Host Induced Gene Silencing - strategies for the improvement of resistance against *Cercospora beticola* in sugar beet (*B. vulgaris* L.) and against *Fusarium graminearum* in wheat (*T. aestivum* L.) and maize (*Z. mays* L.)

Dissertation

A thesis submitted for the degree of Dr. rer. nat. (*rerum naturalium*)
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by

Cornelia Stärkel

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A. Temming

Professor Dr. Axel Temming
Leiter des Fachbereichs Biologie

Universität Hamburg
DER FORSCHUNG | DER LEHRE | DER BILDUNG

UHH · Fachsprachenzentrum (FSZ) · Von-Melle-Park 5, D-20146 Hamburg

Fachsprachenzentrum
Leiterin
Dr. Henriette Javorek

English Language Evaluation

24. März 2011

Tel. 040-428 38 6757 Fax 040-428 38 7218
E-Mail: henriette.javorek@uni-hamburg.de

To whom it may concern,

This letter is to certify that the English in the dissertation "RNAi based strategies for the enhancement of resistance against fungal diseases in sugar beet (*B. vulgaris*), wheat (*T. aestivum*), and maize (*Z. mays*)", submitted for the degree of Dr. rer. nat. (*rerum naturalium*) to the Biology Department, the Faculty of Mathematics, Informatics and Natural Sciences, by Cornelia Stärkel, born 12.11.1981 in Berlin, Germany, fulfills the language requirements of the University of Hamburg.

Universität Hamburg
Fachsprachenzentrum
Von-Melle-Park 5 · 20146 Hamburg

Sincerely,

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Abbreviations

aa	amino acid
alcA	*Aspergillus nidulans* alcohol dehydrogenase A
Amp	ampicillin
ATP	adenosintriphosphate
bar	phosphinothricin acetyl transferase
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CM	complete medium
CamV	cauliflower mosaic virus
Cre	cre-recombinase
CTAB	cetyl trimethyl ammonium bromide
cv	cultivar
2,4-D	2,4-dichlorophenoxy acetic acid
DIC	differential interference contrast
DHS	deoxyhypusine synthase
DEPC	diethylpyrocarbonate
DIG	digoxygenin
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTPs	desoxynucleotide triphosphate (s)
DON	deoxynivalenol
dai	days post inoculation
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
dsRed	*Discosoma* sp. red fluorescent protein
dsRNA	double stranded RNA
dUTP	desoxyuracil triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eIF5A	eukaryotic initiation factor 5A
Fgl1	*Fusarium graminearum Lipase 1*

FHB	Fusarium Head Blight
gDNA	genomic DNA
GFP	green fluorescent protein
Gpmk1	*Gibberella* pathogenicity MAP kinase 1
GUS	glucuronidase
HIGS	Host Induced Gene Silencing
hph/hyg	hygromycin B phosphotransferase
HSP	heat shock promoter
HR	hypersensitivity response
kb	kilo bases
kDa	kilo Dalton
LB	Luria-Bertani medium
MAPK	mitogen activated protein kinase
MCS	multiple cloning site
MIPS	Munich Information Center for Protein Sequences
miRNA	microRNA
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
nptII	neomycin phosphotransferase
OD	optical density
ORF	open reading frame
35s promoter	cauliflower mosaic virus 35s promoter
PCR	polymerase chain reaction
PEG	polyethylene glycol
PTGS	post-transcriptional gene silencing
qPCR	quantitative real time PCR
RIP	repeat induced point mutation; ribosome inactivating protein
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
rpm	rounds per minute

rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
sRNA	short RNA
ssRNA	single stranded RNA
T0, T1, T2	original regenerated plant, first and second daughter generation
T35s terminator	cauliflower mosaic virus 35s terminator
TBE	TRIS-Borate-EDTA
Tri	trichothecene synthase gene
Tris	tris-(hydroxymethyl) aminomethane
tRNA	transfer RNA
ubi promoter	plant specific ubiquitin promoter
UTR	untranslated region
WT	wild type
YPG	yeast extract peptone glucose

Introduction

Fungal plant diseases

Fungi are the largest group among the biological causes of plant diseases. A wide variety of pathogens, especially in the ascomycetes, causes billions of dollars in losses among all cash crops worldwide every year. Fungal diseases are sometimes highly specialized, but have a broad host range in other cases. Common examples of plant pathogenic fungi are *Magnaporthe oryzae*, the rice blast fungus, or *Botrytis cinerea*, the powdery mildew on grapes.

Plant pathogenic fungi face the challenge to overcome the hurdle of the cell wall to enter the host plant. They have subsequently developed very efficient and sophisticated mechanisms to breach that barrier. The way fungi penetrate the surface depends on their mode of living, e.g. rust fungi and powdery mildew are biotrophic and require living tissue. They utilize appressoria which develop on top of stomata and prevent these natural openings from closing while releasing a penetration hypha in the substomatal space where it gives rise to the haustorium (Mendgen & Deising, 1993). Other fungi like *Venturia inaequalis* (Apple Scab), *Colletotrichum graminicola* (Anthracnose Leaf Blight of maize), and *Drechslera tritici-repentis* (Tan Spot of wheat) penetrate the epidermis directly, using appressoria which generate a high turgor pressure and destroy the host tissue punctually. A well studied example of appressoria formation is *M. oryzae*, the rice blast fungus, whose appressoria are melanized and dome shaped and generate a turgor pressure of more than 80 bar (Howard *et al.*, 1991). Another group of fungi have less clearly defined infection structures, like *F. graminearum*, the wheat scab fungus, or *R. solani*, which causes the damping-off syndrome. *F. graminearum* has recently been reported to penetrate host tissue by means of appressorium like infection cushions, developing coralloid subcuticular hyphae and bulbous infection hyphae upon growing intracellularly (Boenisch & Schaefer, 2010, Rittenour & Harris, 2010). *Cercospora beticola*, which causes the leaf spot on sugar beet, has been shown to enter the host leaves by passive invasion through the stomata followed by intercellular growth (Feindt *et al.*, 1981).

The pathogen *Cercospora beticola*

C. beticola belongs to the ascomycetes and is an economically important foliar pathogen of sugar beet (*Beta vulgaris*). The *Cercopora* family has many members that have different hosts like tobacco, soybean, coffee, rice, corn, and peanut (Daub & Ehrenshaft, 2000). *C. beticola* propagates by macroconidia, the asexual spores, which overwinter in plant debris on the field

and are spread in spring by wind and rain splash. Once a spore lands on a leaf, it germinates and penetrates the leaf surface. Inside the leaf, the fungus grows intercellularly in a hemibiotrophic mode. Favourable conditions for the pathogen are a humid and warm climate. When the leaf spots are fully developed, conidiophores emerge from the lesions and disperse new inoculum (Feindt *et al.*, 1981). The life cycle of *C. beticola* is depicted in figure 1.

Characteristic symptoms of *C. beticola* infection are brown leaf spots visible both on the adaxial and abaxial side of the leaf. From spring to summer the spots increase as the disease progresses and finally coalesce so that the entire leaves turn brown and shrunken. The leaf spot disease is economically very important since the destruction of leaves is limiting photosynthesis and compromising yield.

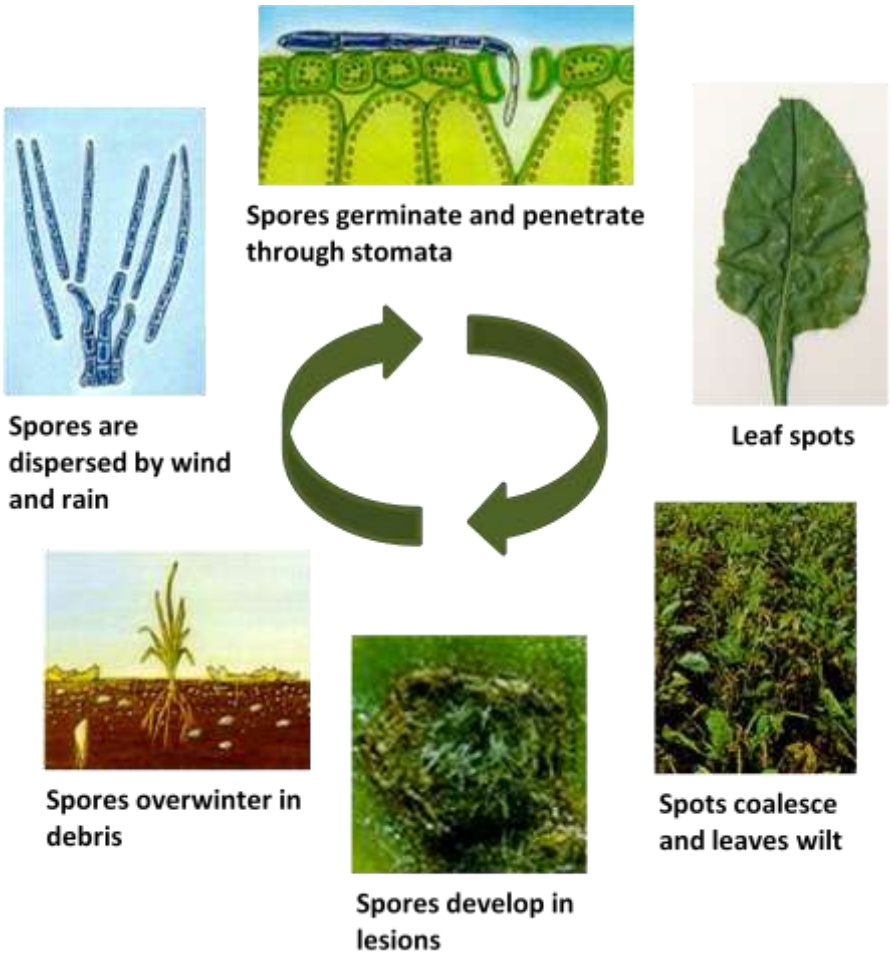


Figure 1 The life cycle of *C. beticola*
C. beticola is a foliar pathogen of sugar beet. Spores germinate on the surface and the emerging hyphae penetrate through the stomata. The mycelium then grows intercellularly and the toxin cercosporin produced by the fungus destroys the leaf tissue. Finally, in the older lesions conidia are formed that overwinter in plant debris (picture adapted from www.sbreb.org).

The cercosporin biosynthesis pathway

Different *Cercospora* species produce cercosporin, a photo-activated toxin which generates reactive oxygen species (ROS) at light exposure. The cercosporin biosynthesis pathway in *C. nicotiana*, a close relative of *C. beticola*, includes eight genes (*CTB1-CTB8*). *CTB1*, a polyketide synthase, condenses and decarboxylates the precursors malonyl-CoA and acetyl-CoA to form a polyketide. It then catalyzes the so called Claisen condensation and ring closure of the molecule. The following steps are oxidation and hydration, executed by *CTB3*, an O-methyltransferase and FAD- dependent monooxygenase, and *CTB5*, a FAD/FMN- dependent oxidoreductase, as well as *CTB6*, a NADPH-dependent oxidoreductase, and *CTB7*, another FAD/FMN-dependent oxidoreductase. The next reaction

is methylation carried out by *CTB2*, a different O-methyltransferase, and *CTB3*. The resulting polyketomethylene is dimerized and then exported from the cells by *CTB4*, a major facilitator superfamily transporter. This export mechanism leads to autoimmunity of *C. beticola* against its own toxin. The cercosporin expression is regulated by a zinc finger transcription factor, *CTB8*. Previous studies have shown that inactivation of *CTB1*, *CTB2*, *CTB3*, or *CTB8*, respectively, leads to a feedback transcriptional inhibition of the entire gene cluster and inhibits cercosporin production in *C. nicotiana* (Chen *et al.*, 2007b). The cercosporin biosynthesis pathway is shown in figure 2.

A cDNA data base revealed various differentially regulated genes among cercosporin resistant and -susceptible *C. nicotiana* strains. Upregulated in the resistant strain were a glutathione-S-transferase and disulfide isomerase, which are necessary for the response to oxidative stress, and an uracil transporter in the immediate vicinity of the cercosporin cluster. Downregulated were a flavohemoprotein, several multidrug transporters, and a cyanide hydratase which is essential for cell detoxification (Herrero *et al.*, 2007).

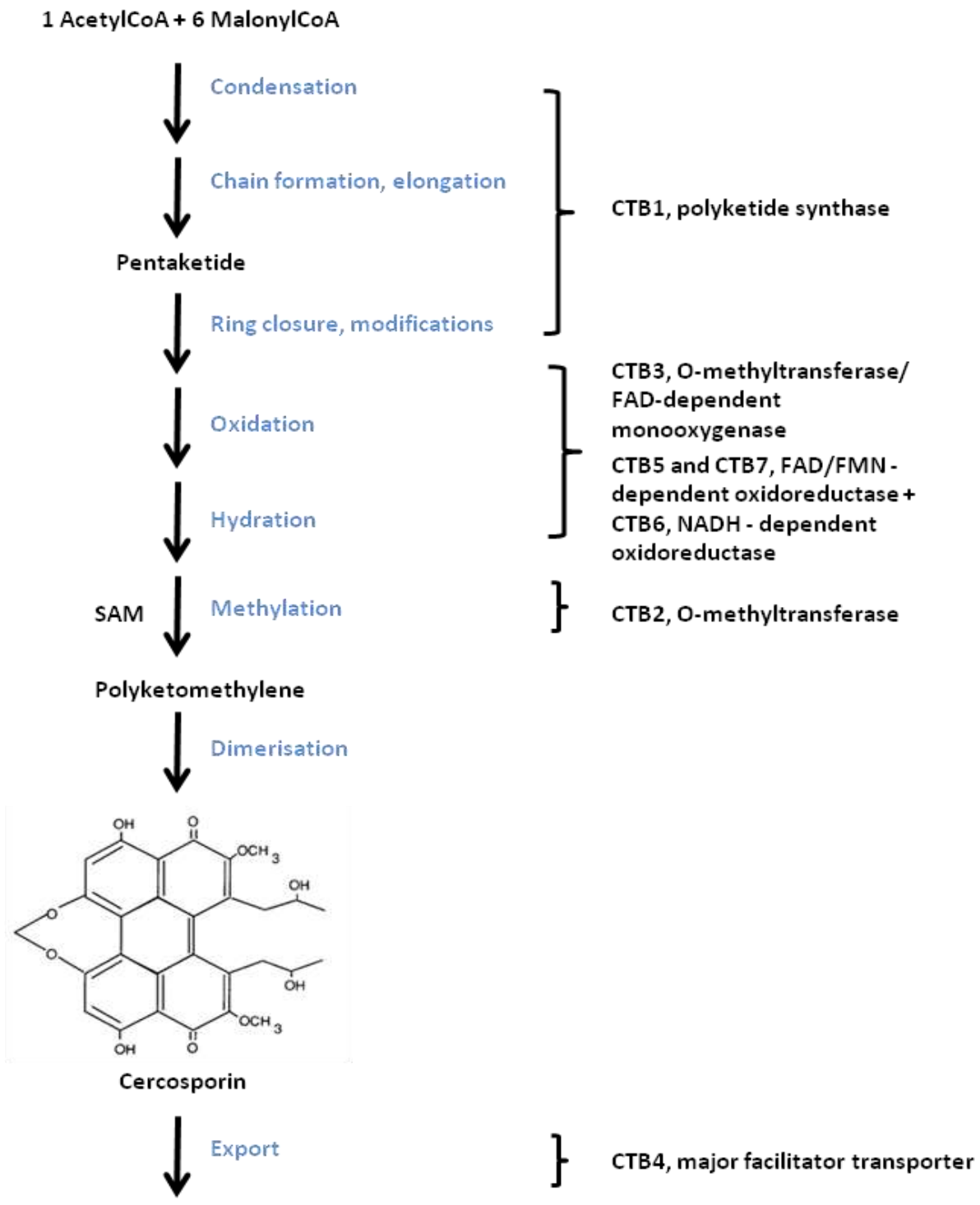


Figure 2 The cercosporin biosynthesis pathway

Cercosporin is a photoactivated toxin produced by many *Cercospora* species. It is produced from acetylCoA and malonylCoA by 6 enzymes (*CTB1*, *CTB3*, *CTB5*, *CTB7*, *CTB6*, and *CTB2*). Cercosporin is secreted by *CTB4*, a specific transporter. The genes in the cercosporin biosynthesis cluster are regulated by *CTB8*, a transcription factor. SAM: S-adenosyl-methionine.

The pathogen *Fusarium graminearum*

The filamentous fungus *F. graminearum* is one of the causal agents of Fusarium Head Blight (FHB), a devastating disease on wheat, barley, and other small grains (Bai & Shaner, 2004, Goswami & Kistler, 2005). The fungus also causes Ear Rot on corn and is responsible for billions of dollars in economic losses in the important cereal production areas like Northern Europe, the USA, the Ukraine, China, and the Middle East (De Wolf *et al.*, 2003, O'Donnell *et al.*, 2004). Infection of wheat causes the kernels to shrivel, leading to small and underweight grains. Additionally, it contaminates the remaining grains with mycotoxins, mainly deoxynivalenol, which inhibits protein biosynthesis, and zearalenone, an estrogenic mycotoxin. These components cause vomiting, liver damage, and reproductive defects in livestock and are harmful to humans through contaminated food.

