, Jena, Germany) in bidistilled H_2O, steril filtrated.

S5: 10x Amino acids mix

21 mg Adenin	85.6 mg *L*-Histidin
85.6 mg *L*-Phenylalanin	85.6 mg *L*-Tryptophan
85.6 mg *L*-Alanin	85.6 mg myo-Inositol
85.6 mg *L*-Asparagin	8.6 mg *p*-Aminobenzoat
85.6 mg *L*-Arginin	85.6 mg *L*-Isoleucin
85.6 mg *L*-Threonin	85.6 mg *L*-Tyrosin
85.6 mg *L*-Aspartat	173.4 mg *L*-Leucin
85.6 mg *L*-Serin	85.6 mg Uracil
85.6 mg *L*-Cystein	85.6 mg *L*-Lysin
85.6 mg *L*-Glutamin	85.6 mg *L*-Valin
85.6 mg *L*-Glutamat	85.6 mg *L*-Prolin
85.6 mg *L*-Glycin	85.6 mg *L*-Methionin

ad 100 ml bidistilled H_2O, autoclaved (1 min).

The solutions were autoclaved, when indicated, for 20 minutes at 121 °C and 2 bar. Steril filtrations were done in sterilbank using a CA-Filtern (pores with diameter of 0.2 μm). The amino-acids mix solution was autoclaved for 1 minute at 121 °C and 2 bar.

VII.1.3 Buffers

B1: DNA Lysis Buffer

4.5 M guanidinium thiocyanate

2% (w / v) N-lauroylsarcosine

(Reset before use) 1% (v / v) β-mercaptoethanol

50 mM Hepes-KOH

pH 7.5

B2: TE-buffer

0.1 mM EDTA,

10 mM Tris-HCl

pH 8.0

B3: DNA extraction buffer I

7 M urea

2% (w / v) SDS

5 mM EDTA

50 mM Tris-HCl

pH 8.0

B4: DNA extraction buffer II

150 mM NaCl

5 mM EDTA

50 mM Tris-HCl

pH 8.0

B5: TAE-buffer

20 mM sodium acetate

2 mM EDTA

40 mM Tris-HCl

pH 8.3

B6: 6 x DNA loading buffer

30% (v / v) glycerol

60% 10x TAE buffer (Buffer 5)

0.25% (w/v) bromophenol

B7: 10 x RNA running buffer

50 mM sodium acetate

10 mM EDTA

20% (v/v)

formaldehyde

200 mM MOPS

pH 7.0

B8: RNA loading buffer

55% (v/v) deionized formamide

10% (v/v) 10x RNA running buffer

6.5% (v/v) formaldehyde

7% (v/v) glycerol

21.35% H_2O bidistilled

0.15% (w/v) bromophenol

B9: Transfer buffer 20 x SSC

3 M NaCl

0.3 M sodium citrate

pH 7.5

B10: Hybridization buffer

5x SSC (Buffer 9)

0.1% (w/v) N-lauroyl sarcosine

0.02% (w/v) SDS

1% (w/v) blocking reagent (Roche Diagnostics, Mannheim, Germany)

Autoclaved

B11: 2 x Wash buffer I

2x SSC (Buffer 9)

0.1% (w/v) SDS

B12: 0.5 x Wash buffer I

0.5 x SSC (Buffer 9)

0.1% (w/v) SDS

B13: 0.25 x Wash buffer I

0.25 x SSC (Buffer 9)

0.1% (w/v) SDS

B14: Blocking buffer

1% (w/v) blocking reagent (Roche Diagnostics, Mannheim)

in wash buffer I

autoclaved

B15: Detection buffer

100 mM NaCl

100 mM Tris-HCl

pH 9.5

autoclaved

B16: Stripping solution

0.05% (w/v) SDS

3 M NaCl

0.3 M sodium citrate

pH 7.5

The buffers, when indicated, were autoclaved for 20 minutes at 121 °C and 2 bar.

VII.2 Oligonucleotides used in this study

Table A1. List oligonucleotides used in this study

Primer name	Sequence	Reference
pAG300-Fw	5'-TCAACTCCGGATCCTCTAGAC-3'	This study
CgGLS1NotI-Fw	5'-GCGCGGCCGCATGTCGGGATACCCAGCC-3'	This study
CgGLS1NotI-Rv	5'-CAGCGGCCGCCTATTAAAACAAGCGCACTCTC-3'	This study
CgGLS1EGFP-Fw	5'- GAGAGTGCGCTTGTTTATGGTGAGCAAGGGCGA GGAGC-3'*	This study
CgGLS1/TAA-Rv	5'-GAGAGTGCGCTTGTTT-3'	This study
EGFPNotI-Rv	5'-CAGCGGCCGCCTACTTGTACAGCTCGTCCATGC-3'	This study
CgPGLS1-Fw	5'-CAGCAACGAGCAAGCAATCC-3'	This study
CgPGLS15'-flank-Rv	5'- GAATGCGGCTCTAGAGCGGTTTGGTGGATAATTA TCTAAGAAGTGGTGT-3'*	This study
CgTGLS13'-flank-Fw	5'- CTTAATGATGATTACTAACAGATATCAAGCTTTAG CGATTGTACCATAAACGC-3'	This study
CgTGLS1-Rv	5'-AGGGAGATGCCGAGGGCAGTCAATG-3'	This study
Nourse1pNR1-Fw	5'-TGACCGGTGCCTGGATCTTCCTATAGAATC-3'	This study
Nourse2pNR1-Rv	5'-GGTCGGCATCTACTCTATTCCTTTGCCCTC-3'	This study
CgPGLS1nest-Fw	5'-CTCTCGTTGCAGGCCGTTCCTTC-3'	This study
CgTGLS1nest-Rv	5'-CCCGAGTGTCTAGGTATGGTACG-3'	This study
CgGLS1test-Fw	5'-GCGAGATTCTCACTCCTCTG-3'	This study
CgGLS1test-Rv	5'-GCGCTCCCAGTCATCGTAAG-3'	This study
RNAi(*GLS1*)-Fw	5'- GGAGATCTCCCGCGAGTACTATTACCTG -3'	This study
RNAi(*GLS1*)-Rv	5'- CCGGGCCCCCGAATTGGCGTAAATCTGG -3'	This study
RNAi(*GLS1*)i-Fw	5'- GGCTCGAGCCGAATTGGCGTAAATCTGG -3'	This study
RNAi(*GLS1*)i-Rv	5'- GCAAGCTTCCCGCGAGTACTATTACCTG -3'	This study
HyhR probe-Fw	5'-TGAACTCACCGCGACGTCTG-3'	This study
HyhR probe-Rv	5'-GAGCTGATGCTTTGGGCCGA-3'	This study
NatR probe-Fw	5'-CTCTTGACGACACGGCTTAC-3'	This study
NatR probe-Rv	5'-GGCAGGGCATGCTCATGTAG-3'	This study
CgGLS1qRT-Fw	5'-CGTGAAACCGCCTCTATCAGG-3'	This study
CgGLS1qRT-Rv	5'-ACTGTTTGGGTCGGCATTGT-3'	This study
CgACTqRT-F1	5'-TCCTACGAGCTTCCTGACGG-3'	Krijger et al., 2008.
CgACTqRT-R1	5'-CCGCTCTCAAGACCAAGGAC-3'	Krijger et al., 2008.
CgH3-qRT.F1	5'-CGAGATCCGTCGCTACCAGA-3'	Krijger et al., 2008.
CgH3-qRT.R1	5'-GGAGGTCGGACTTGAAGTCCT-3'	Krijger et al., 2008.
CgITS2-q1	5'-TGAACGCGAGCTAACTTGACA-3'	Behr et al., 2009.
CgITS2-q2	5'-GGGCATCGAAGATGGAGGA-3'	Behr et al., 2009.
CgPrNmrA-Fw	5'-TTGATATCGTCACTCACTCGATCATCTC-3'	This study
CgPrNmrA-Rv	5'-ATAGCGCTCGGGCTGATGCTAAGCCAAA-3'	This study
CgPrSacDh-Fw	5'-TTGATATCTTCAGTGACCCGGGAGTGGGATG-3'	This study
CgPrSacDh-Rv	5'-ATAGCGCTGAGATCTTGGATGGAGTCAGCTTC-3'	This study
CgPrSecPep-Fw	5'-TTGATATCTGCTAGGTGTCTTCCGATTG-3'	This study
CgPrSecPep-Rv	5'-ATAGCGCTTTTGACTGAGGGTACCGATATGC-3'	This study