Different resistance types in wheat against *F. graminearum* have been identified by classic breeding, some conferring resistance to initial infection, some inhibiting the spread of the fungus in the wheat head (Mesterhazy *et al.*, 2008). Despite great efforts to breed varieties with strong resistance and high yield, no completely resistant wheat cultivar is currently available. Research on the biology of *F. graminearum* is directed towards better understanding the mode of infection and to elucidate ways to inhibit this powerful pathogen.

F. graminearum is a haploid homothallic ascomycete. In the field, it overwinters in plant debris on the soil, where the mycelium grows and gives rise to fruiting bodies, the perithecia. Inside these small black structures, the ascospores develop which are forcibly discharged when the perithecia are mature and break open. The ascospores are dispersed by wind and rain splash and land on the susceptible parts of the host plants, mostly the flowers, to germinate. The scab disease is monocyclic; after one cycle of infection with ascospores, the fungus produces macroconidia by asexual reproduction. They overwinter in the soil or in plant debris on the field and give rise to the mycelium in the next season (figure 3).

F. graminearum infects wheat spikes in a short window of time from anthesis through the soft dough stage of kernel development. The fungus enters the plant mostly through the flowers, then grows from the ovary through the rachis nodes upwards and downwards (Brown *et al.*, 2010, Ilgen *et al.*, 2009). Germ tubes colonize the anthers, but also seem to be able to penetrate the hard, waxy surface of the lemma and palea which protect the flower (Leonard & Bushnell, 2003, Boenisch & Schaefer, 2010). From the infected spikelet, the fungus can grow through the rachis and cause severe damage in a short period of time under favorable conditions of high temperature and humidity. After the spores have germinated on the anthers

and the surface of the developing kernel, hyphae penetrate the epicarp and spread through the seed coat. Successively, the different layers of the seed coat and finally the endosperm are colonized and killed (Jansen *et al.*, 2005). Trichothecene mycotoxins produced by *F. graminearum* are known to inhibit protein biosynthesis in eukaryotes and to contribute to fungal virulence (Desjardins *et al.*, 2000). Several studies proved that loss-of-function mutants defective in trichothecene biosynthesis are normal in growth and development but reduced in virulence on seedlings of wheat and winter rye (Desjardins *et al.*, 1996, Desjardins *et al.*, 2000, Harris, 2005). Further studies indicated that deoxynivalenol (DON) production is not necessary for initial infection but facilitates spread of the fungus within colonized spikes (Bai *et al.*, 2002, Maier *et al.*, 2006). So far it remains unknown which components trigger DON production *in planta*. Recent studies have found that a combination of amine supplements and low pH significantly increases DON production in liquid culture (Gardiner *et al.*, 2009).

The genome sequence of *F. graminearum* is available at www.broad.mit.edu/annotation/fungi/fusarium. Although *F. graminearum* can be genetically modified by homologous recombination (Bowden & Leslie, 1999), the complex molecular mechanisms underlying pathogenesis in *F. graminearum* are not fully understood. Only a limited number of virulence factors in addition to trichothecenes have been identified (Cumagun *et al.*, 2004). Two mitogen-activated protein (MAP) kinase genes, *Mgv1* and *Pmk1*, are important for pathogenicity in *F. graminearum* (Jenczmionka & Schaefer, 2005, Hou *et al.*, 2002, Jenczmionka *et al.*, 2003). The *Gpmk1* mutants are defective in colonization of flowering wheat heads and in spreading from inoculated florets to neighboring spikelets (Jenczmionka *et al.*, 2003). Further analysis indicated that *Gpmk1* regulates the early induction of extracellular endoglucanase as well as xylanolytic and proteolytic activities and is responsible for the overall induction of secreted lipolytic activities (Jenczmionka & Schaefer, 2005). One of the genes regulated by *Gpmk1* is *Fgl1*, which encodes a secreted lipase and is an important virulence factor (Voigt *et al.*, 2005).

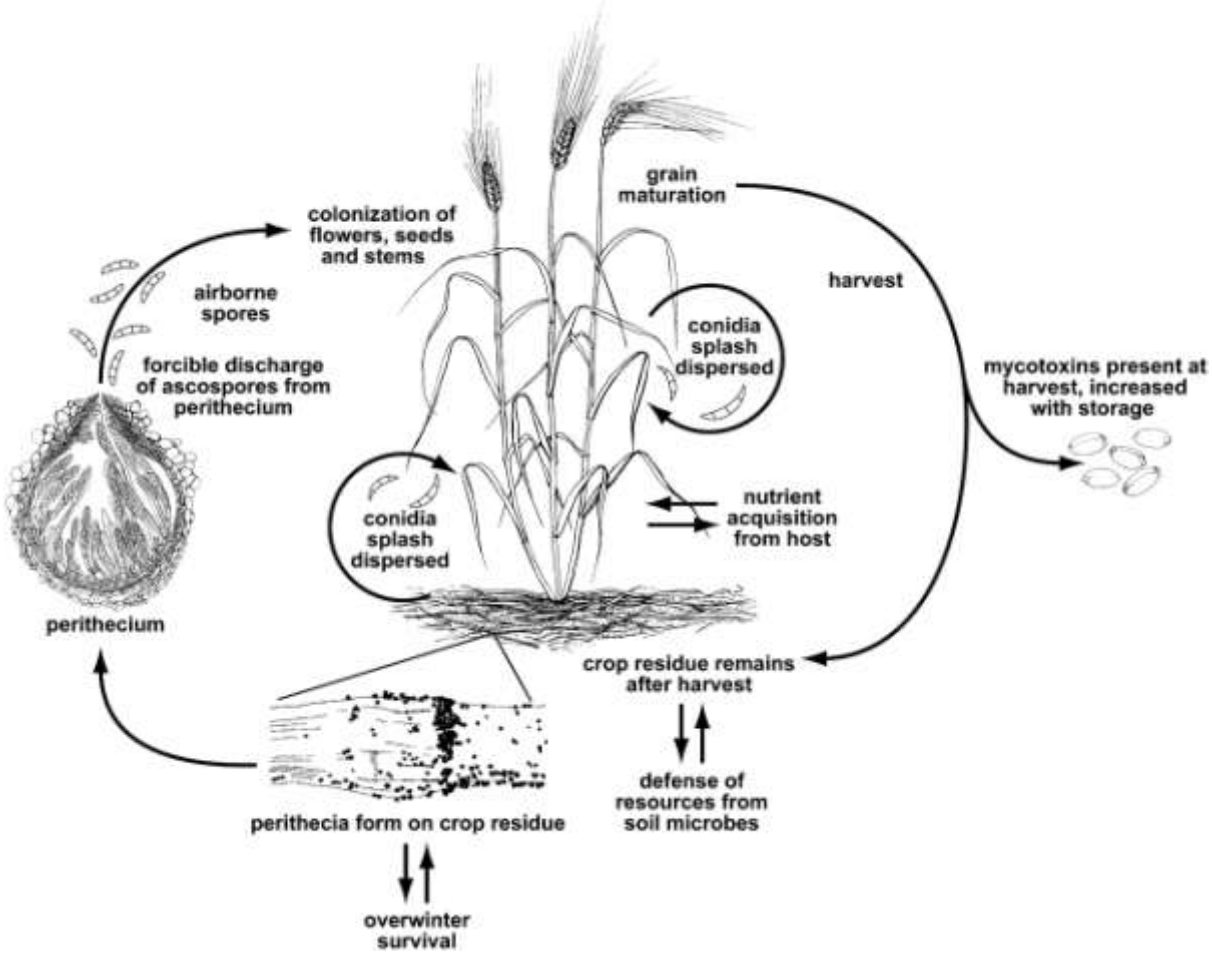


Figure 3 The life cycle of *F. graminearum*
F. graminearum ascospores are discharged from the perithecia and land on flowers of wheat heads, were they germinate and the germ tubes enter the host. The fungus grows in the flowers in a necrotrophic mode, kills the host tissue and contaminates the remaining kernels with mycotoxins. Conidia, the asexual spores, are formed as overwintering structures. New perithecia develop on crop residue and release ascospores in the next season (Trail, 2009).

Lipase 1 (*Fgl1*) is an important virulence factor in *F. graminearum*

Lipases catalyze the hydrolysis and synthesis of ester bonds. They are ubiquitous in nature and widely used for industrial applications, e.g. in pharmaceutical, cosmetic, and cleaning detergent products. For industrial purposes, lipases are often generated as recombinant proteins in fungal cultures, e.g. from *Candida ssp.*or *Aspergillus*. Secreted lipases of *F. graminearum* have previously been characterized as important virulence factors (Voigt *et al.*, 2005).

Plant epidermal cells contain cuticular waxes consisting of a soluble complex mixture of long-chain aliphatic compounds such as fatty acids, aldehydes, alkanes, primary and secondary alcohols, ketones, and wax esters (Agrios, 2005). The role of secreted lipases for fungal

virulence in *B. cinerea* that causes grey mould on various plants has been controversely discussed. While one study found that *Lip1* from *B. cinerea* was required for fungal penetration, infection and lesion formation on tomato leaves and that symptoms caused by *B. cinerea* were completely suppressed when anti-lipase antibodies were added to a conidial suspension prior to inoculation (Commenil *et al.*, 1998), another group showed that *Lip1* is dispensible for leaf penetration (Reis *et al.*, 2005).

The genome of *F. graminearum* contains a significant number of putative lipase genes, especially for secreted lipases. In a previous study, 16 genes encoding secreted lipases in *F. graminearum* strain 8.1 were identified and all were characterized by gene disruption. It turned out that *Lipase 1*, *2*, and *5* are pathogenicity factors in *F. graminearum*, while *Lipase 3* seems to be an essential gene (Nguyen, 2008).

Homoaconitase is an essential gene in *F. graminearum*

Homoaconitase was chosen as a gene of interest in this study because it is an essential gene in *F. graminearum* and unique in its function. *Homoaconitase* is an outstanding enzyme since it enables fungi to generate lysine independently from the diaminopimelate pathway, utilizing the α-aminoadipate pathway instead (Vogel 1960; Bhattacharjee 1985). While plants and other eukaryotes generate lysine from L-aspartate, higher fungi utilize homo-cis-aconitate from the citrate cycle to form the intermediate α-aminoadipate. The turnover from homo-cis-aconitate to homoisocitrate is carried out by *Homoaconitase* (figure 4). *Homoaconitase* has been previously deleted in the barley leaf pathogen *Pyrenophora teres*, rendering the fungus avirulent (Sonnenberger, 2002). This showed that *P. teres* was not able to recruit lysine from the plant for its survival and infection process.

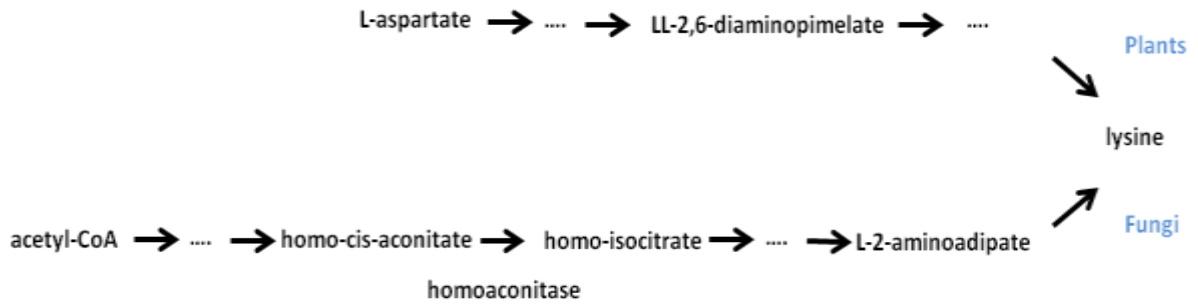


Figure 4 *Homoaconitase* is a key enzyme in the amino adipate pathway
Homoaconitase is a key enzyme in the lysine biosynthesis from acetyl-CoA in fungi. Plants generate lysine from L-aspartate and lack *Homoaconitase*.

Classical resistance breeding and genetic engineering approaches

In modern agriculture, it is a constant challenge to identify resistance genes which can be used in classical breeding approaches. In many intensely cultivated species, genes conferring resistance to the most detrimental diseases are simply missing. In wheat, e.g., there are different quantitative trait loci (QTLs) responsible for resistance against *F. graminearum*. Three main types of resistance can be distinguished: The first confers resistance against initial infection, the second resistance against the spread of the disease in the spikelet (Schroeder & Christensen, 1963), the third is responsible for accumulation of DON in the kernels (Miller *et al.*, 1985). Breeders are constantly trying to identify new loci and stack resistance traits. Initially infected florets are still contaminated with mycotoxins from the fungus. Although breeders worldwide try to cross naturally resistant plants from genetic hot spots with high yield cultivars and marker assisted breeding allows the stacking of quantitative trait loci, classical breeding, which is time consuming and always faces the impediment of drag of undesired traits, is reaching its boundaries.

Genetic engineering makes it possible to selectively overexpress or repress plant endogenous genes involved in the answer to pathogen challenge, or to induce genes conferring desirable traits from different species in a relatively short period of time.

The most obvious genes to employ for genetic engineering of resistance are the ones involved in non host resistance. These genes confer resistance when a pathogen attacks a plant it is not compatible with, which happens most of the time, given the large number of microbes and the relatively small amount which is pathogenic, i.e. compatible with a host plant. Non host resistance includes the enforcement of physical barriers, the release of reactive oxygen species (ROS), and the accumulation of phytoalexins and pathogenesis related (PR) proteins. The hypersensitive response (HR) is an early answer to pathogen attack and includes lignin deposition in the walls of affected cells, ROS release and death of the cell in order to contain the infection.

Plant defense is triggered by elicitor molecules which can be released by the pathogen as products of the avirulence genes or generally from the microbial surface, or by the plant itself during pathogen attack (Agrios, 2005). The elicitors bind to receptors and trigger a signal cascade. Another set of proteins successfully employed for disease resistance are ribosome inactivating proteins (RIPs). These proteins inhibit protein biosynthesis in any species other than their own by glycosylation of 28S rRNA (Mundy *et al.*, 1994). Expression of RIP genes

from different species, e.g. from barley in tobacco, render some plants more resistant to fungi, in this case *R. solani* (Jach *et al.*, 1995).

Phytoalexins are another class of proteins which can be exploited for enhanced resistance. The phytoalexin resveratol from grapes, e.g., is produced by different stilbene synthases. If these genes are transformed into tobacco, they lead to resveratol production in this normally phytoalexin free species and reduce susceptibility to *B. cinerea* significantly (Hain *et al.*, 1993).

RNA interference in plant resistance

The phenomenon of RNA interference (RNAi) has first been described in plants when it was discovered that an additional copy of dihydroflavonol reductase led to the reduction of flavonoid pigments in petunia rather than to the expected enhancement of color of the flowers (Mol *et al.*, 1998). In 1998, Fire and Mellow published their groundbreaking paper on targeted gene silencing via the injection of dsRNA into the gonads of *Caenorhabditis elegans* (Fire *et al.*, 1998). Since then, a lot of time and effort has been dedicated to shed light on this interesting phenomenon.

The RNAi pathway

RNAi is a mechanism to protect animals, plants, insects, and fungi from foreign DNA as well as to regulate endogenous genes. The principle model of gene silencing by double stranded RNA is well established (figure 5). RNAi can silence genes on the translational and transcriptional level. DsRNA from foreign origin or gene duplication is processed into small interfering RNAs (siRNA) by a complex formed by three enzymes, TR-RNA binding protein (TRBP), protein activator of protein kinase (PACT), and *Dicer*. The siRNA then guides argonaute 2 (Ago2) and the RNA induced silencing complex (RISC) to the corresponding mRNA site. Ago2 cleaves and destroys the mRNA. Any siRNA homologous to a promoter region leads to the methylation of the histone 3 lysine 9 and lysine 27, which changes the chromatin structure to a greater density and inhibits transcription. Endogenous primary micro RNAs (pri-miRNAs) are transcribed by RNA polymerase II and processed by a protein complex of drosha- DGCR8 (DiGeorge syndrome critical region 8) to precursor miRNAs (pre-miRNA). Exportin 5 transports the pre-miRNAs out of the nucleus into the cytoplasm, where they bind to the *Dicer* complex. Mature miRNA targets the 3' untranslated region of homologous mRNA and inhibits translation. The mRNA is then degraded in processing-bodies containing the decapping enzymes DCP1 and DCP2 (Kim & Rossi, 2007). In plants,

RNAi plays a crucial role in both gene regulation and immunity. Endogenous small RNAs like miRNA and siRNA have been found to be important for antibacterial defense (Jin, 2008). Plants possess a large number of enzymes involved in RNAi silencing pathways, e.g. *Arabidopsis* has four type III- ribonuclease *Dicer*-like (DCL) proteins, six predicted RNA-dependent RNA polymerases (RDR), and ten predicted argonautes (Ago).

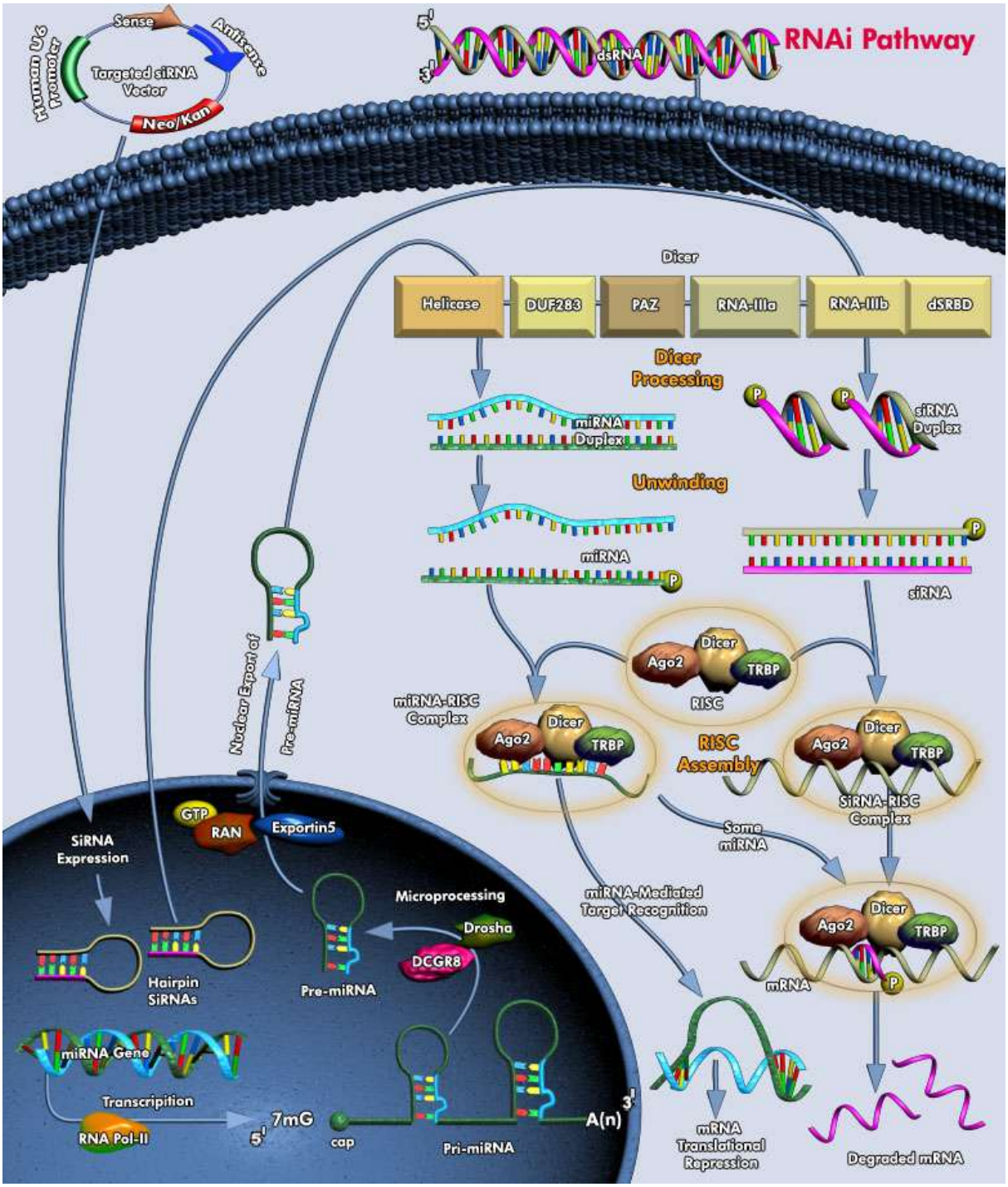


Figure 5 The RNAi and miRNA pathway

DsRNA, e.g. from a virus, is processed by *Dicer* to siRNA. *Dicer*, argonaute (Ago2) and TR-RNA binding protein (TRBP) form the RNA induced silencing complex (RISC), which unwinds the dsRNA, binds the mRNA, and cleaves the target sequence. Vectors with RNAi constructs lead to the formation of hairpin RNA, which is transported out of the nucleus and equally processed by RISC. Endogenous miRNA genes are transcribed by RNA Pol. II, pri-miRNA is formed, processed by drosha and DGCR8 (DiGeorge syndrome critical region 8) to precursor miRNAs (pre-miRNA), and shuttled through Exportin 5 into the cytoplasm, where it is also subjected to RISC (Qiagen, 2011).

Different classes of small RNAs

As the functions of small RNAs are being investigated, more and more classes can be specified and several categories of RNA signaling molecules have been identified so far in different species.

Short interfering RNA (siRNA) is one important group that includes double stranded, 20-24 nt long RNAs which target exogenous intruders, e.g. viruses, as well as endogenous complementary RNA, e.g. from retro-transposons. During the siRNA pathway, RNA dependant RNA polymerases (RdRPs) generate longer dsRNA precursors, which target a partially complementary sequence. Nuclear *Dicer* binds to the endogenous siRNAs, and the complex leaves the nucleus through Exportin 5. In the cytoplasm, RISC and *Dicer* recruit Ago 2, which cleaves the target. The siRNA molecules and *Dicer* can travel systemically, mediated by phloem small RNAi binding protein (PSRP-1) in plants and by systemic RNAi defect protein (SID-1) in animals (Naqvi *et al.*, 2009).

Among the siRNAs, trans-acting siRNA (tasiRNA) is unique to plants because it depends on RdRPs that animals lack. The templates for tasiRNA are endogenous genes.

Repeat-associated siRNA (rasiRNA) arises from transposon transcription and is likewise dependant on RdRPs, which are delivered by the transposon so that rasiRNA can be found in animals. This class of siRNA is important during gametogenesis in flies, worms, and mammals and is capable of silencing viral transcripts (Vagin *et al.*, 2006).

Another prominent type of RNA is micro RNA (miRNA), one major factor in regulatory pathways that work by repression of translation, mainly in animals, or target cleavage, mainly in plants. Translation is usually downregulated by miRNA, although some cases of upregulation seem to occur. The upregulation of translation by miRNAs in quiescent tumor cells has been previously observed (Vasudevan *et al.*, 2007). It was found that in serum starved cells, Ago 2 is associated with fragile X mental retardation protein 1 (FXR1). The miRNA/Ago2/FXR1 complex binds to the 3’ UTR, AU rich region of TNFα mRNA. By placing similar target sites in the mRNA of a reporter gene, the Vasudevan group found a downregulation in proliferating, but an upregulation in quiescent cells, which indicates that miRNA is capable of upregulation of translation under special circumstances and at specific time points during the cell cycle (Vasudevan *et al.*, 2007).

RNAi in fungi

In fungi, the probably best known RNAi related phenomenon is repeat induced point mutation (RIP). This mechanism is employed by *Neurospora crassa* to eliminate foreign RNA and all gene duplications by cytosine methylation leading to a change in chromatin density and G:C to A:T mutation during crosses (Selker & Stevens, 1987). RIP has conserved the *Neurospora* genome free of any mutations caused by gene duplication, a very remarkable reduction of mutations compared to other fungi. RIP and MSUD use two similar, but independent sets of enzymes: In RIP, QDE-1, a DNA helicase closely related to the human Werner-Bloom's-Syndrome protein family, unwinds the repetitive DNA. QDE-1, an RNA dependent RNA polymerase (RdRP) generates the dsRNA, which is processed by DCL-1 and DCL-2, respectively, the two *Dicer*- like homologs which are characteristic of all fungi. The mature siRNA is then bound by the RISC, which consists of QDE-2, an argonaute like protein, and QIP, an exonuclease that removes the passenger strand. During MSUD, aberrant RNA is processed to precursor dsRNA by a complex of SAD-1 (suppressor of ascus dominance) and SAD-2, RdRPs located in the nuclear periphery. The dsRNA is cleaved by DCL-1 in association with SNS-3 (suppressor of meiotic silencing), an argonaute like protein. The RISC processing the resulting siRNA includes SMS-2, another argonaute homolog (Li *et al.*, 2010). Quelling, a process similar to post- transcriptional gene silencing (PTGS) in plants, leads to transient inactivation of homologous sequences (Romano & Macino, 1992). *F. graminearum* employs RNAi and has two *Dicer* like proteins (FG09025.1 and FG04408.1) with similar domains (Segers *et al.*, 2007). If an organism contains more than one *Dicer* protein, each of them is assumed to be specialized, e.g. one is required for the miRNA, the other for the siRNA pathway (Tomari *et al.*, 2007). RNAi silencing has been studied in various fungi like *Podospora anserina, Aspergillus fumigatus, Fusarium oxysporum,* and *Magnaporthe oryzae* and has become of interest as a strategy to observe the function of essential genes by knock down, avoiding the lethal mutation of a gene deletion. RNAi is also being employed in transgenic plants in search for resistant and improved cultivars as well as in the pharmaceutical industry in research of vaccines against different diseases.

The role of *Dicer*

Dicer is an RNAse III type enzyme that processes long dsRNA precursors into 21-25 nt units during silencing. These siRNAs or miRNAs are loaded onto the RNA Induced Silencing Complex (RISC) and guide it to the target mRNA, triggering either mRNA degradation, the repression of translation, or the inhibition of transcription. The structure of *Dicer* is conserved

among eukaryotes and includes two RNAse III domains, a dsRNA binding domain, a RNA helicase domain, and a Piwi-Argonaute-Zwille-domain (PAZ) that binds to ssRNA 3' ends (Naqvi *et al.*, 2009). Different species vary in number of their *Dicer* homologs. Mammals, *C. elegans*, and *Schizzosaccharomyces pombe* have only one *Dicer* gene, while *D. melanogaster* has two and plants have four *Dicer* like (dcl) genes (de Haro *et al.*, 2009). It has been shown that the different *Dicer* genes have distinct functions, e.g. *Dicer* 1 in *D. melanogaster* generates siRNA, while *Dicer* 2 produces miRNA (Lee *et al.*, 2004). In *Arabidopsis*, DCL-1 produces miRNA, DCL-2 generates siRNAs in antiviral defense, DCL3 is necessary for chromatin modification, and DCL-4 leads to 21-nt trans-acting siRNAs (Henderson *et al.*, 2006). However, the separate functions of the *Dicer* genes are not absolute, and the homologs seem to have overlapping or redundant functions in different species.

Host induced gene silencing (HIGS)

Host Induced Gene Silencing (HIGS) is based on Virus Induced Gene Silencing (VIGS), which naturally occurs in plants and has evolved as a means of defense against viral infections (Lu *et al.*, 2003b). The virus produces dsRNA during its genome replication, which triggers the production of siRNA, leading to cleavage of viral dsRNA by the plant *Dicer* pathway. To utilize VIGS in genetic engineering, a ca. 300 bp fragment of the gene of interest is cloned into a DNA copy of the genome of an RNA virus. The plant is then transfected with the construct, which leads to dsRNA production and silencing of the endogenous copy of the gene of interest (Lu *et al.*, 2003a) . The most prominent disadvantage of VIGS is its transient nature (Gilchrist & Haughn, 2010).

HIGS is a further development of VIGS which allows the silencing of genes in plant pathogens by expressing an RNAi construct against specific genes endogenous to the pathogen in the host plant. HIGS is achieved by transformation of plant embryos or calli with a vector containing a sense-linker-antisense hairpin construct with a fragment of the gene of interest from the pathogen or a double promoter construct, which both lead to dsRNA production of pathogen specific sequence. When the pathogen attacks the host expressing a HIGS construct, the gene of interest can be downregulated in the pathogen. So far it is not clear whether the dsRNA is processed in the host plant and taken up by the pathogen or if long dsRNA precursors are transferred from the plant to the pathogen and processed by the RNAi apparatus of the pathogen. HIGS has previously been employed in different systems, e.g. in the barley- *B. graminis* interaction, to study host pathogen reciprocal effects (Nowara *et al.*, 2010). Further developments of HIGS include inducible and tissue specific promoters,

which could make it possible to switch on the silencing effect at a certain time point or to limit the transcription to defined plant organs.

Biolistic plant transformation

Biolistic plant transformation (Klein *et al.*, 1992) was used for wheat transformation in this study. Gold or tungsten particles are covered with the plasmid containing the desired construct and a selectable marker, then delivered under high pressure directly to the nucleus of plant embryo cells.

Agrobacterium mediated plant transformation

Agrobacterium tumefaciens is a gram negative, soil borne bacterium which causes tumors in various host plants by naturally infecting wounds in dicots. Since its discovery in the 1970s, *Agrobacterium* has become a powerful tool in genetic engineering, enabling researchers to shuttle plasmids into plant genomes for stable gene expression. *Agrobacteria* carry a plasmid encoding the so called Ti-plasmid, which includes genes for auxin, cytokinin, and opine synthesis, T-DNA, the transfer region with genes for the DNA shuttling, virulence genes which allow plant infection, and genes for specific opin catabolism. The *Agrobacteria* are able to sense plant roots in their environment in the soil by receptors for certain metabolites like acetosyringone or vanillin. The bacteria then move toward the plant roots and build a pilus from the bacterial to the plant cell (Brock, 1997). This pilus is formed by a membrane channel and different ATPases which drive the transport of the Ti-plasmid from the bacterium to the plant cell. The T- DNA is the only DNA transferred to the plant cell, where it targets the nucleus and inserts into the host genome. Before entering the channel, the T-DNA is restructured into a relaxed coil in order to pass the narrow tunnel (Chen, 2005). Auxin and cytokinin then trigger tumor growth, and the opines produced by the manipulated host tissue are metabolized by the bacteria.

Agrobacterium can be devided into a number of “biovars”: *A. tumefaciens* and *A. rubi* causing crown gall and cane gall disease, *A. rhizogenes* causing the hairy root disease, and *A. vitis* infecting grapevine. When the plasmid of one of these species is moved to another, the species will change to the other biovar (Gelvin, 2003).

Gene transfer mediated by *Agrobacteria* is very common, and protocols have been developed to transform both monocots and dicots. The gene of interest is cloned into a binary vector, which contains the right and left border, but has been modified by removing large sequences which are only necessary for the *Agrobacterium* survival. A selectable marker, usually an

antibiotic or herbicide resistance cassette, is included neighboring the right border. By this procedure it can be ensured that when the marker is delivered, the gene of interest is also very likely to be intact and expressed.

Brachypodium distachyon - a model grass species

Brachypodium distachyon (L.) Beauv is a grass species from the *Poaceae* family that also includes wheat and barley. It is native to the Mediterranean and the Middle East and provides great features as a model plant of the temperate grasses, like a small genome size of approximately 300 MB, a short life cycle of about six month from seed to maturity, a small and compact shape, and simple growth requirements. The genome of *B. distachyon* has been sequenced (Vogel *et al.*, 2010) and the data is available at http://files.brachypodium.org/. *B. distachyon* has also proven to be suitable for transformation and a detailed transformation protocol is available (Alves *et al.*, 2009). Recently, it was shown that *B. distachyon* is a good model for *F. graminearum* infection on wheat. When *B. distachyon* was infected, similar FHB symptoms developed and the spread of the fungus from spikelet to spikelet was clearly visible (Bluemke, unpublished data).

Deoxyhypusine synthase (DHS) as an example of an endogenous silencing target

Deoxyhypusine synthase was chosen as a gene of interest in this study as an interesting candidate for downregulation in wheat and maize. Hypusine is a rare amino acid which can be found among eukaryotes as an essential part of translation initiation factor eIF-5α (Park *et al.*, 1981). The precursor of eIF-5α is post translationally activated by hypusination, which is performed by two enzymes, deoxyhypusine synthase (DHS) that transfers the 4-aminobutyl moiety of a spermidine molecule to the ε-amino group of the eIF-5α-lysyl residue, and deoxyhypusine hydroxylase that carries out an oxidation. Essential for the hypsination of eIF-5α is the availability of spermidine, a compound from the polyamine group. Polyamines are a large family of molecules which are involved in various processes like cell growth and differentiation or apoptosis. Once activated by hypusination, eIF-5α acts as a translation initiation factor for a pool of mRNAs essential to ageing and senescence in *A. thaliana* (Thompson *et al.*, 2004). It plays an important role in shuttling these specific mRNAs out of the nucleus in an Exportin 4 dependant manner (Lipowsky *et al.*, 2000). The factor eIF-5α is highly conserved among eukaryotes and together with hypusine is essential for eukaryotic cell development, e.g. the knockout of eIF-5α in yeast was lethal (Park *et al.*, 2010). Vertebrates have two genes encoding two isoforms of eIF-5α, eIF-5α-1 and eIF-5α-2. The second homolog could be found exclusively in cancer cells (Clement *et al.*, 2006).