PSH1.6-Fw	5’-TTTGTGAGTCGTCGGTGAAG-3’	This study
PSH1.6-Rv	5’-CGTATCACGAGGCCCTTTC-3’	This study
CgGLS1GFP-Fw	5’-TATCGACTACCGCGACTATC-3’	This study
CgGLS1GFP5'flank-Rv	5’- GCTCCTCGCCCTTGCTCACCATAAACAAGCGCA CTCTCTCC-3’	This study
CgTGLS1GFP3'flank-Fw	5’-CAAAGGAATAGAGTAGATGCCGTAGCGATTGTA CCATAAACG-3’	This study
CgTGLS1GFP-Rv	5’-AACCAAGTCGAGGGAGATG-3’	This study
CgGLS1GFPnest-Fw	5’-CGGCAATTCTCGCTCTCAC-3’	This study
CgGLS1GFPnest-Rv	5’-GTCAATGGTACCCGAGTGTC-3’	This study
EGFP-Fw	5’-ATGGCCCCGACTATCATACAGTGG-3’	This study
HyhR-Rv	5’-GGAATAGAGTAGATGCCG-3’	This study
CgPrho1-Fw	5’-GCCAGTGGAATTCTGATTGC-3’	This study
CgPrho15'-flank-Rv	5’- GAATGCGGCTCTAGAGCGGCGGCAGACTCTAAG TGGTGTG -3’*	This study
CgTrho13'-flank-Fw	5’- CTTAATGATGATTACTAACAGATATCAAGCTTAA CTTCAGTCACCGCTCCTC -3’	This study
CgTrho1-Rv	5’-ACCCAATGCTGCTACAGGAG-3’	This study
Nourse12pNR1-Fw	5’-CCGCTCTAGAGCCGCATTC-3’	This study
Nourse22pNR1-Rv	5’-AAGCTTGATATCTGTTAGTAATCATCATTAAG-3’	This study
CgPrho1nest-Fw	5’-GATGGAAGCGTTCCTTCTTG-3’	This study
CgTrho1nest-Rv	5’-CCCGAGTGTCTAGGTATGGTACG-3’	This study
CgRhoIprobe-Fw	5’-TTTACACTTGCCCTCCTTGC-3’	This study
CgRhoIprobe-Rv	5’-CGGCAGACTCTAAGTGGTGTG-3’	This study
CgRho1-RT-PCR-Fw	5’-GGCGACGGTGGTTGCG-3’	This study
CgRho1-RT-PCR-Rv	5’-CCAGACGGCGGGTGTGT -3’	This study
CgGLS1GFP-Fw	5’-TATCGACTACCGCGACTATC-3’	This study
CgGLS1GFP5'-flank-Rv	5’-GCTCCTCGCCCTTGCTCACCATAAACAAGCG CACTCTCTCC-3’	This study
CgTGLS1GFP3'flank-Fw	5’-CAAAGGAATAGAGTAGATGCCGTAGCGATT GTACCATAAACG-3’	This study
CgTGLS1GFP-Rv	5’-AACCAAGTCGAGGGAGATG-3’	This study
CgGLS1GFPnest-Fw	5’-CGGCAATTCTCGCTCTCAC-3’	This study
CgGLS1GFPnest-Rv	5’-GTCAATGGTACCCGAGTGTC-3’	This study
EGFP-Fw	5’-ATGGCCCCGACTATCATACAGTGG-3’	This study
HyhR-Rv	5’-GGAATAGAGTAGATGCCG-3’	This study
PtrpC-SacI-Fw	5’-GGGGAGCTCCTTCCGGCTCGTATGTTGTG-3’	This study
PtrpC-SacI-Rv	5’-CGCGAGCTCTTTTCATTTGGATGCTTGGGTAG-3’	This study
PtoxB-SacI-Fw	5’-GGGGAGCTCAGGCCACGTGTCTTGTCCAG-3’	This study
PtoxB-SacI-Rv	5’-CGCGAGCTCGGAGCACAAGACTGGCCT-3’	This study
CgTGLS1NotI-Rv	5’-GAGCGGCCGCCCCGAGTGTCTAGGTATGGTA CG-3’	This study
NatR-PtrpC-Fw	5’-CTTAATGATGATTACTAACAGATATCAAGCT TACGTTAACTGATATTGAAGGAGC-3’	This study
NatR-PtoxB-Fw	5’-CTTAATGATGATTACTAACAGATATCAAGCT TAGGCCACGTGTCTTGTCCAG-3’	This study
		This study

P-TrpC-GLS1-Rv	5’-GGCTGGGTATCCCGACATTTTTCATTTGGAT GCTTGGGTAG-3’	This study
P-ToxB-GLS1-Rv	5’-GGCTGGGTATCCCGACATAGGCCAGTCTTGT GCTCCAG-3’	This study
P-TrpCGLS5'flank-Fw	5’-CCAAGGAGGTTACCTACAC-3’	This study
P-TrpCGLS5'flank-Rv	5’-GAATGCGGCTCTAGAGCGGTTTCGTGACTGC CTGGGTAG-3’	This study
CgGLS3’flank -Fw	5’-ATGTCGGGATACCCAGCC-3’	This study
P-TrpCGLS3'flank-Rv	5’-CAAGCATTCCGGCATAAAGC-3’	This study
P-TrpCGLS1nest-Fw	5’-CCAAAGGAGCCTGTCTGTTC-3’	This study
P-TrpCGLS1nest-Rv	5’-AGATACAGCGCAAGCTGACG-3’	This study

* Sequence overlaps and restriction sites are underline.

VII.3 Vectors used in this study

Table A2. List of vectors used in this study

Vector name	Description	Reference
pAG300	Plasmid obtained from Alexander Graf (Dipl.-Biol.), used to transform yeast. The plasmid has an ADH1 constitutive promoter and terminator. *Not*I restriction sites allow the ligation of the Cg*GLS1* gene in this plasmid. Selection markers: ampicillin (*Escherichia coli*) and uracil (*S. cereviseae*).	Horbach *et al*., 2009.
pRedi	Plasmid used in the RNAi approach. The plasmid has a trpC constitutive promoter, the sense of *dsRed* gene separed for 135 bp of antisense of *dsRed* gene and a *trpC* terminator. Selection markers: ampicillin (*E.coli*) and nourseothricin (*C. graminicola*).	Janus *et al*., 2007.
pJET2.1/blunt	(Fermentas, St. Leon-Rot) „High copy“: Plasmid for cloning PCR-fragments with blunt ends and amplification in *E. coli*. Selection marker: ampicillin.	Fermentas, St. Leon-Rot
pJET2.1/CgGLS1GFP	Plasmid containing the *CgGLS1*:*eGF*P cassette fragment. Resistance markers: ampicillin (*E.coli*) and hygromycin B (*C. graminicola*).	This study
pJET2.1/CgGLS1KO	Plasmid containing the *CgGLS1* deletion cassette fragment. Resistance markers: ampicillin (*E.coli*) and nourseothricin (*C. graminicola*).	This study

pJET2.1/CgRho1KO	Plasmid containing the *CgRHO1* deletion cassette fragment. Resistance markers: ampicillin (*E.coli*) and nourseothricin (*C. graminicola*).	This study
pSH1.6eGFP	Plasmid provided by Prof. Amir Sharon (Tel Aviv University, Israel). The vector contains *eGFP* gene and a hygromycin B phosphotransferase (hph) gene cassette used for construction of the *GLS1*:*eGFP* cassette. Selection markers: ampicillin (*E.coli*) and hygromycin B (*C. graminicola*).	Prof. Amir Sharon (Tel Aviv University, Israel).
pNR1:	Plasmid provided by Prof. Bettina Tudzynski, Westfälische Wilhelms-Universität Münster, Germany. The vector contains a nourseothricin acetyltransferase 1 (nat1) gene cassette used for construction of the Cg*GLS1* and Cg*RHO1* deletion cassette. Selection markers: ampicillin (*E.coli*) and nourseothricin (*C. graminicola*).	Malonek *et al*., 2004
pOG1-overexp	Plasmid containing an *Aspergillus midulans trpC* promoter used to dive overexpression of Cg*GLS1*. Selection markers: ampicillin (*E.coli*) and nourseothricin (*C. graminicola*).	This study
pOG2-overexp	Plasmid containing a *Pyrenophora tritici-repentis toxB* promoter used to dive overexpression of Cg*GLS1*. Selection markers: ampicillin (*E.coli*) and nourseothricin (*C. graminicola*).	This study
pSM1	Plasmid provided by Stefanie Pöggeler and Ulrich Kück, Ruhr-Universität Bochum, Germany. Plasmid used to amplify the *Aspergillus midulans trpC* promoter used to dive overexpression of Cg*GLS1*. Selection markers: ampicillin (*E.coli*) and hygromycin (*C. graminicola*).	Pöggeler *et al.*, 2003
pSH1.6eGFP-Biotrophic Markers	Plasmids containing *eGFP* gene dive by different biotrophic reporter promoter regions of the genes NmrA-like family protein, a saccharopine dehydrogenase, a pentafunctional AROM protein, an argininosuccinate lyase, an argininosuccinate synthase, and of the non-annotated unigenes 3, 125, 143. Selection markers: ampicillin (*E.coli*) and hygromycin (*C. graminicola*).	This study
pSH1.6eGFP-Necrotrophic Markers	Plasmids containing *eGFP* gene dive by different necrotrophic reporter promoter regions of the genes extracellular serine-rich protein and secreted peptidase gene. Selection markers: ampicillin (*E.coli*) and hygromycin (*C. graminicola*).	This study