Moreover, eIF-5α is involved in pathogenesis of HIV. It activates the trans- activator- protein Rev that is located in the nucleus and interacts with the Rev- Responsive- Elements (RRE) in the viral genome in order to establish infection (Ruhl *et al.*, 1993). The eIF-5α mutants which block Rev activity inhibit HIV replication in human T-cells (Bevec *et al.*, 1996).

Goals of this study

The major goal of this study was to elucidate ways to use host induced gene silencing (HIGS) in the wheat - *F. graminearum* and sugar beet - *C. beticola* pathosystems. Several different approaches were taken:

Firstly, a gene silencing method was to be established in *C. beticola*, using reporter strains with GFP as an easily quantifiable target.

Additionally, *CTB2*, a gene in the toxin biosynthesis pathway, was to be characterized by gene deletion and to be evaluated as a target for gene silencing.

Most importantly, silencing constructs targeting *Fgl1*, a secreted lipase and major virulence factor of *F. graminearum*, and *Homoaonitase*, an essential gene in the lysine biosynthesis pathway, were to be cloned and transformed into wheat with particle bombardment for HIGS in *F. graminearum*.

Since *B. distachyon* is an important model plant for grasses which allows easier transformation and faster reproduction than wheat, *B. distachyon* transformation by *Agrobacterium* was to be carried out with an RNAi construct against *Fgl1*. Also, a silencing construct for maize transformation targeting *Fgl1* was to be cloned for a HIGS approach in maize.

During these experiments, *Homoaconitase* was to be further characterized in *F. graminearum* by deletion and downregulation.

Moreover, *Dicer 1* was to be deleted or silenced in *F. graminearum* to elucidate the role of fungal *Dicer* during HIGS and clarify whether the plant host or the fungus process the dsRNA generated by HIGS constructs.

Finally, previously generated transgenic wheat plants expressing an inducible RNAi construct against an endogenous gene involved in stress response and senescence, deoxy hypusine synthase (DHS), were to be characterized. To further investigate the role of DHS in plant development and stress resistance, a DHS silencing construct for maize was to be cloned and transformed into maize. Previously generated transgenic maize plants overexpressing DHS were to be analyzed to complete the picture of DHS function in maize.

Materials and Methods

Primers

The following primers were used in this study:

GFP silencing construct

JB pSilentGFPXhoI fw	CCGctcgagCGGGACCCTGAAGTTCATTTGCAC
JB pSilentGFPHindIII rev	CCCaagcttGGGGTGTTCTGCTGGTAGTGGTC
JB pSilentGFPKpnI fw	CGGggtaccCCGGACCCTGAAGTTCATTTGCAC
JB pSilentGFPBglII rev	GGAagatctTCCGTGTTCTGCTGGTAGTGGTC

These primers were designed by J. Boennighausen, University of Hamburg.

CTB2 gene disruption (underlined are the hygromycin overlapping parts)

CTB2 1F	CGCTAGATTTAGGTGTTGGA
CTB2 2R	agatgccgaccgaacaagagctgtccccGCAATCTTTCTTCCTATGCT
CTB2 3F	caatgctacatcacccacctcgctccccCGTTTCCAAGTCCAAGATCTG
CTB2 4R	CTCTTTCGTCCCTCGTATCTC
CTB2 5F	AACCTCCTTTGCGTATTCTC
CTB2 6R	ATGTTTCCGAGTTCTTGATGTG
CTB2 int F	AGCATAGGAAGAAAGATTGC
CTB2 int R	CAGATCTTGGACTTGGAAACG

Internal hygromycin primers

YgF	GTTGGCGACCTCGTATTGG
HyR	CTTACCACCTGCTCATCACCT

Fgl1 silencing in wheat

CS Fgl1 35S F SpeI	CTAGactagtCTGCCTTTGTCTCGAACCAG
CS Fgl1 sense R HindIII	CCCaagcttGGCGTGAGTCTTGATATACTCC

Fgl1 silencing in *B. distachyon*

LH1.2	CGAAGGCGGGAAACGACAAT
Pr2	CAAAATCCAGTGACCTGC

Homoaconitase silencing in wheat

Homoaconitase**35s2F**	ctagactagtACTTTCTCCCATTGTCGCTG
Homoaconitase**35s2R**	cccaagcttCTGTGCCCTGTTCAATAGTC

Inducible *Fgl1* RNAi construct

CSFgl1FXmaI	atgccccgggCTGCCTTTGTCTCGAACCAG
CSFgl1RMluI	atgcacgcgtGGCGTGAGTCTTGATATACTCC

Homoaconitase gene disruption (underlined are the hygromycin overlapping parts)

CS Homoaconitase **ko 1F**	CCCATCATTACCGAGTTCTG
CS Homoaconitase **ko 2R**	agatgccgaccgaacaagagctgtccccTGTCGATTATGTGATTGGTCG
CS Homoaconitase **ko 3F**	caatgctacatcacccacctcgctccccTACCGTTTCACTATTTGCTCGT
CS Homoaconitase **ko 4R**	ACACTGGAACACAATGGGAG
CS Homoaconitase **ko nestF**	CTTTGAGATTGCCAAGTCGTG
CS Homoaconitase **ko nestR**	GCTACCCTCCTGACATACCT
CS Homoaconitase **ko int F**	GGAAAGGATGTTATTATTGCCCTC
CS Homoaconitase**k ko int R**	CAGTCCGATACAAGGTCCAC

Inducible *Homoaconitase* silencing construct

CSHomoaconitase**pSW08BamHIF**	cgc ggatccACTTTCTCCCATTGTCGCTG
CSHomoaconitase**pSW08BamHIR**	cgc ggatccCTGTGCCCTGTTCAATAGTC

Dicer 1 gene disruption (underlined are the hygromycin overlapping parts)

CS *Dicer* 1F	CATGCCCAGGATAGATACCC
CS *Dicer* 2R	caatgctacatcacccacctcgctccccTGTGTCAGTAATTGATCCGT
CS *Dicer* 3F	agatgccgaccgaacaagagctgtccccGATCAGGGACAAGATTGCGA
CS *Dicer* 4R	CAGCGAATGGAGTTATTGGA
CS *Dicer* 5F	CCCAGCAATATCATCGATCC
CS *Dicer* 6R	CAGTCACTATCTGAATAGTCGT
CS *Dicer* int F	CCTGTAAGTGAAAGACTCTTCTG
CS *Dicer* int R	ACGGCCATGTTCAAATTGTC
CS gen XbaI F	gctctagaAATTCATGCCAGTTGTTCCC
CS gen BglII R	gaagatctTGGGTAAACGACTCATAGGA
CS gen NdeI F	gggaattccatatgAATTCATGCCAGTTGTTCCC
CS gen KpnIR	ggggatccTGGGTAAACGACTCATAGGA

Dicer 1 silencing

CS gen SacIF	cgagctcAATTCATGCCAGTTGTTCCC
CS gen SacIR	cgagctcTGGGTAAACGACTCATAGGA
CS D1RNAiBamHI F	cgcggatccAAAGTATTGCGGATGTCTGTG
CS D1RNAiBamHI R	cgcggatccGTTGCGATAGAGATATTCGAC

Maize DHS RNAi construct

CS DHS Zm s RNAi F EcoRI	cggaattcTCAACCAGATGTTAGACTGGA
CS DHS Zm s RNAi R AflII	agtcttaagATGTCTCCAAGTGATCCATCAG
CS DHS Zmas RNAi F BamHI	cgcggatccATGTCTCCAAGTGATCCATCAG
CS DHS Zm as RNAi R FseI	atcggccggccTCAACCAGATGTTAGACTGGA

DHS verification PCR

Cre_F	CCATCGCTCGACCAGTTTAG
Cre_R	TCGACCAGGTTCGTTCACTC
Bar_F	GGTCTGCACCATCGTCAACC
Bar_R	ACCACGTCATGCCAGTTCC

GUS probe for wheat RNAi construct

CS gus 1F	ACTGTGGAATTGATCAGCGT
CS gus 2R	CAGTTCATAGAGATAACCTTCACC

DHS RNAi construct PCR control

CS Ubi int F	CCTGTTGTTTGGTGTTACTTCTG
CS spacer GUS 3‘ R	ACCAACGCTGATCAATTCCA
CS AS2 F	GTTCTTGTATGCCCAATAAAGG
CS Ubi int F	CCTGTTGTTTGGTGTTACTTCTG

Wheat DHS qPCR

CS W Actin qF	CTCTTAGCACTTTCCAGCAG
CS W Actin qR	GAGGGTACACATCTTCTACAG
CS W DHS qF	AAATAAATGATGAAAGCTCCTACC
CS W DHS qR	TTGAGCCGTGTTGATATAGAC

DHS overexpression construct PCR control

DHS 1F	ATCCTGGCCTTATTATTGAC
DHS 1R	GTTTGAACGATCTCATTTGG
DHS 2F	GATCACTTGGAGACATGCTG
DHS 2R	TGCCAAATGTTTGAACGATCTC
CS Ubi int F	CCTGTTGTTTGGTGTTACTTCTG

Maize DHS qPCR

CS Maize DHS q F	TCCTGACACTGAAGTACCCGATTGA
CS Maize DHS q R	AACTGGCATCACACCTTCTACAACG
CS Actin Maize q F	GGCATACAAGAATAACATCCCT
CS Actin Maize q R	CTCCACCAAGAACTATAATCCC

The author's initials are abbreviated by "CS" for primer ordering according to current laboratory practices. Primers were ordered from MWG Operon, Hamburg, Germany, and sequencing was carried out by Starseq, Mainz, Germany.

Plasmids

The following plasmids were used in this study: pGEMT (Promega, Germany), pIGPapa (Lee *et al.*, 2003), pII99dsRed (Namiki *et al.*, 2001), pSilent-1 (FGSC), pSW08 (Dr. Rolf Prade, Oklahoma State University), pDB35SGus35S, p6U, p7iHspCre, pKSII, p153ActRNAi, pD1ubibarDHSRNAi, pCaNeo (Callis *et al.*, 1987), pMonGFP and pActIGus (all kindly provided by Dr. Dirk Becker, University of Hamburg). Vectors pActI-D and pMON349 were employed for Gus and GFP control transformation in wheat, respectively.

Plasmids were named according to their original appellation given by the person who first created the plasmid. The author added denominations of genes specifically cloned in this study. The use of the word "cassette" implies that the gene and a promoter and terminator were cloned in one piece.

Fungal and bacterial strains

For molecular cloning we used *E. coli* strain XL1-blue (Stratagene, LaJolla, CA). *Agrobacterium* strain GV31 was kindly provided by Dr. C. Voigt, University of Hamburg. The *C. beticola* isolates Ahlburg and Ferrara were kindly provided by Dr. D. Stahl, Planta GmbH. *F. graminearum* strain PH-1 (FGSC 9075, NRRL 31084) was kindly provided by Dr. H. Giese, Århus, Denmark. *Cochliobolus heterostrophus* C4 was kindly provided by K.Kroeger, University of Hamburg. *Colletotrichum graminicola* and *Setosphaeria turcica* were purchased from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Media

Bacteria were cultured in PDM liquid media or on LB plates with the appropriate selective antibiotics. *F. graminearum* and *C. beticola* were kept on CM agar or V8 (200 ml/l tomato juice) agar plates. While *F. graminearum* was cultivated in YPG liquid media for DNA

extraction, *C. beticola* was cultured in CM liquid media. For the selection of transformants, 50 µg/ml and 100 µg/ml hygromycin were used for *C. beticola* and *F. graminearum*, while 100 µg/ml and 200 µg/ml geneticin (Invitrogen, Darmstadt, Germany) were used for *C. beticola* and *F. graminearum*. Hygromycin was purchased from Duchefa, Haarlem, the Netherlands, and geneticin was ordered from Invitrogen, Darmstadt, Germany. Agrobacterium strain GV31 was grown on LB media with 100 µg/ml gentamycin, spectromycin, and rifampicin (Sigma Aldrich, Munich, Germany).

F. graminearum conidia formation was induced in carboxymethyl cellulose liquid culture at ambient light for 10 days. *C. beticola* conidia were produced on potato dextrose agar (Difco, Beckton and Dickinson, Heidelberg, Germany) with pH 5.6. *C. heterostrophus* was grown on CM, *C. graminicola* on oatmeal agar (50 g of oats per l), and *S. turcica* on lactose casein hydrolysate media (Dhingra & Sinclair, 1994).

Media recipes can be found in appendix A.

Special supplements

Media for *Homoaconitase* transformations were supplemented with 50 mM lysine.

Regeneration media for pSW08 transformants was supplemented with 50 mM threonin for induction.

Plant cultivars

Florida (KWS, Germany), a cultivar suitable for biolistic transformation, was used in wheat transformation experiments. Sugar beet cv 4D-0029 was kindly provided by Dr. D. Stahl, Planta GmbH, KWS AG, Germany. *B. distachyon* wild type was line Bd21 (Dr. C. Brown, Berkely University, CA, USA), and maize cvs were A188 (Green & Phillips, 1975) and HiIIA/HiIIB (Armstrong *et al.*, 1991).

Fusion PCR and double homologous integration

Fusion PCR is a method that allows fast and efficient cloning of several fragments (Szewczyk *et al.*, 2006). It has become a common method for generating gene deletion constructs in fungi, which consist of flanking parts of the gene of interest fused to an antibiotic marker that replaces the gene when the flanking regions pair with their endogenous match in the genome. In general, up to 1 kb flanks are amplified with two primer sets from genomic DNA. The marker cassette is released from a plasmid by restriction or amplified by PCR. The products

are purified and fused in a second PCR reaction without primers and the following PCR program:

95 °C	5 min	
95 °C	1 min	20 cycles
55 °C to 60 °C gradient	1 min	
72 °C	1 min per kb	
72 °C	10 min	

The PCR product is then used as the template in a third PCR with nested primers located ca. 100 bp inside the originally amplified flanks in order to facilitate amplification.

The final fusion product can be ligated into pGEMT by TA overhangs and released by restriction for fungal transformation.

Fungal transformation

Transformation of *F. graminearum* was carried out according to a modified protocol, integrating two approaches. Protoplasts were prepared as published before (Jenczmionka *et al.*, 2003) and PEG mediated transformation was carried out as previously described (Proctor, 1995). *C. beticola* was transformed with the same protocol as *F. graminearum*, but with buffers as used in the *C. nicotiana* transformation (Chung *et al.*, 2002). The recipes are listed in appendix A and the protocol is given in appendix B. Fungal transformations were carried out by B. Hadeler and C. Kroeger, University of Hamburg.

Toxin extraction from *Cercospora beticola* cultures

Cercosporin was extracted with 5 N KOH (Chung, 2003). PDA plates with pH 5.6 were evenly inoculated with *C. beticola* conidia and kept at ambient light and room temperature for 2 weeks. The agar was cut into small squares and covered with 5 N KOH overnight. Agar pieces were removed with a household sieve the next day. A 5 mM cercosporin standard was kindly provided by Dr. D. Stahl, Planta GmbH. The absorbance was measured in a spectrometer (Ultrospec 3000, Pharmacia Biotech) at 480 nm and the cercosporin content calculated by the molecular weight of 534.51 g/Mol (Sigma). After concentrating the extract in a vacuum evaporator and re- dissolving it in water, the toxin lost its activity. For testing on plants, cercosporin was extracted from PDA plates directly with water, which worked as well as extraction with KOH.

Plant inoculation

Sugar beet

Sugar beet plants were grown in a growth chamber at 18 °C with 18 hours light (20 000 lux, 400-600 nm = 4000 to 6000 K, Philips Master TLD 56 Watt Reflex Eco lamps) and infected at 3 month of age. To produce conidia for the plant inoculation, PDA plates (pH 5.6) were equally seeded with mashed mycelium and incubated in the dark for two weeks. The conidia were then carefully scraped off the surface, adding some sterile water and using a spatula. The scraped material was filtered through a small household sieve, one layer of miracloth, and a 200 µm Wilson sieve to completely remove all agar particles. The conidia were then counted in a Fuchs- Rosenthal hemocytometer.Ten week old sugar beet plants were inoculated by thoroughly applying a conidia suspension of 20 000 conidia/ ml and a drop of Tween 20 onto the adaxial and abaxial leaf surface by a spray bottle, using 50 ml of the suspension on each plant. The plants were covered in a foil tent for ten days and kept at 18 hours light, 24° C day, 18 °C night. The foil was removed after 10 days. For testing the effects of toxin on *in vitro* plants, one week old *in vitro* cultured plants were dipped into cell free plate extract, using water as the negative control.

Wheat

Wheat plants were cultivated in a green house and transferred to an infection chamber with 24 °C at day and 16 °C at night with 16 hours of light. A suspension of 200 conidia in a total volume of 20 µl was inoculated on each of the two central spikelets at the early stages of anthesis. The inoculated spikes were enclosed in small plastic bags misted with water during the first three days and monitored for three weeks.

Brachypodium distachyon

Plants were cultivated in a growth chamber with 24 °C at day and 16 °C at night, applying 16 hours of light. Spikelets with a developing flower were infected at about 6 weeks of plant age, at early flowering before the set of seeds. In 1 µl of suspension, 80 conidia were used per flower. Inoculated plants were kept under a plastic bag without additional misting for 2 days. The infection progress was monitored over 2 weeks.

Maize

The maize leaf assay was carried out with detached leaves of 6 week old maize plants. From the middle of the leaves, 8 cm long sections were taken, surface sterilized and placed in Petri dishes with a moist sterile filter paper. Conidia of *C. graminicola*, *C. heterostrophus*, and

S. turcica were produced by keeping plates for 3 weeks under near-UV light (TLD 36 W-08; Philips, Eindhoven, The Netherlands) and white light (TL 40 W-33 RS; Philips), harvested from plates with sterile water and a sterile glass rod, counted with Fuchs-Rosenthal hemocytometer, and adjusted to a concentration of 50 conidia per µl. One drop of 10 µl of the conidia suspension was placed in the middle of each leaf section. The plates were then sealed carefully with parafilm without disturbing the drop. The plates were kept at 24 °C for 7 days.

Disease rating in sugar beet

Disease ratings were carried out following a method that counts the spots and correlates the diseased surface area with the disease severity (Rossi & Battilani, 1989). Leaves were rated according to the following disease index table:

Necrotic leaf surface	Index
Spots < 1%	1
Spots 2-5%	2
Spots 6-10%	3
Spots 11-20%	4
Spots 21-40%	5
Spots 41-60%	6
Spots 61-80%	7
Spots 81-100%	8
Leaf dead	9

Microscopic analysis

Micrographs were taken using a stereo fluorescence microscope (Leica MZ FLIII) with the appropriate filter sets for dsRed or GFP.

DNA extraction from fungal material

Fungal DNA was extracted with the cetyl trimethyl ammonium bromide (CTAB) method (Cubero *et al.*, 1999).

DNA extraction from plant material

DNA was isolated from plant material following a previously established protocol (Pallotta *et al.*, 2000). In short, ca. 300 mg leaf material were put in a screw cap tube and frozen in liquid nitrogen after adding two steel spheres to each tube. The samples were ground in the Retsch mill at highest speed for 3 minutes and kept under liquid nitrogen before further processing. To each tube 800 µl extraction buffer (1% N-lauryl-sarcosin, 100 mM TrisHCl, pH 8, 10 mM

EDTA, pH 8, 100 mM NaCl) were added and the samples were vortexed thoroughly. Subsequently, 800 µl phenol- chloroform- isoamylalkohol (25:24:1) were added to each tube, samples were mixed vigorously, spun for 2 minutes at 5000 rpm, and the supernatant was transferred to a fresh tube. Next, 800 µl isopropanol and 80 µl 3 M sodium acetate were added to each sample followed by 3 minutes of centrifugation at 5000 rpm. The supernatant was discarded and pellets were washes once with 100 µl 75% ethanol, then air dried and resuspended in 50 – 100 µl of TE buffer, pH 8, with 40 µg/ml RNAse.

Optimal PCR results were achieved using the Phire Direct Plant PCR Kit (Finnzymes, Espoo, Finland).

Southern and western blot

Southern and western blots were carried out following standard procedures (Sambrook & Russel, 2001). Southern blots were probed according to the manufacturer's instructions using the digoxygenin dUTP (DIG) system and positively charged membranes (Roche, Mannheim, Germany). In each lane of the gel 5 µg of fungal DNA and 20 µg of plant DNA were separated. Plant DNA was restricted applying 5 µM spermidine (Sigma Aldrich, Munich, Germany). In the case of fungal samples, membranes were exposed to X-ray film for 30 minutes, in the case of plant samples for 4 to 8 hours.

RNA extraction and cDNA synthesis

RNA was efficiently isolated according to the following protocol (Hsieh, 2010): 200 mg leaf material were ground under liquid nitrogen in a Retsch Mill, extracted with 500 µl extraction buffer (200 mM NaCl, 50 mM Tris pH 8,8, 5 mM EDTA pH 8, ad 100 µl DEPC water, 1% SDS), mixed with 750 ml freshly prepared phenol: chloroform: isoamylalcohol (25 : 24 : 1) and centrifuged at full speed for 5 min. The supernatant was transferred to 1250 µl ethanol and the nucleic acids were precipitated for 30 min on ice. The mixture was then centrifuged for 20 min at full speed and 4 °C, and the pellet washed with 70% ethanol, air dried, and resolved in 400 µl DEPC water. In the next step, 400 µl 8 M LiCl was added and the mixture was incubated at -20 °C for 30 min before centrifuging for 30 min at full speed at 4 °C. The pellet was again washed with 70% ethanol and air dried, and then resolved in 40 µl DEPC water. RNA integrity was examined on a standard 3-(N-morpholino)-propansulfonic acid (MOPS) gel.

Synthesis of cDNA was carried out using components from Fermentas, St. Leon-Rot, Germany.

Quantitative Real Time PCR (qPCR)

QPCR was conducted in a Roche Light Cycler 480, using Roche SYBR Green Master Mix.

Plant transformation

Wheat

Wheat transformation was carried out with the Biorad PDS-1000/He-system (Biorad, figure 6) according to the manufacturer's instructions.

Figure 6 PDS 100/He system gene gun from Biorad
The rupture disc is located above the carrier disc with DNA coated gold particles. The wheat embryos are placed in a Petri dish with osmotic media and placed on the sample holder in the vacuum chamber. Helium gas is pumped into the chamber from above until the rupture disc bursts and the gold particles are forced from the carrier disc towards the embryos.

The protocol for preparation of wheat embryos, media and DNA coated gold particles was established and kindly provided by Dr. D. Becker, University of Hamburg (see appendix B). A detailed description of wheat transformation can be found in a recent review (Jones & Sparks, 2009). Before the bombardment, the embryos are isolated from the caryopses and pre-cultured, then moved to high osmotic pressure media. The plasmid covered gold particles are spread on a carrier, which is located between the rupture disc and the stopping mesh over the target embryos in a vacuum chamber. After drawing the vacuum, Helium gas is pumped into the space above the rupture disc to a critical pressure point, upon which the rupture disc bursts and releases the pressure to the carrier, pushing it to collide with the mesh and evenly force the gold particles towards and into the targeted cells. After the bombardment, the plants are allowed to regenerate in the dark before being moved to selection media with increasing stringency under light. Regenerated plants are finally moved to the greenhouse for further analysis (figure 7).

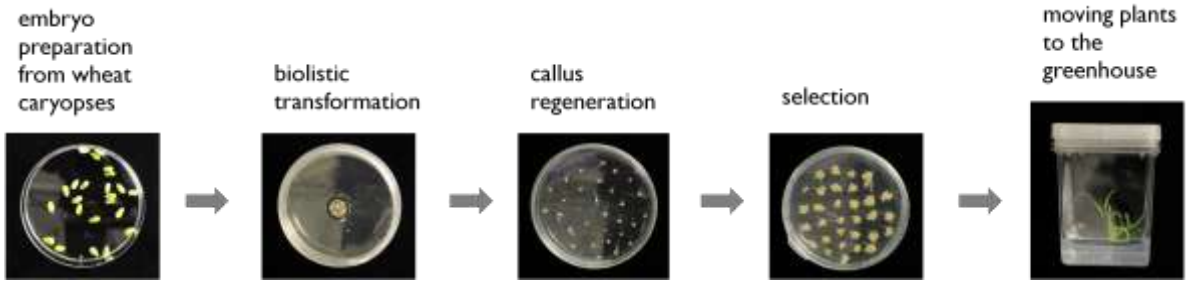


Figure 7 Biolistic transformation of wheat embryos
Wheat embryos are dissected from caryopses, collected on osmotic media, and transformed with the particle gun. The embryos are allowed to regenerate and developing calli are selected media with Basta. Surviving plants can be transplanted to soil.

Maize

Maize transformation was carried out by S. Amati, University of Hamburg, following an established protocol (Frame *et al.*, 2002). All potentially transgenic plants were sprayed with 250 mg/l Basta four weeks after transplanting to soil three times to verify Basta resistance. Plants were pollinated by harvesting pollen from the flowers in a paper bag and dispersing it equally over the freshly emerged silks. The procedure was repeated daily for one week to ensure sufficient pollination and seed set.

Brachypodium distachyon

The procedure of *Agrobacterium* mediated transformation in *B. distachyon* has been established recently (Alves *et al.*, 2009) and was carried out by K. Wolff, University of Hamburg. Embryos are isolated and precultured, then cocultivated with the transgenic *Agrobacteria*. Subsequently, they are moved to timentin (Duchefa, the Netherlands) containing media in the dark to stop *Agrobacteria* growth, then to media with timentin and the respective selectable marker in the light. After sufficient callus development and root as well as shoot induction, the small plants are transferred to the greenhouse. The advantages of *Agrobacterium* mediated transformation lie in the delivery of single, intact copies of the gene of interest, and the high efficiency in dicots. Seeds were planted in a 1 : 2 sand : soil mixture and stratified for 1 week at 4 °C before moving pots to the climate chamber.

Heat shock conditions in wheat and maize

Before undergoing heat shock, seeds were surface sterilized with 2% NaOH for 30 minutes, rinsed thoroughly with sterile water, and placed between two wet sterile filter papers in a Petri dish. The Petri dishes were sealed with parafilm and placed in a growth chamber in darkness and 22 °C for two days to allow pre-germination of the seeds. After that, the Petri dishes were placed in a chamber with 42 °C, 80% humidity and light for 4 hours in the case of maize and for 2 hours in the case of wheat, followed by 2 hours at 37 °C to facilitate the activity of the cre-recombinase, and 22 °C in the dark for the remaining time. This procedure was repeated on 4 consecutive days. On the next day, the seeds were transferred to soil in the greenhouse and covered with a plastic hood for one week to aid germination.

As a second method in wheat, seedlings were grown for one week before exposing them to 42 °C, 80% humidity and light for 2 hours on 4 subsequent days. Since this procedure was not efficient enough, the heat exposure time was extended to 4 hours at 42 °C, 80% humidity and light on 4 consecutive days.

Results

RNA interference based silencing in fungi

A preliminary goal of this study was to establish RNA interference based silencing in *C. beticola* to proof the concept of RNAi based silencing in this fungus.

Cercospora beticola GFP reporter strain

To generate the GFP reporter strain, protoplasts were transformed with the pIGPapa plasmid (Lee *et al.*, 2003) carrying the GFP cassette. The plasmid was linearized with *Hind*III. After 1 week, 40 transformants were isolated and more than 20 were confirmed to have GFP expression by fluorescence microscopy at 480 nm.

Cloning strategy for pSilent GFP geneticin

The vector pSilentGFPgeneticin was cloned by J. Boennighausen in his MSc thesis. The geneticin cassette was excised from pGEM-T (kindly provided by Le Thi Thu Giang, University of Hamburg) and ligated into the pSilent-1 vector backbone (FGSC, Kansas City, MO, USA) with *Aat*II and *Nde*I. The GFP sense and antisense fragments were amplified by PCR from pIGPapa with primers including the restriction sites for *Xho*I, *Hind*III, *Bgl*II, and *Kpn*I, respectively. Fragments were subcloned in pGEMT (Promega), released by digesting 40 µg overnight in a 500 µl reaction and consecutively cloned into pSilent GFP. The vector map of pSilentGFPgeneticin is shown in figure 8 (Boennighausen, 2010). For fungal transformation, the plasmid was opened with *Nde*I.

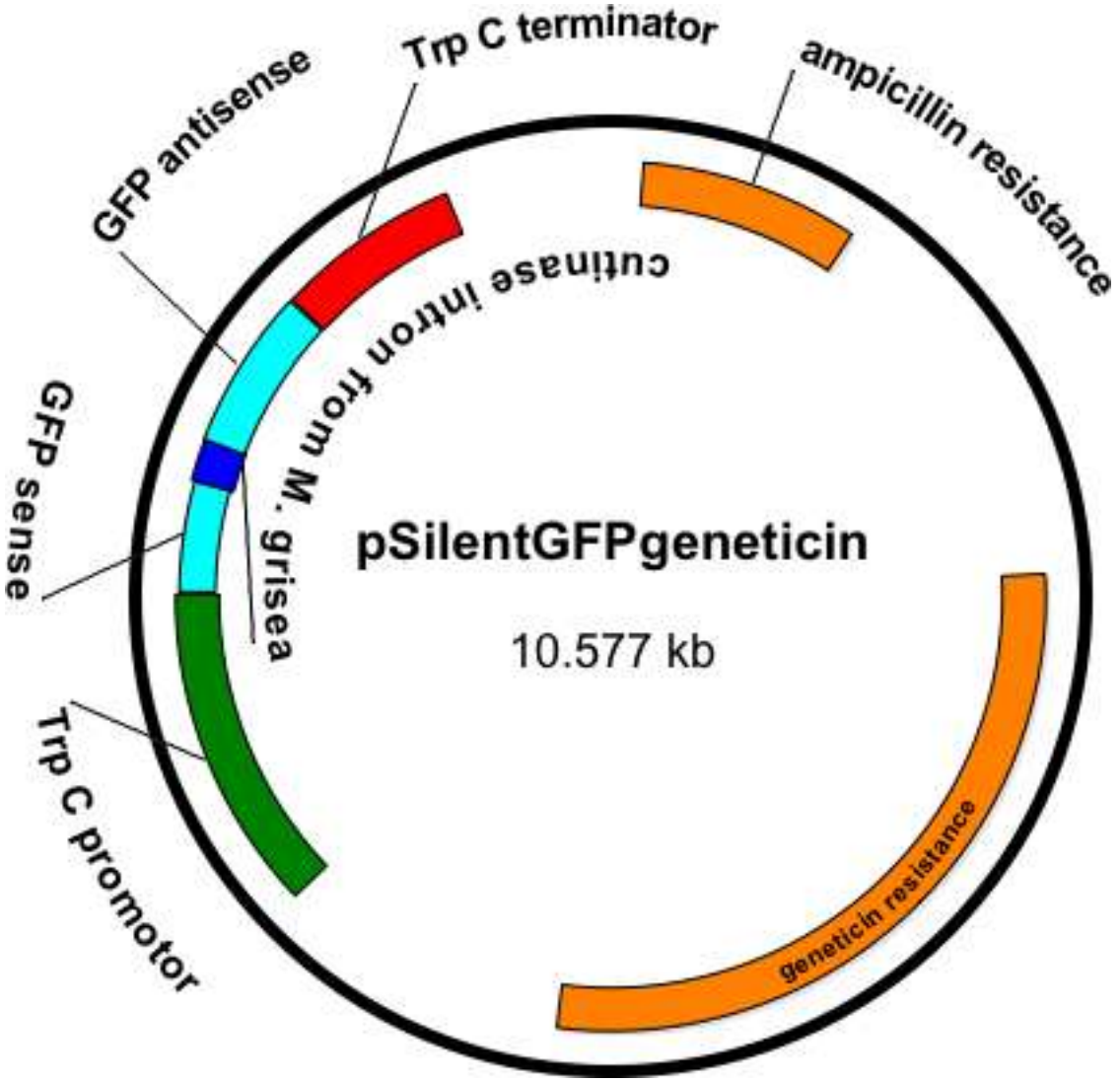


Figure 8 Vector pSilent GFP geneticin
In order to silence GFP in fungal reporter strains, a GFP wild type strain containing pIGPapa (kindly provided by Paul Boyer, UK) with a GFP cassette and the hygromycin resistance cassette was used. For this reason, geneticin was used in the silencing vector as a second marker. The geneticin cassette was excised from pGEMTgeneticin (kindly provided by Le Thi Thu Giang) and cloned with *Aat*II and *Nde*I into the pSilent-1 vector backbone (FGSC). The GFP sense and antisense fragment were amplified from pIGPapa with the enzyme sites *Xho*I, *Hind*III, *Bgl*II, and *Kpn*I. Fragments were subcloned in pGEMT (Promega), then released by enzymatic digestion and consecutively cloned into pSilent GFP. Trp C promoter: *Aspergillus nidulans* tryptophan C promoter. This vector was cloned by J. Boennighausen as a part of his MSc work.

GFP silencing in *Cercospora beticola*

To conduct this experiment, a GFP reporter strain of *C. beticola* was generated by transformation of *C. beticola* with pIGPapa. Retransformation of *C. beticola* isolate Ferrara GFP strain 993.14 with the GFP silencing vector rendered 4 transformants, which were verified by PCR. Different degrees of silencing effect were visible in transformants 1105.1

and 1105.4 under fluorescent light. Strain 1105.3 had an equal amount of GFP like the wild type (figure 9).