VII.4 Alignment *C. graminicola GLS1, Z. mays CSFL3* and *CESA11*

CLUSTAL 2.0.10 multiple sequence alignment

```
Z._mays_CSLF3_NP_001147926.1    ------------------------------------------------------------
Z._mays_CESA11_NM_001111766     ----------------------------------------------------ATGATGG-
C._graminicola_GLS1_EFQ30502    ATGTCGGGATACCCAGCCGGTGGTGCCGGTGCCGGTGGCCACCAGGATTACGACGATGGT


Z._mays_CSLF3_NP_001147926.1    -------AGT---GCCACTGC-----------------ACA---C---------------
Z._mays_CESA11_NM_001111766     -------AGTC-GGCGGCGGC-----------------CCAGTCC---------------
C._graminicola_GLS1_EFQ30502    TACGGCCAGCACGGCCGCGGCCAGGGAAACGGTACCCAACAGACCGATTCCTACTACCAG
                                       **    **  * **                  **   *


Z._mays_CSLF3_NP_001147926.1    --TGGCTAG-AGTGT-----------------CAGTGC-----------CCCCCCA----
Z._mays_CESA11_NM_001111766     --TGCGCGG-CGTGCGGGGA-----------CGACGCGCGCG------CTGCCTG----
C._graminicola_GLS1_EFQ30502    GATGATCAGCAGTATTACGATCAAAACGGTTACGACACCCACGGCCAACCTCCCCAGGGC
                                  **    *  **                   *    *          *  **


Z._mays_CSLF3_NP_001147926.1    --------------------CCCCAC------CTAC---CTCT-----G---GTCTTGC
Z._mays_CESA11_NM_001111766     --------------------CCGCGCGTGCAGCTACGCGCTCT-----GCAGGGCGTGC
C._graminicola_GLS1_EFQ30502    GGCGATGGCTACTATGACGAATCGTACGTGAAACCGC---CTCTATGAAGGCAGTCTAGC
                                                   *   *      *  *   ****     *   * *  **


Z._mays_CSLF3_NP_001147926.1    ---------------------------------------------CAAAGGA---------
Z._mays_CESA11_NM_001111766     ---------------------------------------------CTCGACG---------
C._graminicola_GLS1_EFQ30502    ATTACTGACCACCCTTCGCTTCTAGTGGCTATTACAATGCCGACCCAAACAGTGCCTATC
                                                                            *


Z._mays_CSLF3_NP_001147926.1    ---AGGA--------------------AAAGGTAGTAGCTAG-CTAGGAC--------CT
Z._mays_CESA11_NM_001111766     ---AGGACGCCGCC-------------GAGGGCCGCACCACA-TGCGCGCG-------CT
C._graminicola_GLS1_EFQ30502    ACCAGGACGGCGGCTACTACGACAACAATGAGCAGTATCAGGATGAGTACTACAACAACG
                                   ****                          *  * * *       *  *        *


Z._mays_CSLF3_NP_001147926.1    GCAA----GGC----------------------------ATGGCTTCGCCG---------
Z._mays_CESA11_NM_001111766     GCGG----AG-----------------------------GGGACTACGCCGCTAT---CA
C._graminicola_GLS1_EFQ30502    GCAACAATGGCAATGGGTATTATGACCAAGACTACAATCAGGGCTATGCTGGTGCACCTC
                                **       *                                * **  ** *


Z._mays_CSLF3_NP_001147926.1    ---------GCACCGGCGGG-----CGATGCC----GTG-------TATGCGGCC---AA
Z._mays_CESA11_NM_001111766     ACCCAGCGCGCGCCAGCGAGGGAACCGAGGCG----GAG-------GAGGAGGT----GG
C._graminicola_GLS1_EFQ30502    GCCGACAAGGTTCCGAGGAGGATTCCGAGACTTTCAGCGATTTCACCATGAGGTCCGATA
                                         *  **   * *     ***  *     * *        * * **


Z._mays_CSLF3_NP_001147926.1    TGGC--GGCCT------------CACTG----------------------ACCCGCT---
Z._mays_CESA11_NM_001111766     TGGA--GAAC-------------CACCA----------------------CACCGCC---
C._graminicola_GLS1_EFQ30502    TGGCTCGAGCTACTGACATGGACTACTACGGCCGTGGCGATGAGCGTTATAACAGCTACG
                                ***   *  *             **                           * **


Z._mays_CSLF3_NP_001147926.1    -----TCTAGTGAGCGCGA-----------------------------------------
Z._mays_CESA11_NM_001111766     -----GGTGGCCTGCGTGA-----------------------------------------
C._graminicola_GLS1_EFQ30502    ACCAGGGTGGTGCTCGTGGTTTCAGGCCCCCTTCTTCTCAGATCTCGTACGGCGGTAACC
                                       * *    ** *


Z._mays_CSLF3_NP_001147926.1    --------------------------------ACGG----C-----CATG--GT--GCCG
Z._mays_CESA11_NM_001111766     --------------------------------GAGGGTCAC-----CATG--GGCAGCCA
C._graminicola_GLS1_EFQ30502    GTTCTTCCGGTGCTTCAACCCCCAACTATGGCATGGACTACGCCAACATGATGTCGGCCG
                                                                **    *     ****  *   ***


Z._mays_CSLF3_NP_001147926.1    CCGC----------------------------------------------------GAGG
Z._mays_CESA11_NM_001111766     CCTC----------------------------------------------------AATG
C._graminicola_GLS1_EFQ30502    CCTCTCGTGAGCCTTATCCTGCGTGGACCTCGGATGCTCAGATTCCTCTCTCCAAGGAGG
                                ** *                                                    * *
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```
Z._mays_CSLF3_NP_001147926.1    A-----AGGCTG-----------------GCCA-----------CGGCG---C--CAGGG
Z._mays_CESA11_NM_001111766     ATCGCCAGGATGAAGT-----------AAGCCA-----------CGCCAGGAC--CATGA
C._graminicola_GLS1_EFQ30502    AAATTGAGGACATTTTCCTCGACCTCTGCGCCAAATTTGGTTTTCAGCGGGACAGCATGC
                                *     ***                     ****          *  *    *  ** *

Z._mays_CSLF3_NP_001147926.1    GCA-------------------------------------------------------
Z._mays_CESA11_NM_001111766     GCA-------------------------------------------------------
C._graminicola_GLS1_EFQ30502    GCAACATGTACGACCACTTCATGATCTTGCTGGATTCGAGGGCTTCGCGCATGACTCCTA
                               ***

Z._mays_CSLF3_NP_001147926.1    -----GGT-------------------ACTG----GGTG----------------------
Z._mays_CESA11_NM_001111766     -----GCTTGTCGGGAATTGGTAGTGAATTG----AATGATGAATCTGGTAA--------
C._graminicola_GLS1_EFQ30502    ACCAGGCTCTGCTGTCTCTCCATGCAGACTACATTGGTGGCGACAACGCCAACTACCGCA
                                     * *                   * *       **

Z._mays_CSLF3_NP_001147926.1    --------------GCC--TCCG-------------------------------------
Z._mays_CESA11_NM_001111766     --------------GCCCATCTGGA-------------------------------------
C._graminicola_GLS1_EFQ30502    AGTGGTACTTTGCTGCCCATCTGGACTTGGACGACGCTGTCGGATTTGCCAATATGAAGG
                                             ***  ** *

Z._mays_CSLF3_NP_001147926.1    ---------------ACAAGGCCGAGAGGCG-----------------------------
Z._mays_CESA11_NM_001111766     ------------AGAACAGGGTGGAGAGTTGGAAGGAAAAGAAGAATGAG------AAGA
C._graminicola_GLS1_EFQ30502    GCAAAGGTCTGCGGCGCAAGGCCAAGAACAAGAAGAAGAGTAAGGGCGAAGCCGACAATG
                                          ** **   ***