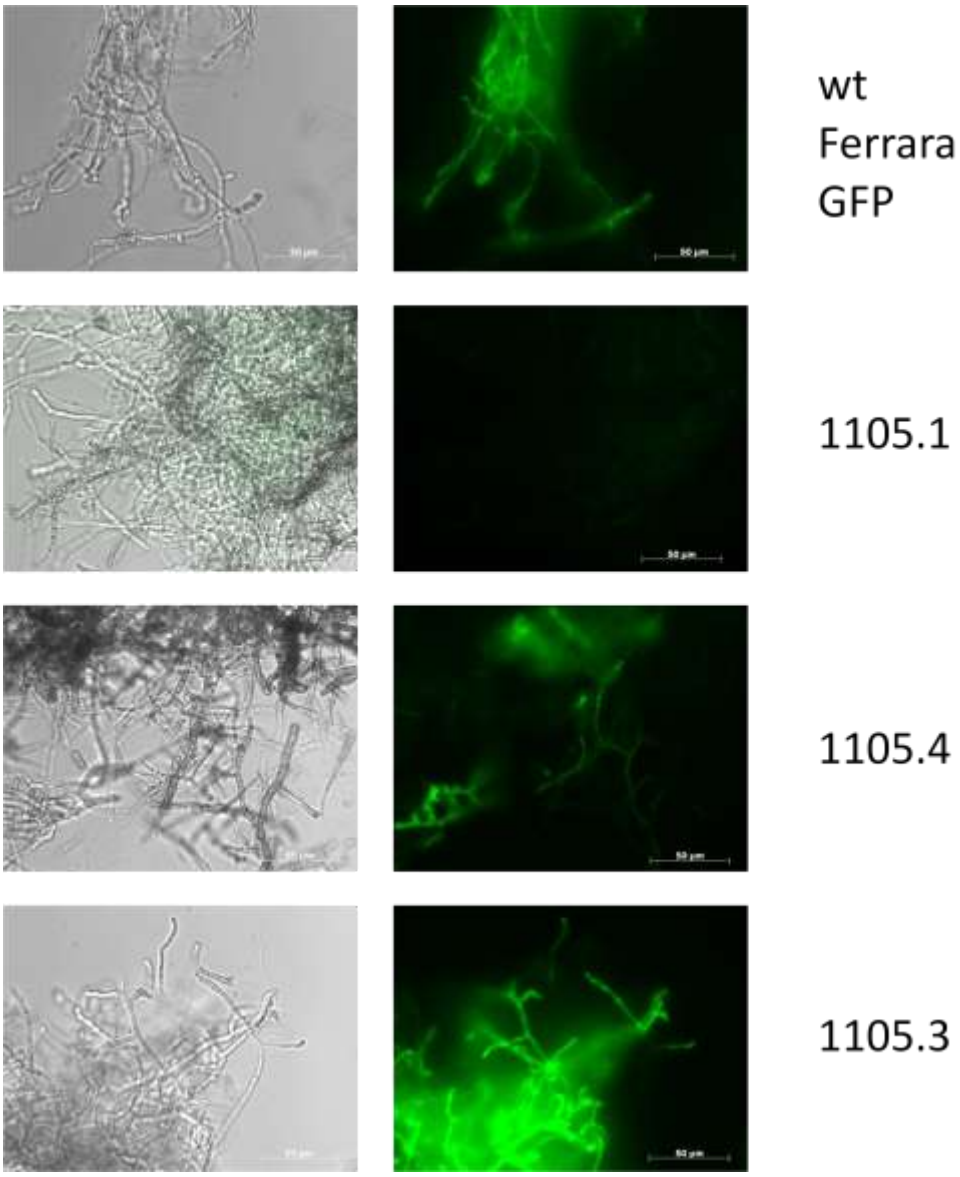


Figure 9 GFP silencing in *C. beticola*
The wild type GFP strain was transformed with pSilentGFPgeneticin. The GFP content of the transformants varied due to different levels of downregulation from no GFP in 1105.1 to as much GFP as the wild type in 1105.3. Left panel: DIC, right panel: GFP. Wt: wild type.

Western blot was carried out with equal amounts of protein extract. The membrane was probed with GFP specific antibodies (Invitrogen, Carlsbad, CA, USA). Different amounts of GFP were detected in the transformants in unison with the GFP level visible under the fluorescence microscope. Transformant 1105.1 had the weakest band corresponding with the least amount of GFP (figure 10).

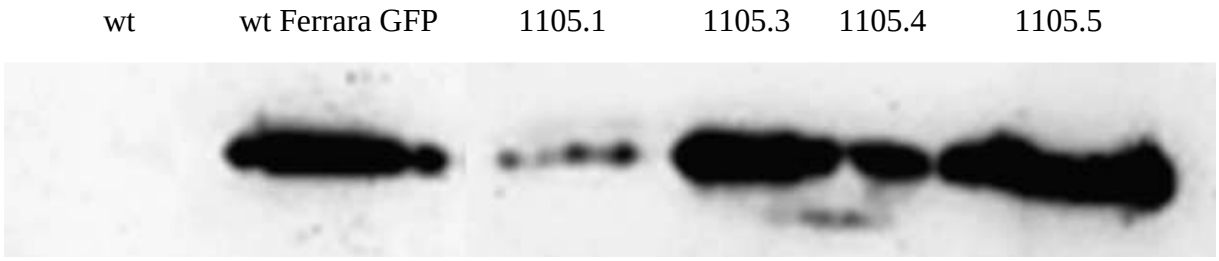


Figure 10 Western blot detecting GFP in the *C. beticola* pSilentGFPgeneticin transformants
Strain 1105.1 contained the least GFP, corresponding to the fluorescence micrographs. Wt: Ferrara wild type.

Identification of *CTB2* as a valuable target for host induced gene silencing

In this part of the study, *CTB2* was evaluated as a target for HIGS and its function in the pathogenicity of the fungus was elucidated. *CTB2* encodes the second enzyme, an O- methyl transferase, in the cercosporin synthesis pathway of *C. beticola*. This gene is essential for the production of cercosporin, a light induced toxin and major virulence factor of *C. beticola*.

Two isolates of the fungus *C. beticola* called Ahlburg and Ferrara were chosen for genetic manipulation to compare the results between different strains.

Cercospora beticola dsRed reporter strains

DsRed reporter strains were obtained by transformation with pII99dsRed (Namiki *et al.*, 2001), which was linearized with X*ho*I. Of 25 Ahlburg and 36 Ferrara transformants, more than 15 of each strain were confirmed to have dsRed fluorescence.

Gene disruption of *CTB2* in isolate Ahlburg

Primers were designed using the homologous sequence from *C. nicotiana* (accession number DQ991505). Sequencing revealed that *CTB2* in *C. beticola* and *C. nicotiana* are identical.

CTB2 was disrupted with the fusion PCR approach as illustrated in figure 11 A by double homologous recombination using fusion PCR to generate a construct consisting of a ca. 800 bp 3' part of the gene, the hygromycin resistance cassette, and a ca. 800 bp 5' part of the gene. The hygromycin cassette was released from vector gGEMThyg (Le Thi Thu Giang, University of Hamburg) with *Sma*I, while the upper and lower part of *CTB2* were amplified from genomic DNA with primers CTB2 1F, CTB2 2R, CTB2 3F, and CTB2 4R, respectively. In the fusion PCR reaction, equal amounts of the upper fragment, hygromycin cassette, and lower fragment were fused and the resulting 3.7 kb fragment was amplified with primers CTB2 nestF and CTB2 nestR. The final PCR product was cloned into pGEMT and released with *Not*I and *Apa*I. These restriction sites were added to the nested primers. More than 20 transformants were selected, but only 2, transformants number 1070.4 and 1070.8, were proven deletion mutants (figure 11 B). Transformants 1070.19 and 1070.38 were ectopic transformants that have a hygromycin integration, but not in the expected locus. The *ΔCTB2* transformants are of white color, while the ectopic and wild type colonies are dark grey. Transformant 1070.4 was chosen for transformation with pII99dsRed, which resulted in over 20 fluorescent transformants of which number 1080.1 was chosen for further work.

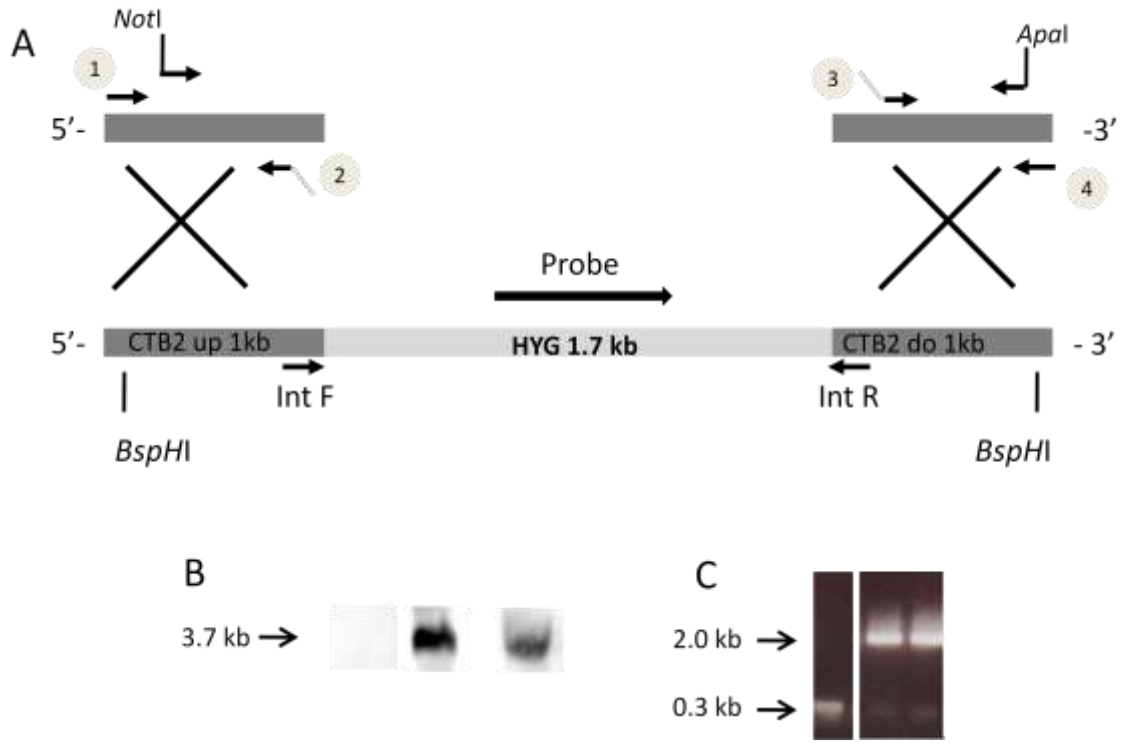


Figure 11 Gene disruption strategy for *CTB2* in *C. beticola* Ahlburg and Ferrara
This figure shows the schematic design of the disruption construct and an exemplary Southern blot. The 800 bp 5' and 3' fragments of *CTB2* were amplified separately with primers 1 and 2, 3 and 4, fused with the hygromycin cassette by overlapping regions, and the resulting construct was amplified with the nested primers (including *Not*I and *Apa*I) before being cloned to pGEMT. B Exemplary picture of Southern blot with a hygromycin probe, showing from left to right: wt (no band), gene disruption strains 1070.4 and 1029.16 (band at 3.7 kb). C Confirmative PCR with *CTB2* internal primers located adjacently to the integration locus (IntF and IntR), showing from left to right: wt (band at 0.3 kb) and gene disruption strains 1070.4 and 1029.16 (band at 2 kb).

Gene disruption of *CTB2* in isolate Ferrara

CTB2 was disrupted with the fusion PCR approach as described above. Transformation resulted in 44 transformants. Strains 1029. 16 and 1029.27 were proven gene disruptions, and 1029.1 and 1029.2 were ectopics. Transformant 1029.16 was picked for dsRed transformation, which resulted in more than 20 red fluorescent strains of which strain 1071.9 was used for further studies.

***ΔCTB2* is reduced in pigmentation**

The gene disruption strains lost the characteristic dark pigmentation and looked white (figure 12). Other properties like conidiation and growth rate remained unchanged. The loss of color corresponds with the phenotype of other gene deletion mutants involved in the cercosporin biosynthesis pathway in *C. nicotiana* (Dekkers *et al.*, 2006). The loss of function in this gene

leads to downregulation of the cercosporin synthesis as well, resulting in a non pigmented mutant. Deletion mutants transformed with dsRED had a pink color because the red protein is visible in the white hyphae.

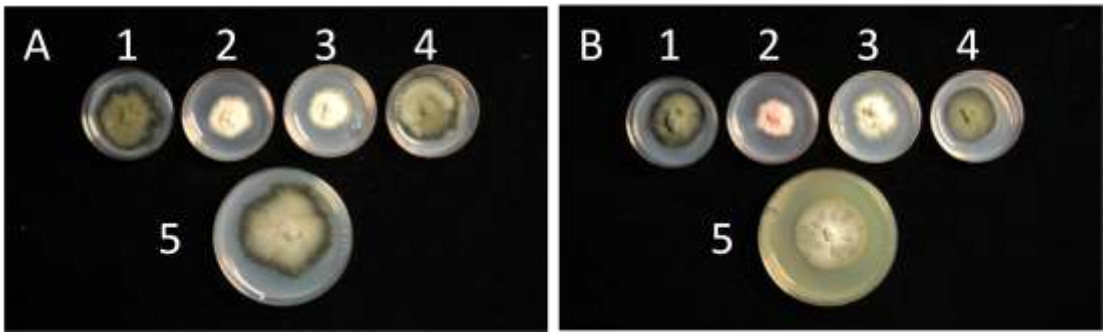


Figure 12 Phenotype of the *CTB2* null mutants
All strains were grown on complete media for 1 week. A Isolate Ahlburg: 1 ectopic strain (1070.19), 2 *ΔCTB2* dsRed (1080.1), 3 *ΔCTB2* (1070.4), 4 ectopic strain (1070.38), 5 wt. B Isolate Ferrara: 1 ectopic strain (1029.1), 2 *ΔCTB2* ds Red (1071.9), 3 *ΔCTB2* (1029.16), 4 ectopic strain (1029.2), 5 wild type. The deletion strains have lost their black pigmentation and appear white. The wild type strains were grown on bigger plates.

ΔCTB2 is reduced in toxin production

The toxin content was measured after extracting toxin from PDA plates cultured for two weeks in ambient light. The plates were soaked overnight in 5 M KOH and the absorption was measured at 480 nm in the spectrometer. A standard curve was used to calculate the toxin concentration. Each measurement was repeated three times from three independent extractions. As shown in figure 13, Ahlburg had a higher toxin content than Ferrara, which may explain why Ahlburg is the more aggressive isolate. The gene disruption strains produced no toxin. Transformants with empty vector (pGEMThyg) were used as control.

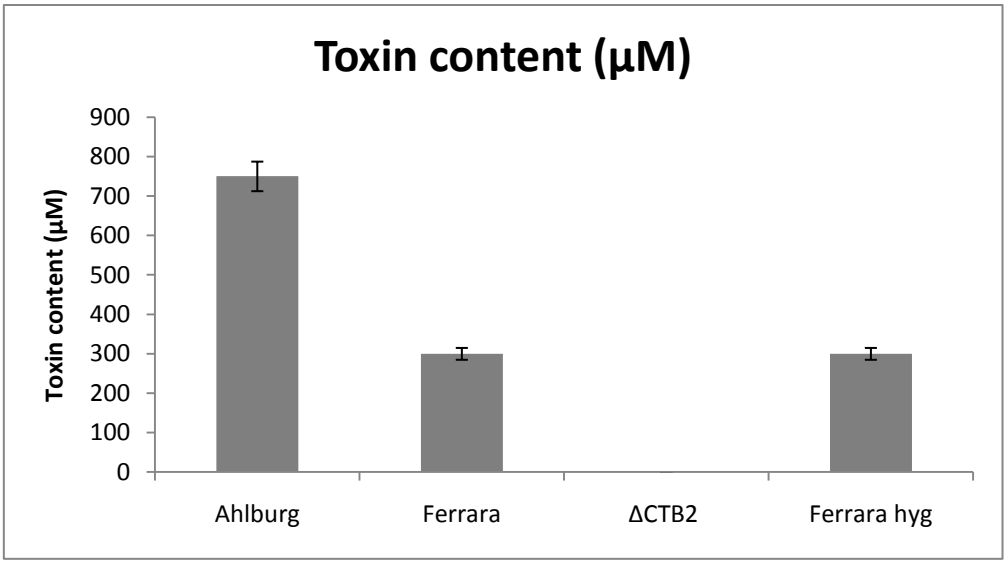


Figure 13 *ΔCTB2* is reduced in toxin production
Ahlburg, the more aggressive wild type, produced more toxin than Ferrara, the less aggressive isolate. The gene disruption strains in both isolates were completely free of toxin. Ferrara hyg is the control strain with a vector carrying hygromycin (pGEMThyg).

CTB2 is essential for pathogenicity of *Cercospora beticola*

Infection of plants with the wild type and gene disruption strain showed that the *ΔCTB2* strain caused no lesions on the leaves, and the entire plants appeared healthy three weeks after infection. Both wild type strains Ferrara and Ahlburg heavily infected the plants and caused severe lesions that finally killed the plants (figure 14). These results indicate that the *CTB2* gene is essential for pathogenicity of *C. beticola*.

Figure 14 The *ΔCTB2* strain failed to cause disease symptoms on sugar beet leaves
The picture shows enlarged sections of the surface of sugar beet leaves. While the wild type strain led to the characteristic leaf spot symptoms 21 days after infection, the gene disruption strain did not affect plant health. Ahlburg hyg is the control strain transformed with a vector carrying hygromycin (pGEMThyg).

When *in vitro* cultured plants were treated with plate extract, the ones dipped in wild type extract died after one day while plants treated with the water control and the *ΔCTB2* extract remained healthy and green (figure 15). These data show that culture extract of the gene disruption strain lacks a key component needed to damage plant tissue. The experiment was repeated three times with five plants treated in each plate extract.

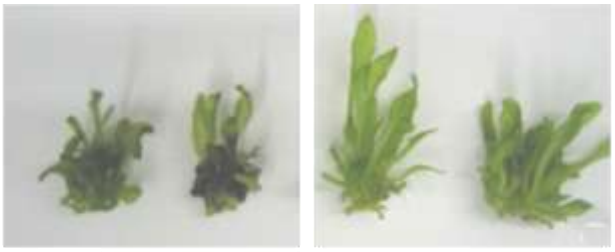


Figure 15 Sugar beet plants grown *in vitro* and treated with toxin extract
Plants suffered different degrees of damage one day after the treatment with plate extract. Plants treated with wild type plate extract became necrotic and black, but plants treated with *ΔCTB2* plate extract remained green and healthy. Water was used as negative control.

Disease rating was carried out by counting the percentage of diseased leaf surface (Rossi & Battilani, 1989). For each fungal strain, 25 3 month old sugar beet plants were used in the infection assay. The disease rating at 21 days after infection (dai) proved that the plants infected with the gene disruption strain were symptom free, while the plants infected with the wild type strain had a disease index of 4 on a scale of 9 (figure 16). However, at 24 dai, 12% of the leaves of plants infected with the gene disruption strain showed single spots (disease index 1). When these lesions were examined histologically, it was observed that the mutant strain had not spread between the lesions inside the leaf. These lesions were around 2-3 mm in diameter and did not enlarge after 21 dai.

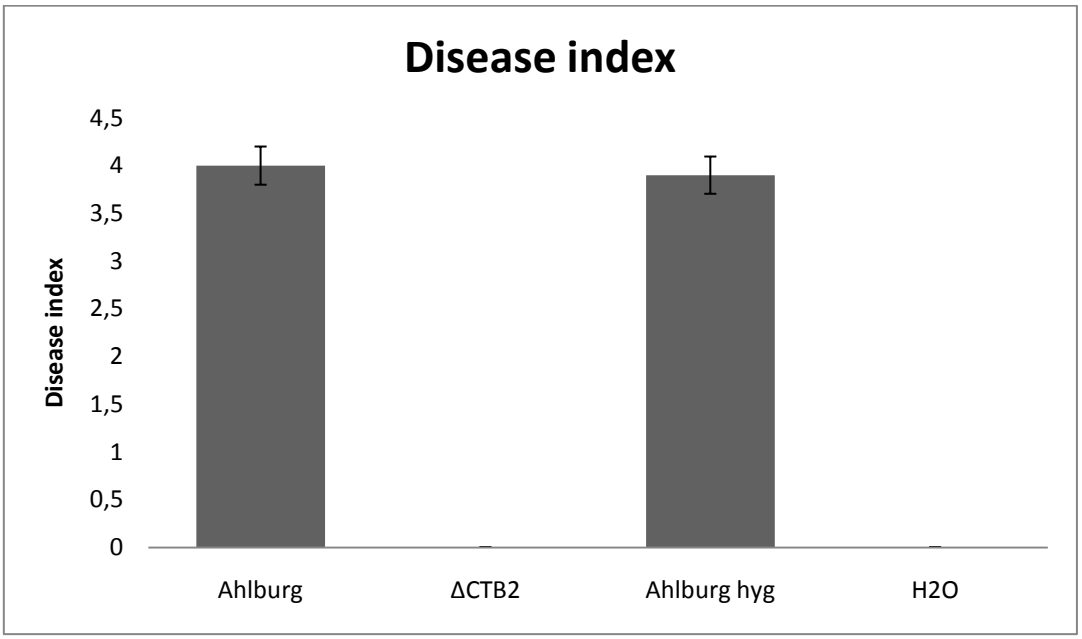


Figure 16 Disease index of sugar beet
Plants were infected with conidia of the wild type and knock out strain and the disease severity was rated after 21 days. Plants infected with the *ΔCTB2* strain had no symptoms of infection. Ahlburg hyg: control strain transformed with a vector carrying hygromycin (pGEMThyg)

ΔCTB2 is impaired in penetration of host leaves

The micrographs in this section were taken by M. Boenisch, University of Hamburg. For fluorescence microscopy, the dsRed reporter strains resulting from transformation with pII99dsRed were used. Fluorescence microscopy on both adaxial and abaxial leaf surfaces and on thin sections of leaves at different stages of the infection revealed that the wild type strain was beginning to infect through the stomata at 8 dai. While the wild type had progressed intercellularly from the penetration site and was beginning to cause necrosis at 14 dai, the gene disruption strain stopped at the epidermis layer and did not penetrate the host tissue. The wild type spread massively inside the leaves until 21 dai, when necrotic lesions became visible on the surface. The gene disruption strain had ceased to grow at this point and apparently died on the leaf surface (figure 17).

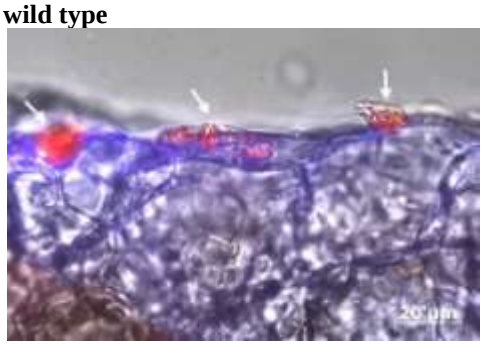
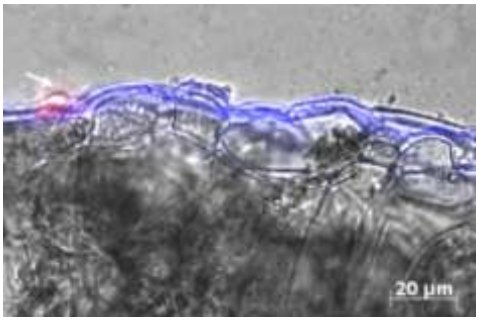
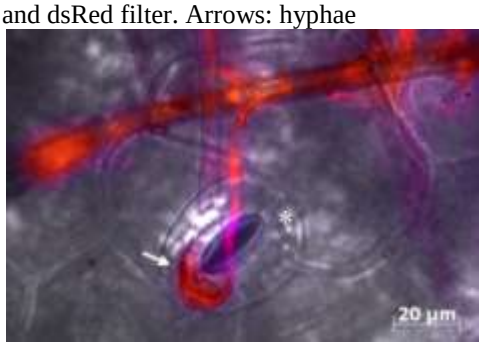
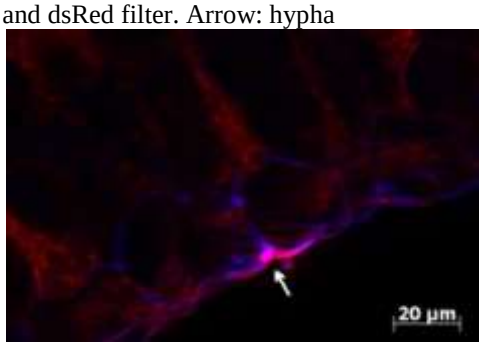
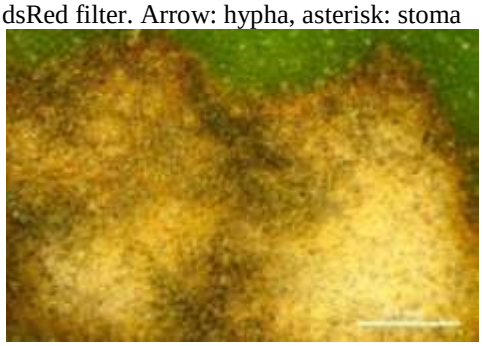
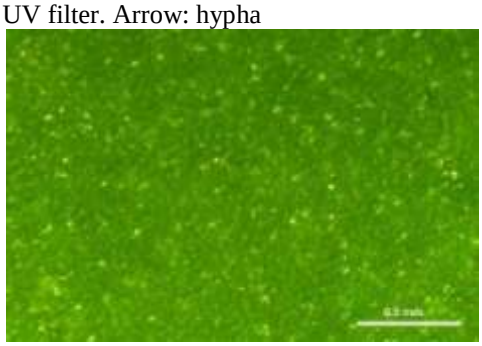
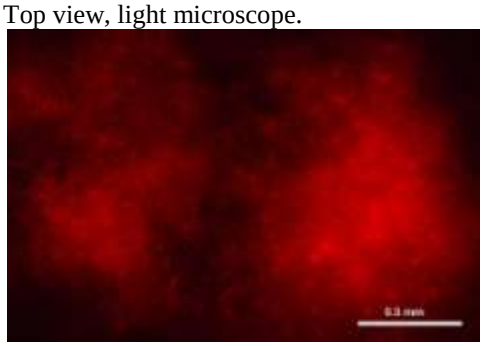
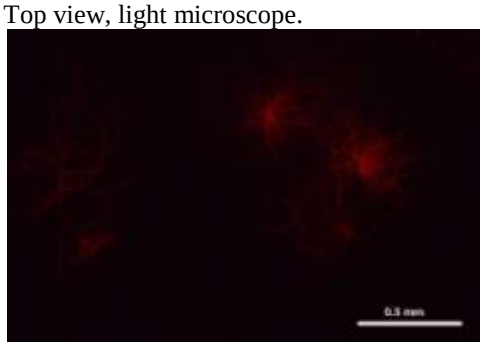
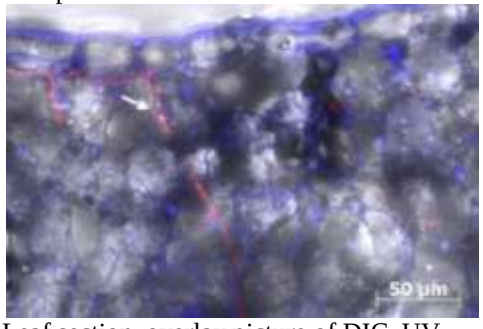
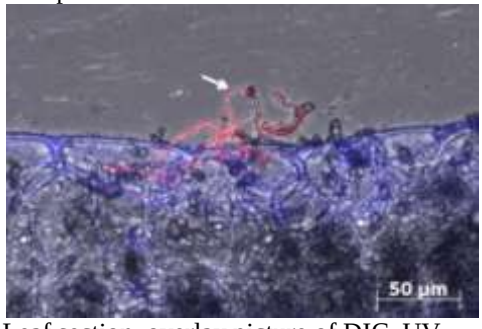
	wild type	***ΔCTB2***
0 dai		
	Leaf section, overlay picture of DIC, UV, and dsRed filter. Arrows: hyphae	Leaf section, overlay picture of DIC, UV, and dsRed filter. Arrow: hypha
4 dai		
	Top view, overlay picture of DIC, UV, and dsRed filter. Arrow: hypha, asterisk: stoma	Leaf section, overlay picture of dsRed and UV filter. Arrow: hypha
21 dai		
	Top view, light microscope.	Top view, light microscope.
21 dai		
	Top view, dsRed filter, corresponding field with picture above.	Top view, dsRed filter, corresponding field with picture above.
21 dai		
	Leaf section, overlay picture of DIC, UV, and dsRed filter. Arrow: hypha	Leaf section, overlay picture of DIC, UV, and dsRed filter. Arrow: hypha

Figure 17 Infection process monitored by fluorescence microscopy
For fluorescence imaging, the dsRed strains of wild type and *ΔCTB2* were used. Images were taken with a Zeiss fluorescence microscope, using DIC for light microscopy, the dsRed filter for fluorescence of the fungus and the UV filter for autofluorescence of the cell walls. At the day of inoculation, both wild type and mutant conidia and mycelium particles were visible on the surface of leaves. At 4 dai, the wild type approached stomata and began to penetrate, while the gene disruption strain was still found on the surface. At 21 dai, the infection was at its peak and symptoms were prominent on the leaves infected with wild type, while the gene disruption strain did not cause any leaf spots. The remaining hyphae were not closely attached to the surface and easily dislocated. Arrows point to hyphae and the asteriscs emphasize stomata. The micrographs were taken by M. Boenisch, University of Hamburg.

Host Induced Gene Silencing in *Brachypodium distachyon* and wheat

Host Induced Gene Silencing (HIGS) is an emerging tool in plant resistance engineering. By expressing RNAi targeting a fungal virulence factor, the host plant triggers silencing of the respective gene in the attacking pathogen.

Biolistic transformation with HIGS constructs in wheat

For Host Induced Gene Silencing, an important virulence factor of *F. graminearum*, *Lipase 1* (*Fgl1*), a secreted lipase, and an essential gene, *Homoaconitase*, an enzyme in the lysine synthesis pathway, were chosen as targets because they are essential for the virulence and survival of the fungus and their downregulation should lead to reduced fungal infection of wheat plants. A 400 bp fragment of *Fgl1* and *Homoaconitase* were each cloned into vector pDB35SGus35S. This vector is suitable for biolistic transformation of wheat and includes two 35s promoters in sense and anstisense direction, flanking a multiple cloning site for insertion of the gene of interest. The original vector was designed by Dr. D. Becker. The resulting plasmids are shown in figure 18 and figure 19.

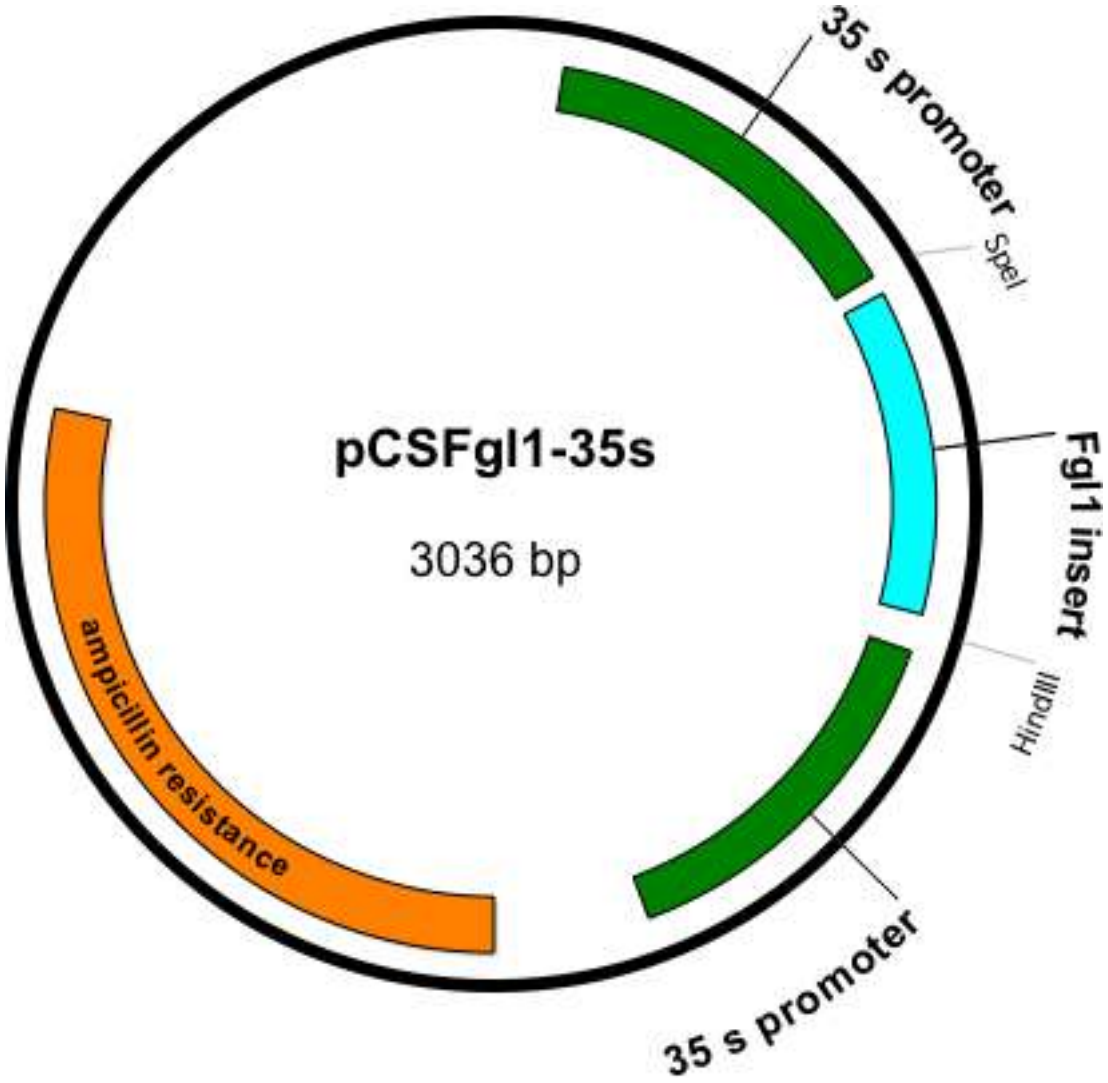


Figure 18 Vector pCSfgl1-35s
This plasmid was designed to be transformed into wheat for silencing of *Fgl1* in *F. graminearum* (HIGS). The 400 bp *Fgl1* fragment was inserted between the two 35 s promoters. The original plasmid was designed by Dr. D. Becker. 35s promoter: Cauliflower Mosaic Virus (CamV) 35s promoter