Z._mays_CSLF3_NP_001147926.1    ----CGCTGC---CAAGGAGTCCGGCGGTGAGG-ACGGCCGCG-CGCTGCTG--------
Z._mays_CESA11_NM_001111766     AAGCCTCGGC---CAAAAAGACTGCAGCTAAAGCACAGCCTCC-GCCTGTCGAAGAACA-
C._graminicola_GLS1_EFQ30502    AGGCCGATGCGCTCGAGGATCTCGAAGGTGACA-ACAGTCTCGAGGCTGCTGAATACCGC
                                        *   **   * *  *    *  * * *   ** * * *    ***  *

Z._mays_CSLF3_NP_001147926.1    ----------------------------------TTCCG--------------------
Z._mays_CESA11_NM_001111766     --------------------------------GATCATG--------------------
C._graminicola_GLS1_EFQ30502    TGGAAGACTCGCATGAACCGCATGTCCCAGCATGATCGTGTCCGTCAGCTTGCGCTGTAT
                                                              *   *

Z._mays_CSLF3_NP_001147926.1    ---------------GAAG--TATAAGGT-----------------------------
Z._mays_CESA11_NM_001111766     ---------------GATG--AAAAAGACTTGA-------------------------
C._graminicola_GLS1_EFQ30502    CTTCTCTGCTGGGGTGAAGCCAATCAGGTTCGCTTTATGCCGGAATGCTTGTGCTTCATC
                                         ** *   *  **

Z._mays_CSLF3_NP_001147926.1    --CAAAGGCG---------CCCTCCTG---CACCCC------------------------
Z._mays_CESA11_NM_001111766     --CAGATGCATATGAGCCACTCTCCCGGGTCATCCCAATATCAAAGAACAAGCTCACACC
C._graminicola_GLS1_EFQ30502    TTCAAATGCGC--CGACGACTACCTCAACTCACCTGCTTGCCAAAACATGGTTGAGCCGG
                                  ** * **          *   *      ** *

Z._mays_CSLF3_NP_001147926.1    -TACAGG-----------TTGCTGA--TCATCATCC-GATT----AGTCGCTGT------
Z._mays_CESA11_NM_001111766     TTACAGA-----------GCAGTGA--TCATTATGC-GGTT----AAT---TGT------
C._graminicola_GLS1_EFQ30502    TCGAAGAGTTCACTTTCCTCAACAACGTCATTACCCCGATCTACCAGTT-CTGTAGAAAT
                                    **                  *  **** *  * * *     * *   ***

Z._mays_CSLF3_NP_001147926.1    -----CCTCG---CCTTC-------TTCGCGT--GGCG---------------------C
Z._mays_CESA11_NM_001111766     -----TCTTGGGCTCTTC-------TTTCACT--ACCG---------------------T
C._graminicola_GLS1_EFQ30502    CAGGGCTACGAAATCTCCGACGGTGTTTATGTTCGCCGGGAACGCGACCACGACAAGGTC
                                          *    ** *       **    *    **

Z._mays_CSLF3_NP_001147926.1    ATCAGGCACAACAAATCCGACATCA-----TGTGGT-TCTGGACAATGTCCA-----TCG
Z._mays_CESA11_NM_001111766     ATCAC------CAATCCTGTTAACAGTGCCTTTGGTCTCTGGATGACATCAG-----TTA
C._graminicola_GLS1_EFQ30502    ATCGGTTACGACGA--CTGTAATCAGCTCTTCTGGTATCCCGAAGGAATCGAGAAGATCG
                                ***        * *  * *  * **     * **** **  **     **       *

Z._mays_CSLF3_NP_001147926.1    TCGGCGACGTC----------TGGTTCG-GCTTCTCG---TGGCTCCTCAACCAGCTCCC
Z._mays_CESA11_NM_001111766     TATGTGAGATC----------TGGTTTG-GTTTCTCC---TGGATATTGGATCAATTCCC
C._graminicola_GLS1_EFQ30502    TCCTCGAGGACAAGTCGAAGTTGGTTGACGTTCCTCCCGCTGAGCGCTATCTCAAGTTCA
                                *    **   *          *****   * * ***    **     *    **  * *

Z._mays_CSLF3_NP_001147926.1    AAAGT-TCAAC----------------------CCTGTGAAGA---CCATCCCAGATCT-
Z._mays_CESA11_NM_001111766     GAAGT-GGTAT----------------------CCTATCA--A---TCGT--GAGACTTA
C._graminicola_GLS1_EFQ30502    AAGATATCAACTGGAAGAAGTGTTTCTTCAAGACTTACAAGGAGACTCGGTCGTGGTTTC
                                 *  *    *                        * *   *  *    *      *   *
```

```
Z._mays_CSLF3_NP_001147926.1    ---CGCCGCCCT-C--CAGCGGCACTTTGGCTACCC---TGAC--------GGCGGCGCC
Z._mays_CESA11_NM_001111766     ---TGTTGATAGGC--TGATTGCAC----GATATGG---AGAT--------GGTGAAGAA
C._graminicola_GLS1_EFQ30502    ACATGCTGGTGAACTTCAACCGTATCTGGATCATTCACTTGACCATGTTCTGGTTCTACA
                                    *  *     *       * *        *       **         **

Z._mays_CSLF3_NP_001147926.1    TCTAGGCT--CCCCGGTATCGATGTTTTCG--TC--ACCACT--------GCCGAC--CC
Z._mays_CESA11_NM_001111766     TCTGGGTTAGCACCTGTA--GATTTCTTTG--TC--AGTACA--------GTGGAT--CC
C._graminicola_GLS1_EFQ30502    CCTCGGCCAACGCCCCCAGTGTTATCCTCGGTTCCAAGTACGAGCAGCAGGCCAACAACC
                                 ** **    * **   *  * * *  * *  **  *  **         *   *   **

Z._mays_CSLF3_NP_001147926.1    CATTGAT--GAGC---------CCATACT-----------------CTACACCATGAACT
Z._mays_CESA11_NM_001111766     -ATTGAAA-GAGC---------CTCCACT-----------------AATCACTGCAAACA
C._graminicola_GLS1_EFQ30502    AGCCGACAAAAGCGCAGCAGTTCTCCATCGTCGGTTTTGGTGGTACCATCGCCGCACTCA
                                    **    ***         *   *                        * *      *

Z._mays_CSLF3_NP_001147926.1    GT---GTCCTCTCCATCCTGTCTG--TCGAT---TACCCAG-------------------
Z._mays_CESA11_NM_001111766     CT---GTGCTGTCTATTCTTGCTG--TGGAC---TATCCCG-------------------
C._graminicola_GLS1_EFQ30502    TTCAGGTTTTGGCAACTTTGGCCGAGTGGGCGTACGTCCCGCGCAAGTGGGCAGGCGCAC
                                 *   **  *  * *   *  * *  * *         ** *

Z._mays_CSLF3_NP_001147926.1    -----TCGATCGGCTAGCCTGCTATCTCT----CAGATGATAGT-------GGGGCGCTG
Z._mays_CESA11_NM_001111766     -----TTGAGAAGATCTCATGCTATGTAT----CTGATGATGGT-------TCTGCTATG
C._graminicola_GLS1_EFQ30502    AACACTTGACCAAAC-GCATGTTGTTCTTGATCCTGATCCTGGTCATCAACGTTGCGCCT
                                     * **        * ** * *   *    * ***  * **          **

Z._mays_CSLF3_NP_001147926.1    GTTCTCTATG--------------AGGC----------ACTGGCCGAGGTC---------
Z._mays_CESA11_NM_001111766     CTCACATTTG--------------AATC----------GCTCGCAGAGACT---------
C._graminicola_GLS1_EFQ30502    TTCGTCTACGTTTTCGTCCTCCCCAATCCGAACGAGAAGATTGCCGAGATTTTGGCCATT
                                 *    *  *             *  *           * ** ***

Z._mays_CSLF3_NP_001147926.1    GGAAAGTTTG--------------------CGCCTCTATGGG----TTCCGTTCTGCCG-
Z._mays_CESA11_NM_001111766     GCAGAATATG--------------------CTAGAAAGTGGG----TGCCGTTTTGCAA-
C._graminicola_GLS1_EFQ30502    GTTGAGTTCGTTATTGCACTTATCACCTTCCTCTTTTATTCGATCATGCCCCTTGGCGGT
                                *   * *  *                    *       *  *    * **  *  **

Z._mays_CSLF3_NP_001147926.1    --------------------CAAGTACTC---------CATTG--AGCCAAGGGCAC-CA
Z._mays_CESA11_NM_001111766     --------------------GAAGTACGC---------CATTG--AGCCACGAGCTC-CT
C._graminicola_GLS1_EFQ30502    CTCTTCGGAAGCTACCTTACCAAGAACTCGCGCAAGTACGTTGCCAGCCAGACGTTCACG
                                                  *** ** *         * ***  *****   *  * *

Z._mays_CSLF3_NP_001147926.1    GAAAGCTATTTTGAGC------------ATGTGGC-------------------ACCGCC
Z._mays_CESA11_NM_001111766     GAGTTCTACTTCT--C------------ACAGAAA-------------------ATTGAC
C._graminicola_GLS1_EFQ30502    GCCAGCTACCCCCGTCTCAAGGGCAACGACATGGCCATGTCCTACGGTCTCTGGGTTGCC
                                *    ***       *            *                           * *

Z._mays_CSLF3_NP_001147926.1    G----------CAGGCTGGAAGGGTGACGCAGG---AGTTC---TTG------AATGATT
Z._mays_CESA11_NM_001111766     -------------TACTTGAAGGACAAGATACACCCATCTT---TTGTC----AAGGAGC
C._graminicola_GLS1_EFQ30502    GTTTTCGGTGCCAAGTTCGGAGAGTCATACGTG--TACCTCACTCTGTCCTTCAGGGATC
                                             * * **    *         *  *     **      *  **

Z._mays_CSLF3_NP_001147926.1    -------ATAG-----GAGGGTGCAGATGGAGTA------CGATGAG-------------
Z._mays_CESA11_NM_001111766     -------GTAG-----GGCTATGAAGAGAGACTA------TGAAGAG-------------
C._graminicola_GLS1_EFQ30502    CCATCCGGTACCTCTCGATCATGCAGATCGATTGTATGGGCGACAAGATTATTGGCAACA
                                       **      *    ** ***  ** *        **  **

Z._mays_CSLF3_NP_001147926.1    -----TTCAAG-----------------GCGCGCTTGGACAACCT---------------
Z._mays_CESA11_NM_001111766     -----TACAAG-----------------GTGAG----GATAA-----------------
C._graminicola_GLS1_EFQ30502    TACTCTGCAAAAACCAGCATTATGTTCTGCTAGCCTTGATGACCTTCACAGACTTGATCT
                                     * ***                   *   *    **  *

Z._mays_CSLF3_NP_001147926.1    -------CCCTGATGCCATACGCAAGAGATCCGATGTTTA-----------CAATAGCG
Z._mays_CESA11_NM_001111766     ------------ATGCT----------------TTGGTTG-----------CCAAGGCT
C._graminicola_GLS1_EFQ30502    TCTTCTTCCTTGATACG-TATCTGTGGTACGTTCTGATCAACGCCCTCTTCTCCATCGCC
                                           ** *                 ** *            * *  **

Z._mays_CSLF3_NP_001147926.1    TGA-------------------------GAGATGCAGGAGGAGC---------------
Z._mays_CESA11_NM_001111766     CAA-------------------------AAGACACCTGATGA----------------
C._graminicola_GLS1_EFQ30502    AGATCGTTCTACATCGGTTCCTCCATCATGACACCCTGGAGGAACGTCTTTTCACGCCTT
                                  *                         * *  *  ** **
```

```
Z._mays_CSLF3_NP_001147926.1    TCAAAA--------------------------GGCAACTTGGA----------------
Z._mays_CESA11_NM_001111766     -----A--------------------------GGC----TGGA----------------
C._graminicola_GLS1_EFQ30502    CCGAAACGTATCTACTCCAAGATTCTCGCTACTGGCGACATGGAAATCAAGTACAAGCCG
                                     *                           ***    ****

Z._mays_CSLF3_NP_001147926.1    ----------TG--GCAA--ATGGGACGCAGTGGCC------------------------
Z._mays_CESA11_NM_001111766     ----------TCATGCAAG-ACGGTACACCATGGCC------------------------
C._graminicola_GLS1_EFQ30502    AAGGTACTGATTTCGCAAGTCTGGAACGCTATCGTCATTTCCATGTACAGAGAGCACCTG
                                          *   ****    ** ** *  * * *

Z._mays_CSLF3_NP_001147926.1    -CGGCACATGGATTG-ATCCA---------------------------------------
Z._mays_CESA11_NM_001111766     -TGG------GAACA-ATCCT---------------------------------------
C._graminicola_GLS1_EFQ30502    TTGGC-CATCGATCACGTCCAAAAGCTTCTCTATCACCAGGTCCCTTCGGAGCAAGAAGG
                                  **      **     ***

Z._mays_CSLF3_NP_001147926.1    GCAGAGAACCATAGGA----------------------AAGGACACCA-TGCTCCAATTG
Z._mays_CESA11_NM_001111766     CGTGACCACCCTGG-------------------------------CA-TGATCCA----
C._graminicola_GLS1_EFQ30502    CAAGAGAACCCTGAGAGCACCTACCTTCTTCGTCTCCCAGGAAGATCACTCATTCAAG-A
                                   **  *** *                                  ** *  * **

Z._mays_CSLF3_NP_001147926.1    CCAAGGTTGTGCTGAACCATCCAAGCCG----TGGACAACACCC------TATTACTGAA
Z._mays_CESA11_NM_001111766     ----GGTTTTCCTGGGTGA---GACTGG----TGCACGGGACT---------TTGATGGA
C._graminicola_GLS1_EFQ30502    CCGGAGTTCTTCCCCACGAAC-AAGCGGAGGCTGAACGACGCCTGTCCTTCTTTGCTCAG
                                    *** * *      *    *   *    ** **    *          **  *

Z._mays_CSLF3_NP_001147926.1    AGCAATCCCAGCATTGCCACCACTGAT----GAGCGCCTCCCA---------ATGCTTGT
Z._mays_CESA11_NM_001111766     A----------------------AT----GAAC---TTCCT---------CGGTTAGT
C._graminicola_GLS1_EFQ30502    T----------CACTGTCCACTCCTATTCCGGAGCCTGTCCCAGTTGACAATATGCCTAC
                                           **    ** *   * **           *

Z._mays_CSLF3_NP_001147926.1    TTACG----TCTC-----------TCGTGAGAAGAACC--CAG--GTTATG----ACCA-
Z._mays_CESA11_NM_001111766     GTATG----TGTC-----------AAGAGAGAAAAGAC--CAG--GCTACC----AACA-
C._graminicola_GLS1_EFQ30502    TTTCACTGTTCTCATCCCGCACTATAGTGAGAAAATCCTCCTGTCGTTGCGTGAGATCAT
                                 *       * **             * ***** *  *  * *  * *       * **

Z._mays_CSLF3_NP_001147926.1    CAACAAGAAGGCA------------GGTGCCCT----GAATGC-----ACAGCT------
Z._mays_CESA11_NM_001111766     CCACAAGAAGGCA------------GGGGCTAT----GAATGC-----TCTGGT------
C._graminicola_GLS1_EFQ30502    CCGCGAGGATGAGCCTTACTCCCGTGTTACCCTCCTGGAGTACCTCAAGCAACTTCACCC
                                *  * ** * *              *   *  *    ** * *      *   *

Z._mays_CSLF3_NP_001147926.1    --GCGGGCCT--CTGCTC-----------------TCCTCTCTAATG------CCCAACT
Z._mays_CESA11_NM_001111766     --CCGAGTGT--CTGCTG-----------------TTCTGACAAATG------CCCCTTA
C._graminicola_GLS1_EFQ30502    TCACGAGTGGGACTGCTTCGTCAAGGACACGAAGATCCTGGCGGATGAGACATCTCAGTT
                                   ** *     *****                  * **  *  ***      * *

Z._mays_CSLF3_NP_001147926.1    CATCATCAACTTCGA---------------------------------CTGCGACC----
Z._mays_CESA11_NM_001111766     CATTCTTAATCTTGA---------------------------------TTGTGATC----
C._graminicola_GLS1_EFQ30502    CAACGGCGACAGCGAAAAGAACGAGAAGGATACAGTCAAGAGCAAGATCGACGACCTTCC
                                **      *    **                                    ** *

Z._mays_CSLF3_NP_001147926.1    -----ACTACATCAA---CAA--------CTCTCAAG----CCCTAAG--------CTCG
Z._mays_CESA11_NM_001111766     -----ACTATGTTAA---CAA--------CAGCAAAG----CTGTTCG--------TGAA
C._graminicola_GLS1_EFQ30502    TTTCTACTGCATTGGGTTCAAATCCTCTGCCCCAGAGTACACCCTACGGACGCGTATTTG
                                     ***   *      ***        *     **    *  *  *

Z._mays_CSLF3_NP_001147926.1    G----CTGTGTGCTTC---ATGCTGGATCA-ACGG--------------------GACGG
Z._mays_CESA11_NM_001111766     G----CAATGTGCTTC---ATGATGGACCCTACTGTT------------------GGCAG
C._graminicola_GLS1_EFQ30502    GGCCTCTCTGCGTTTCCAGACCCTTTACCGCACGATTTCTGGTTTCATGAATTATAGCAG
                               *    *  ** * ***   *   *  * *  **                        * *

Z._mays_CSLF3_NP_001147926.1    TGATAACACCGCATTTGTTCAGT----------------TCCCACAGCGCTTCGACAATG
Z._mays_CESA11_NM_001111766     AGATGTC--TGC-TATGTACAAT----------------TCCCCCAGAGGTTCGATGGCA
C._graminicola_GLS1_EFQ30502    AGCCATCA-AGCTTTTGTATCGTGTGGAGAACCCAGAGGTCGTCCAGATGTTCGGCGGCA
                                 *    *   ** * ***    *                **   ***   ****

Z._mays_CSLF3_NP_001147926.1    ------------------------TTGACCCCACGGACCGC-----------------TA
Z._mays_CESA11_NM_001111766     ------------------------TTGATCGCAGTGATCGA-----------------TA
C._graminicola_GLS1_EFQ30502    ACTCTGACAAACTAGAGCGTGAGCTCGAGCGCATGGCCCGTCGCAAGTTCAAGCTTTGTG
                                          * ** * **  *  **                   *
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Z._mays_CSLF3_NP_001147926.1    TGGTAACCACAATCGTGTC-----TTCTTTGATGGGACCATG--------CTTGCCCTTA
Z._mays_CESA11_NM_001111766     TGCCAATAGGAACGTTGTG-----TTCTTTGATGTTAATATG--------AAAGGACTTG
C._graminicola_GLS1_EFQ30502    TGTCAAT-GCAGCGTTATGCCAAGTTCAAAAAGGAGGAGATGGAGAACGCCGAGTTCTTG
                                **  **    *    * *      ***    * *     ***           *  ***

Z._mays_CSLF3_NP_001147926.1    ATG---GTTTGCAAGGGCC-------CTCATACCTCG-----GTAC------TGGTTGCA
Z._mays_CESA11_NM_001111766     ATG---GCCTCCAAGGCCC-------AGTTTATGTGG-----GAAC------TGGTTGT-
C._graminicola_GLS1_EFQ30502    CTTCGCGCCTACCCCGACCTCCAGATCGCTTACCTCGACGAAGAGCCCCCCTTGGCTGAA
                                 *    *  * *   * **           **  * *     *  *      *** **

Z._mays_CSLF3_NP_001147926.1    TGTTCCG----TCGCTTAGCACTCTATGGT-ATTGACC--CACCCC--------------
Z._mays_CESA11_NM_001111766     TGTTTCAA---TAGGCAAGCACTTTATGGTTATGGGCCTCCATCTCTGCCCGCACTTCCA
C._graminicola_GLS1_EFQ30502    GGTGAAGAACCTCGCCT-GTACTCTGCGCTCATCGACGGTCA-CTCGGAAATTATGGAGA
                                 **        * *    * *** *  * * ** * *   ** * *

Z._mays_CSLF3_NP_001147926.1    -------------------------------ACTGT------------------AGAGCA
Z._mays_CESA11_NM_001111766     AAGTCTTCGATTTGTTCCTGGTGTTGCTGCTGCTGTCCCAAGA---------AAAAGGTT
C._graminicola_GLS1_EFQ30502    ATGGCATGCGCAAGCCGAAATTCCGTATTCAGCTCTCCGGCAACCCGATTTTGGGAGACG
                                                              ** *

Z._mays_CSLF3_NP_001147926.1    GAAAACATCACGGCCGAAGCTAGCAGG----TTT-------GGCAA------CTCCA---
Z._mays_CESA11_NM_001111766     GAAAGAAGTGAGAGGGAAATCAACAGAGACTCTC-------GGCGAGAAGACCTCGAGTC
C._graminicola_GLS1_EFQ30502    GCAAATCCGACAACCAGAACCATTCGATCATCTTCTACCGTGGCGA--ATATATCCAGCT
                                * **             *   *   *      *        *** *       ** *

Z._mays_CSLF3_NP_001147926.1    ---------CAATC--------------------TTC-------------CTA----GAT
Z._mays_CESA11_NM_001111766     TGCCATTTTTAACC--------------------TTCGCGAAATTGACAACTA----CGA
C._graminicola_GLS1_EFQ30502    TATCGACGCCAACCAGGATAACTACCTAGAGGAATGCCTCAAGATCAGATCTGTTCTGGC
                                          ** *                    * *           **

Z._mays_CSLF3_NP_001147926.1    TCAGTGTCAAAAG--------------------CCC----------------TGAAAAAT
Z._mays_CESA11_NM_001111766     TGAGTACGAGAGG--------------------TCCATGCTCATCTCTCAGATGAGCTTC
C._graminicola_GLS1_EFQ30502    CGAGTTTGAGGAGATGAAGACTGATAACGTCAGCCCCTACACGCCTG--GAGTCAAGAAC
                                  ***   *   *                    **               * *

Z._mays_CSLF3_NP_001147926.1    GACA-GGACAATCACACCGCCACCA------ATTGA--TGACACAT--------------
Z._mays_CESA11_NM_001111766     GAGA-AGTCTTTTGGGCTGTCCTCGGTCTTTATTGAATCGACCCTTATGGAGAATGGGGG
C._graminicola_GLS1_EFQ30502    AAGATGGTCAACCCCGTTGCCATCCTGGGTGCTCGCGAGTACATTT--------------
                                 * *  * *         * *  *        * *     **   *

Z._mays_CSLF3_NP_001147926.1    --TCCTTG------CTG----------------AGCT------AGAGAG---------GG
Z._mays_CESA11_NM_001111766     CGTCCCTGAAT---CTGCAAACCCATCTACCCTAATT------AAAGAA---------GC
C._graminicola_GLS1_EFQ30502    --TCTCCGAGAACATCGGTATTCTAGGTGACGTGGCCGCCGGCAAGGAACAGACGTTCGG
                                  **   *        *                          *  **          *

Z._mays_CSLF3_NP_001147926.1    ---TTGTGACAT--GTTCT---T-----ATGACAAAGGTACTG-ACTGGGG---------
Z._mays_CESA11_NM_001111766     CATTCATGTCATTAGCTGTGGAT-----ATGAAGAGAAAACTG-AATGGGG---------
C._graminicola_GLS1_EFQ30502    TACTCTCTTCGCTCGTACTCTATCCCAGATTGGAGGAAAGCTGCACTACGGTCACCCCGA
                                   *     *    *   *   *     **          *** * *  **

Z._mays_CSLF3_NP_001147926.1    ------CAAGGGTGT-----AG------------GGTACATCTATGACATAG--------
Z._mays_CESA11_NM_001111766     ------AAAAGAGAT-----TG------------GCTGGATCTATGGTTCAG--------
C._graminicola_GLS1_EFQ30502    TTTCCTCAACGGTATCTTCATGACTACTCGCGGTGGTGTATCAAAGGCTCAGAAGGGCTT
                                      ** *   *      *           * *  *** * *    **

Z._mays_CSLF3_NP_001147926.1    -----CCACTGAAGATATAGTAACAGGATTCCGCATCCACGGGCAGG-GGTGGC-GCTCT
Z._mays_CESA11_NM_001111766     -----TTACAGAGGATATTCTGACTGGGTTTAAGATGCACTGCCGTG-GCTGGA-GATCC
C._graminicola_GLS1_EFQ30502    GCACCTTAACGAAGATATTTATGCTGG--TATGAACGCCCTGTTGCGTGGTGGCAGAATT
                                       *  ** *****     * **  *    *  * * *    * * ***  *

Z._mays_CSLF3_NP_001147926.1    ATGTATTGCACA----------ATGGAGCACGATGCGTTCTGTGGCGTTGCACCAATC--
Z._mays_CESA11_NM_001111766     ATCTACTGCATG----------CCGGTGAGACCTGCATTCAAGGGATCAGCCCCAATC--
C._graminicola_GLS1_EFQ30502    AAGCACTGCGAGTACTACCAGTGCGGTAAAGGTCGTGATTTGGGTTTCGGCTCCATTCTG
                                *   * ***               **        *   *    *     ** *** **

Z._mays_CSLF3_NP_001147926.1    AACCTAACT-----GAACGCCTCC----------ATCAAAT---TGTGCGCTGGT-CTGG
Z._mays_CESA11_NM_001111766     AATCTTTCC-----GATCGTCTTC----------ACCAAGT---TCTCCGGTGGG-CTCT
C._graminicola_GLS1_EFQ30502    AACTTTACCACCAAGATCGGCACCGGTATGGGTGAGCAGATGCTTTCCCGCGAGTACTAT
                                **  *  *      ** ** *  *          * **  *   *   **   *  **
```

```
Z._mays_CSLF3_NP_001147926.1    TGGGT--------------CTTTAGA--GATGTTCTTCT----------CCCAC------
Z._mays_CESA11_NM_001111766     TGTTT--------------CTGTCGA--GATCTTCTTCAGTCGGCACTGCCCGC------
C._graminicola_GLS1_EFQ30502    TACCTGGGAACTCAGCTTCCTCTGGATCGATTCCTCTCTTTCTACTACGCCCATCCTGGC
                                    *   *              ** * **  ***     **           ***

Z._mays_CSLF3_NP_001147926.1    ---------AACAATCCATTCAT--------CGGT-------------------------
Z._mays_CESA11_NM_001111766     ---------TGTGGTACGGTTA---------CGGT-------------------------
C._graminicola_GLS1_EFQ30502    TTTCACCTTAACAACATGTTCATCATGTTGTCAGTCCAGATGTTCATGATCTGTCTCCTC
                                          * *         * **

Z._mays_CSLF3_NP_001147926.1    -----GGTCGCC------------------GGATTCAA---------------CCCCTTC
Z._mays_CESA11_NM_001111766     -----GGCGGCC------------------GTCTGAAA---------------TGGCTCC
C._graminicola_GLS1_EFQ30502    AGCTTGGGCGCCCTTCGACACGAGACCAAGGCATGCAATTACAACCGCGACGTCCCCATC
                                     **  ***                  *  *  **                  *  *

Z._mays_CSLF3_NP_001147926.1    AGCGTGTCTC-------------CTACCTCAACATG-------------AC--AGTCTAC
Z._mays_CESA11_NM_001111766     AGAGGCTCTC-------------CTACATCAACACC-------------AT--CGTGTAC
C._graminicola_GLS1_EFQ30502    ACCGATCCTCTCTTCCCCACCGGCTGCCAGAACACGGATGCGCTCATGGACTGGGTGTAC
                                *  *   ***             ** *   ****                *    ** ***

Z._mays_CSLF3_NP_001147926.1    CCAGTCACATCAGTCTTCATCCTAATCTATGCTCTAAGC--CCG----------------
Z._mays_CESA11_NM_001111766     CCGTTCACTTC--TCTTCCTCTCGTTGCCTACTGTTGCCTGCCT----------------
C._graminicola_GLS1_EFQ30502    CGCTGCATCTTATCCATTATCTTTGTGT-TGCTGTTGGCCTTCGTCCCCTTGGTGGTCCA
                                *    **  *    * *  **    *   * ** *   *   *

Z._mays_CSLF3_NP_001147926.1    ----------------GTGATGTGGCTTATC---------CCCGATGAA-----------
Z._mays_CESA11_NM_001111766     ----------------GCCATTTG-CCTGCT---------CACAGGAAA-----------
C._graminicola_GLS1_EFQ30502    GGAATTGACCGAGAGGGGCATCTGGCGTGCCGCCAAGCGTCTCGGCAAGCAGTTCGGGTC
                                          *  ** ** * *           * *    *

Z._mays_CSLF3_NP_001147926.1    ------------------------GTATAC-ATCCAGAGACCA-TTCA---C-------
Z._mays_CESA11_NM_001111766     ------------------------GTTCATTATACCTACGCTG-TCCAACGC-------
C._graminicola_GLS1_EFQ30502    TCTTTCACCGTTCTTCGAGGTCTTCGTTTGCCAGATTTACGCCAATTCGGTACAACAGGA
                                              **     *     *  *   * *    *

Z._mays_CSLF3_NP_001147926.1    ----------------------TA----GGTA-------TG---TCGTGTAC-CT-----
Z._mays_CESA11_NM_001111766     ----------------------TGCAACGATA-------TGGTTTCTTGGCCTCT-----
C._graminicola_GLS1_EFQ30502    TCTCTCGTTTGGCGGTGCCCGGTACATTGGTACCGGTCGTGGTTTTGCGACCGCTCGCAT
                                           *     * **       **   *   *  * **

 Z.mays_CSLF3_NP_001147926.1    -----------TCTTGTA-------ATCATCG----------------------------
 Z.mays_CESA11_NM_001111766     -----------TCATGTCC------ATCATCG----------------------------
 C.graminicola_GLS1_EFQ30502    TCCCTTCGGCGTCTTGTACTCCCGTTTCGCCGGTCCTTCGATTTATTTTGGTTCGCGCCT
                                          ** ***         **  **

Z._mays_CSLF3_NP_001147926.1    --TGATGATTCACATGATTG-----GCTGGCTTGAGATAAAGTGG-GCGG---------G
Z._mays_CESA11_NM_001111766     --TGACGA---GCGTG-TTG-----G--AGCT-GCGGTGGAGTGGCATCG---------G
C._graminicola_GLS1_EFQ30502    GCTGATGATGCTTCTTTTTGCCACCGTTACCATCTGGCAAGGTGCCCTCGTGTACTTCTG
                                  *** **      *  ***     *    *    *     ***     *         *

Z._mays_CSLF3_NP_001147926.1    GGTCACATGGCTGG-------------ACTATTG--GCGCAAT-----------------
Z._mays_CESA11_NM_001111766     GATCGA------GG-------------ACTGGTG--GCGCAAC-----------------
C._graminicola_GLS1_EFQ30502    GATCTCTCTCCTGGCTCTGGTCATTTCGCCGTTCTTGTACAACCCACACCAGTTCGCTTG
                                * **        **              *   *   *  ***

Z._mays_CSLF3_NP_001147926.1    GAGCAGTTCTTT--------------ATGATC------GGCTC------AACAAGT----
Z._mays_CESA11_NM_001111766     GAGCAGTTCTGG--------------GTCATC------GGA--------GGCGTGTC---
C._graminicola_GLS1_EFQ30502    GAGCGATTTTTTTATCGACTACCGCGACTATCTCCGATGGCTCTCTCGCGGCAATTCTCG
                                ****  ** *                     ***      **          *   *

Z._mays_CSLF3_NP_001147926.1    -----GCATATCC-----AATGGCAGTGCTGCACATGGCAGTGAACCTC-CTCACAAAGA
Z._mays_CESA11_NM_001111766     ----CGCGCACCT-----GTTCGCCGTGTTCCAGGGTATCCTCAAGATG-ATTGCCGGGC
C._graminicola_GLS1_EFQ30502    CTCTCACGCTTCCTCCTGGATCGC-GTTCTGCCGTCTGTCTCGAACTCGTATTACAGGTT
                                      *    *        * ** **  * *           **      *  *

Z._mays_CSLF3_NP_001147926.1    A-GGGCATACACTTCAGGGTCACTTCCAAGCAAACA-------------GCCGCGGACGA
Z._mays_CESA11_NM_001111766     T-GGACACCAACTTCACGGTCAC-----GGCAAA-G-------------GCCACGGACGA
C._graminicola_GLS1_EFQ30502    ACAAGCGCAAGGCCCTCGGTGACCCATCGGCCAAGATGTCATCTGACGTACCGCGTGCGG
                                     *        *  *** **      ** **                ** **  **
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```
Z._mays_CSLF3_NP_001147926.1    C----AATGACAAGTTC---GCCGACCTTTATGATTTTCGA----TGGGTGCCAATGC--
Z._mays_CESA11_NM_001111766     C----ACTGA---GTTC---GGGGAGCTGTACCTGTTCAAG----TGGACGACGGTGC--
C._graminicola_GLS1_EFQ30502    CCATCACCAACATGTTTTGGGGCGAGATTCTCACTCCTCTGCTCCTCGTTGCTACTACCG
                                *    *   *   ***    *  **  *                 * *  *    * *

Z._mays_CSLF3_NP_001147926.1    TCATCCCAA----------CAATGGCAGTTCTGAT-CTGCA------------------
Z._mays_CESA11_NM_001111766     TGATCCCGC----------CCACAAGCATCCTGGTGCTGAA------------------
C._graminicola_GLS1_EFQ30502    TCATCCCATACCTCTTCATCAACGCCCAGACTGGTGTTGAAGGAGCGAACAACAGTGACA
                                * *****            * *        *** *  ** *

Z._mays_CSLF3_NP_001147926.1    ----------------------------AT--GTTGGTGCCATTGGTGTAGCCCTGG---
Z._mays_CESA11_NM_001111766     ----------------------------CCTGGTGGGCGTGGTGGCTGGGTTCTCGGCCG
C._graminicola_GLS1_EFQ30502    TCTCGGAACCGCCTAAGCCAACCAACTCCTTGATCAGAGTCGGAGTCGTAGCGCTCGCCC
                                                           *  * *     *  *        *

Z._mays_CSLF3_NP_001147926.1    -----GCAA--AACTGTGG-----TA---------------------TACAT--TGGAAC
Z._mays_CESA11_NM_001111766     C----GCTC--AACAGCGGC----TA---------------------CGAGTCCTGGGGC
C._graminicola_GLS1_EFQ30502    CTATCGCAATCAACGCCGGTTTGCTAATGGTAATGTTCTTCATGGCCTGCTTCATGGGAC
                                     **    ***   **     **                          *  ***  *

Z._mays_CSLF3_NP_001147926.1    A--TGGACAGCG------GCGAAGAA-------------------GAT-G--CATG---
Z._mays_CESA11_NM_001111766     C--CGCTCTTCG------GTAAGGTGTTCTTCGCCATGTG------GGT-GATCATG---
C._graminicola_GLS1_EFQ30502    CCCTCCTCAGCATGTGCTGCAAAAAGTTTGGCAGCGTGCTTGCGGCGATCGCTCATGGTG
                                       *  *       *  *                         * * *  ****

Z._mays_CSLF3_NP_001147926.1    -------CA---GCACT---------------GG----GTCT--------GCTGTTCAA-
Z._mays_CESA11_NM_001111766     -------CACCTGTACCCGTT-----CCTCAAGG----GTCTCA---TGGGCCGCCAGAA
C._graminicola_GLS1_EFQ30502    TTGCTGTCAT-TATGCTGCTTGTCTTCTTCGAGGTTATGTTCCTGCTTGAGTCGTTCGAG
                                       **      *               **    **          *  *    *

Z._mays_CSLF3_NP_001147926.1    ---CATATGGATCAT--------------------GTT--TCTCCT---CTACCCATTTG
Z._mays_CESA11_NM_001111766     CCGCACGCCGACCATCGT--------GGTGCTCTGGTC--CGTCCT---CCTCGCCTCCG
C._graminicola_GLS1_EFQ30502    TT-CGCGAGGA-CATTGCTCGGTATGATTGCTGTAGTTGCCATCCAGCGCTTCGTCTTCA
                                   *     ** ***                     **     ***    *  *   *

Z._mays_CSLF3_NP_001147926.1    ------------CACTAGCGATCATGGG---G-----------------CGG--------
Z._mays_CESA11_NM_001111766     ------------TCTTCTCCCTCCTGTG---GGTCAAGATCGACCCATTCGT--------
C._graminicola_GLS1_EFQ30502    AACTGATCATCTCACTCACCCTCACACGTGAGATTAAGACGGACGCGGCCAACATTGCAT
                                             *  *  **    *   *               *

Z._mays_CSLF3_NP_001147926.1    --TGGGCG---AA--GAGGCC------A--ATCATCC----TTGTAGT------------
Z._mays_CESA11_NM_001111766     --TGGAGG---AACCGAGACC------GTCAACACCAACAACTGCAACACACA-------
C._graminicola_GLS1_EFQ30502    TCTGGACGGGCAAGTGGTACTCTATGGGATGGCACTCGATCTCGCAACCCGCTCGCGAGT
                                  ***  *   **  *   *           **         * *

Z._mays_CSLF3_NP_001147926.1    ---TCTGC-------------TG-------------------------CCGGTTGTCT-T
Z._mays_CESA11_NM_001111766     ---TCTGC-------------TGA-----------------TTCACCATCGGTCAGCTGC
C._graminicola_GLS1_EFQ30502    TCCTCTGCAAGATTACGGAGCTGAGCATGTTTGCGGCTGACTTCATCTTGGGTCACTTTT
                                   *****             **                              ***    *

Z._mays_CSLF3_NP_001147926.1    TGCACTT-----------------------------------------------------
Z._mays_CESA11_NM_001111766     TGT-CGT-----------------------------------------------------
C._graminicola_GLS1_EFQ30502    TGCTCTTTATCATGCTACCGGTCATTCTCATTCCCAAGATTGACATGCTGCATTCCATGA
                                 **  * *

Z._mays_CSLF3_NP_001147926.1    ----------GTCGCG------------CT------------------------------
Z._mays_CESA11_NM_001111766     ----------GCCGCGGCGGACGTGTTTCT------------------------------
C._graminicola_GLS1_EFQ30502    TGCTCTTCTGGCTGCGCCCTGGGTATGTCTTCATTCTGATTCTGAACTCCTTGAGCAAAC
                                          *  ***           **

Z._mays_CSLF3_NP_001147926.1    ----------------GTTGTA---------------------------------------
Z._mays_CESA11_NM_001111766     ----------------GGTGT---------------------------------------
C._graminicola_GLS1_EFQ30502    TACTAACATTTTCTCAGTCGTCAAATCCGCCCGCCCATCTACTCGATGAAGCAGTCCAAG
                                             *  **

Z._mays_CSLF3_NP_001147926.1    ------TGTAGGTATCCATATC----------------CTACTTGCTG---GTCTGAT--
Z._mays_CESA11_NM_001111766     ------TGCAAACGTGGGTTGC----------------CTGCCTGATGCGGGTCTCCTC-
C._graminicola_GLS1_EFQ30502    CTGAGGCGCAAGCGCGTATTCCGTTACGCCATCCTCTACTTCCTCATGTTCATCCTCTTC
                                       * *        *  *                ** * *  **    **   *
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```
Z._mays_CSLF3_NP_001147926.1    ----------------------------TC-----CAT------TCTAGGT---------
Z._mays_CESA11_NM_001111766     --------------------------TGTC-----TATCTCGCATCTGGGCTTTTGCCCC
C._graminicola_GLS1_EFQ30502    ATGGCCCTCATGATTGGGCCCGCTGTTGTCGGTGGCATGATTCCTATGGACACTT-TCAA
                                                        **      **      * * *

Z._mays_CSLF3_NP_001147926.1    AGTATAGAAAACAT----TGTAATTT---CT--------TGTGTA---------------
Z._mays_CESA11_NM_001111766     AGGATCTGAAGCGGGTGGTGTAGGTTAGCTT--------TATTTT---------------
C._graminicola_GLS1_EFQ30502    GATGTTGAACACGGCGGATCTGGCTCTCATTCAACCCACTATTTACAACAACGACAACAC
                                    *   *  *      * *   *     *        * * *

Z._mays_CSLF3_NP_001147926.1    -------------------GTTACC--AAGGA---ACCAAT--------TATGTTTT---
Z._mays_CESA11_NM_001111766     -------------------GCGTCC--AAGTGTTGATTGATGTTG--TCTGTGTTATGAA
C._graminicola_GLS1_EFQ30502    GCATGAATCATCGGCAACGGGTACCGGAAGACCAGACTACTCTGGAGCCTATCTTACGAT
                                                  *   **  ***     *    *        * * **

Z._mays_CSLF3_NP_001147926.1    ---TT--------------TTAG-----------------------------------
Z._mays_CESA11_NM_001111766     AAGTT--------------TTGGTGGTGA--AACCTGA--------------------
C._graminicola_GLS1_EFQ30502    GACTGGGAGCGCCTCCGCATCGGCGACGAGCAACTCGGAGAGAGTGCGCTTGTTTTAA
                                   *                *  *
```

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List of scientific publications and abstracts

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Declaration (Erklärung)

Hiermit erkläre ich an Eides Statt, dass ich mich mit der vorliegenden wissenchaftlichen Arbeit ertmals um die Erlangung des Doktorgrades bewerbe und diese Arbeit noch bei keiner anderen Universität oder Fakultät eingereicht wurde. Außerdem erkläre ich, dass diese Arbeit von mir selbst und nur unter der Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde. Alle aus anderen Arbeiten und Veröffentlichungen verwendeten Wortlaute, Erkenntnisse und Ergebnisse wurden mit entsprechenden Quellenangaben gekennzeichnet.

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