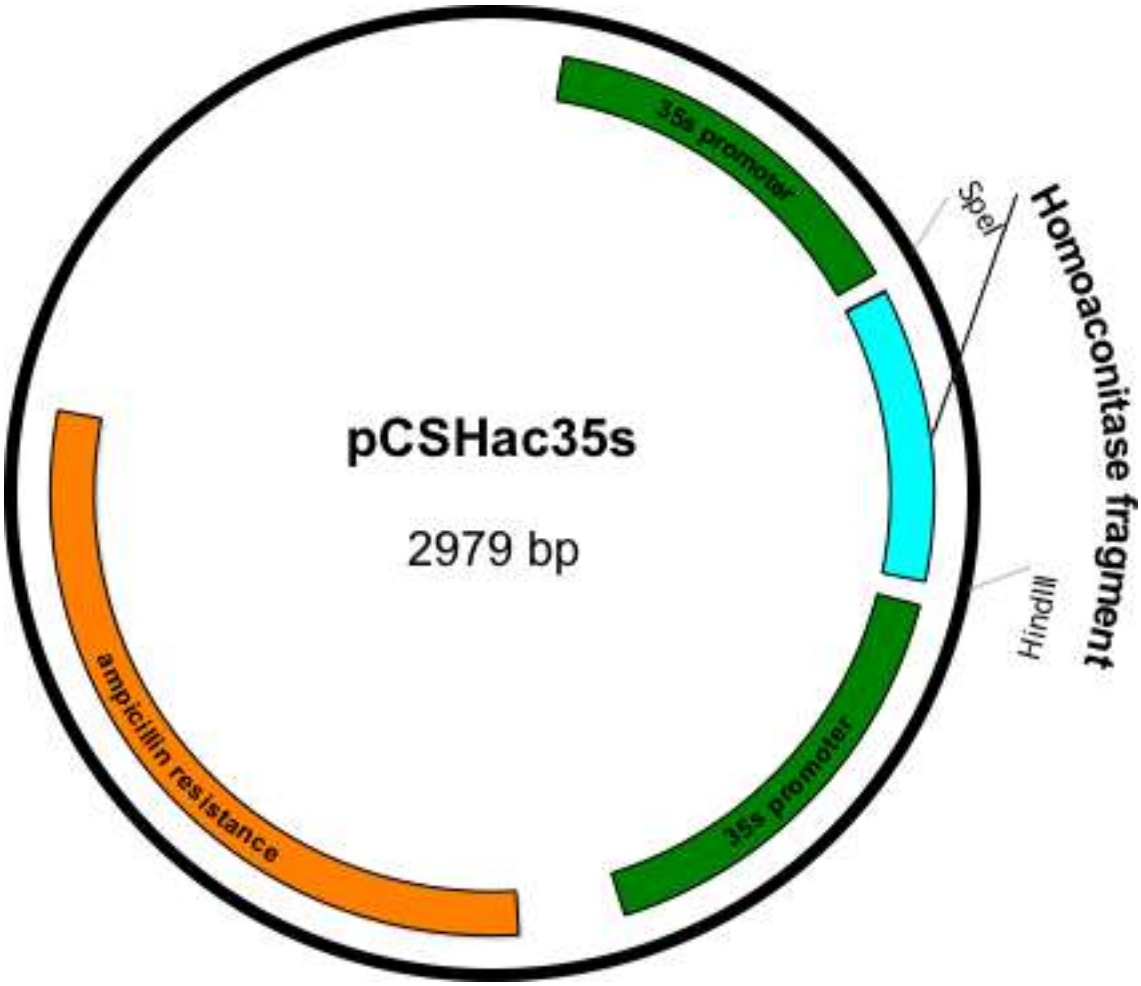


Figure 19 Vector pCSHac35s
This plasmid was designed to be transformed into wheat for silencing of *Homoaconitase* in *F. graminearum* (HIGS). The 400 bp *Homoaconitase* fragment was inserted between the two 35s promoters. The original plasmid was designed by Dr. D. Becker. 35s promoter: Cauliflower Mosaic Virus (CamV) 35s promoter.

The vectors pCSfgl1-35s and pCSHac35s were cotransformed with pucBar (provided by Dr. D. Becker) for Basta resistance. Although more than 8000 embryos were transformed, no transgenic plants could be regenerated. Simultaneous control transformation of wheat embryos with plasmids pActI-D carrying the GFP gene or pMon349 carrying the GUS gene (both vectors provided by Dr. D. Becker) lead to stable GFP (figure 20) or GUS expression, verifying that the transformation procedure as such was technically working. However, these vectors lack the Basta resistance gene and because of that the resulting calli were only grown for two month on non selective media since transgenic tissue is lost over time without selection pressure.

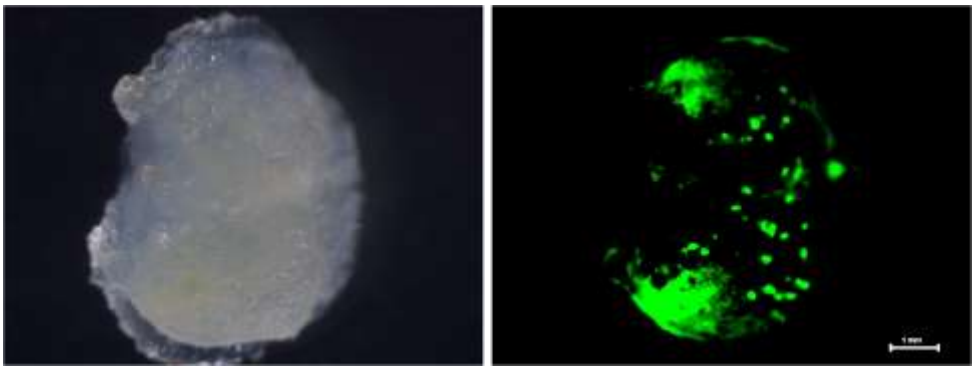


Figure 20 Transformation control with GFP
The embryo was transformed with pMON 349 carrying GFP and photographed after 4 weeks of regeneration. On the left: bright field image, on the right: Fluorescence image with GFP spots. Scale bar: 1mm.

Inducible RNAi constructs for wheat transformation

To create an inducible system for the *Fgl1* HIGS construct in wheat, vector p153ActRNAi, designed by Dr. D. Becker, University of Hamburg, was employed. The inducible system works as follows: A fragment of the gene of interest *Fgl1* was cloned between the *Mlu*I and *Xma*I site into vector p153ActRNAi (figure 21). This vector contains two actin promoters and lox sites and is co-transformed with vector pCSHspCreBar.

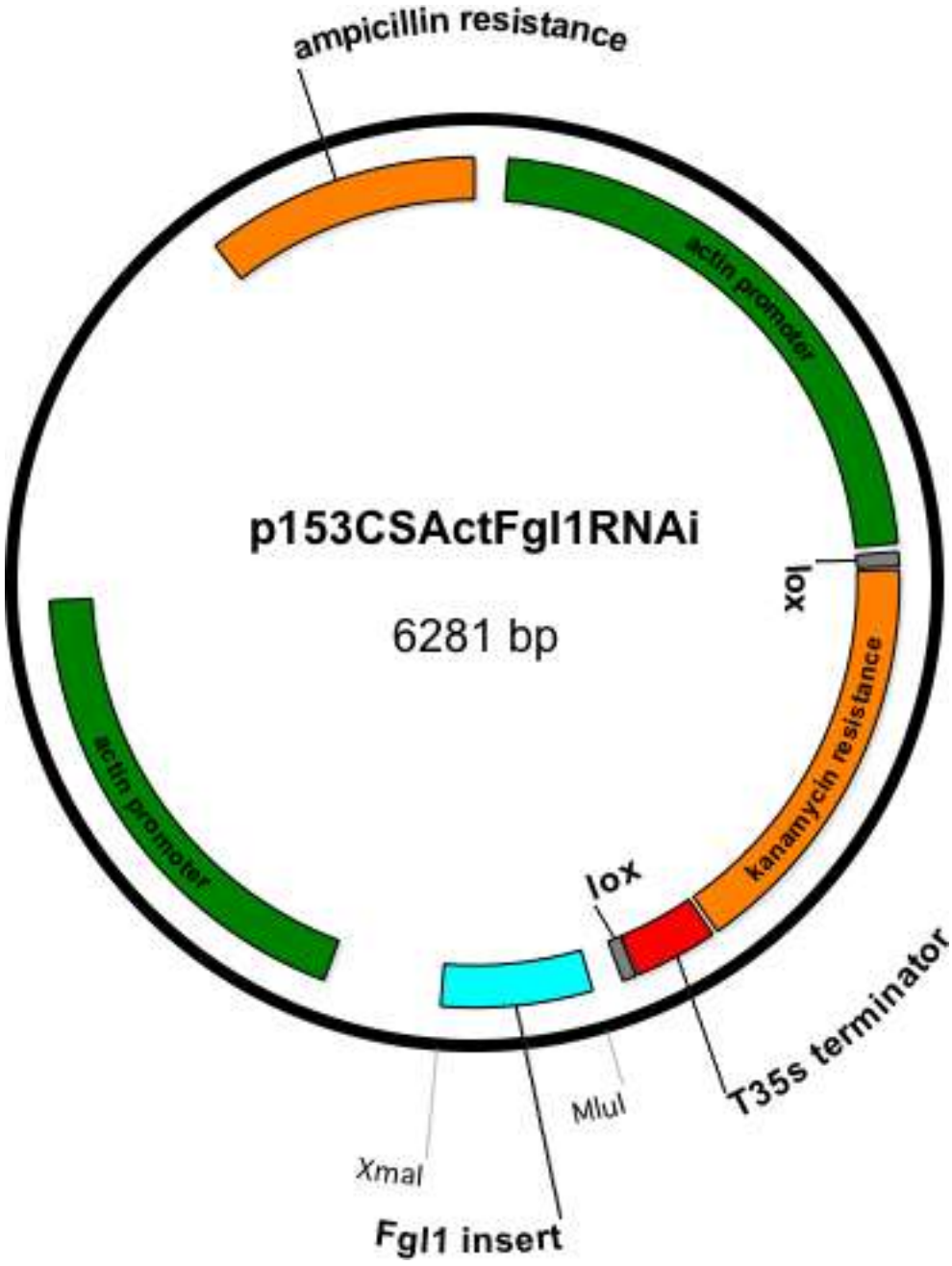


Figure 21 Vector p153CSActFgl1RNAi
This plasmid was designed to be transformed into wheat for inducible silencing of *Fgl1* in *F. graminearum* (HIGS). The *Fgl1* fragment was cloned between the first actin promoter and the lox site. When co-transformed with vector pCSHspCreBar, the cre-recombinase would release the kanamycin resistance gene and move the second actin promoter right behind the *Fgl1* fragment, creating a double promoter construct. The original plasmid was designed by Dr. D. Becker. T35s terminator: CamV 35s terminator.

Vector pCSHspCreBar (figure 22) was assembled from precursors p153ActRNAi and PIIKS, a pUC type vector (both vectors provided by Dr. D. Becker), using the *Eco*RI and *Xba*I restriction sites, to generate a construct with the Basta resistance gene and the cre-recombinase with a heat shock promoter. After successful co-transformation, the cre-

recombinase can be activated by heat shock and will cut at the lox sites in vector p153ActRNAi, leading to a recombination of this vector that moves the second actin promoter behind the gene of interest and starts the transcription of the RNAi construct. Transformation with these constructs is currently under way.

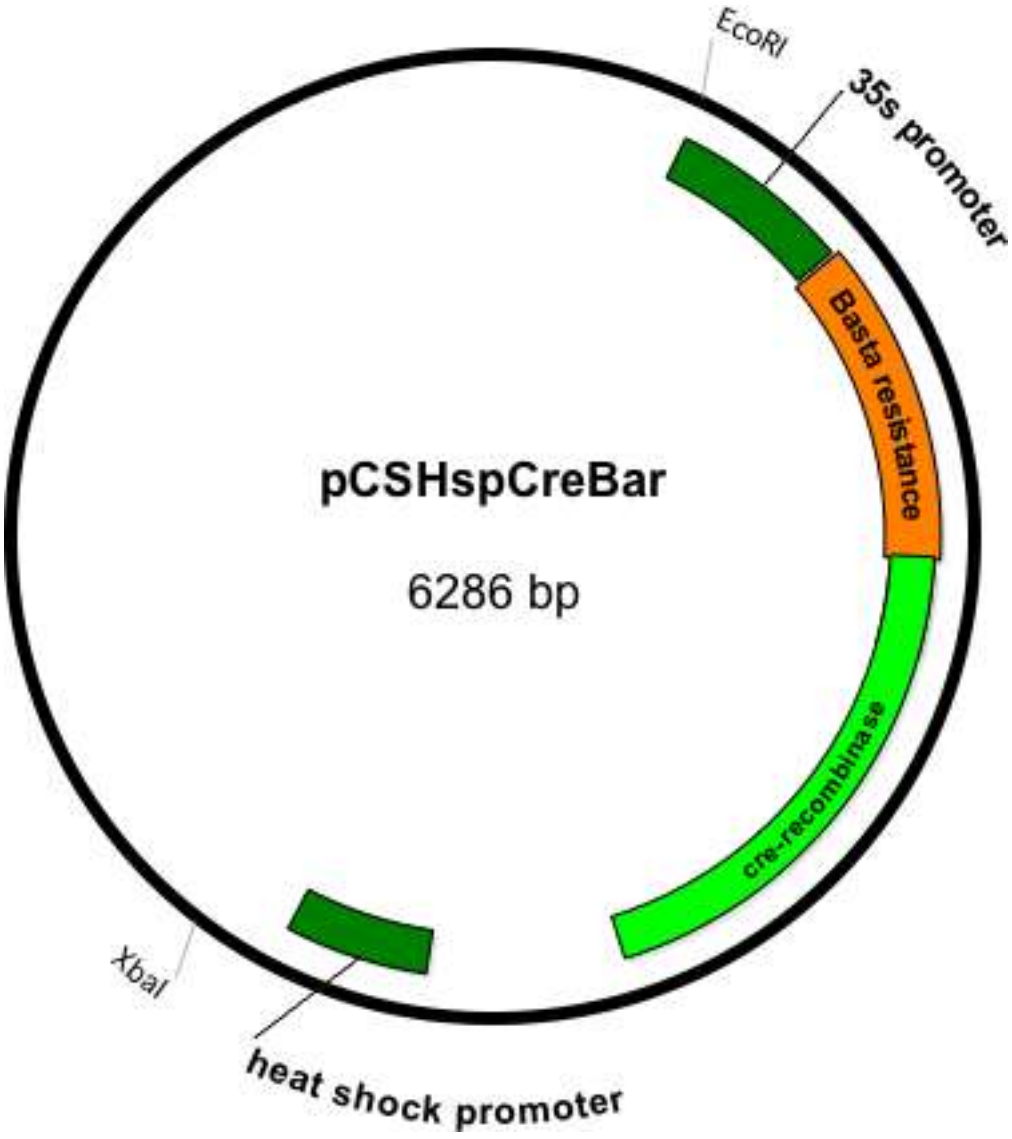


Figure 22 Vector pCSHspCreBar
This plasmid was used for co-transformation with vector p153CSActFgl1RNAi. After heat shock, the cre-recombinase was supposed to be expressed and cut at the lox sites of p153CSActFgl1RNAi, moving the second actin promoter behind the *Fgl1* fragment and starting the transcription of the RNAi construct. This vector was provided by DNA Cloning Services, University of Hamburg. The Basta resistance was to be used for transgenic plant selection. 35s promoter: CamV 35s promoter.

Agrobacterium mediated transformation with a HIGS construct in *Brachypodium distachyon*

In this experiment, the *Agrobacterium* method was used to transform *B. distachyon* plants with an *Fgl1* RNAi construct. Vector pCS6UFgl135s is a binary vector for the transformation of *B. distachyon* and was created by releasing the *Fgl1* fragment flanked by the 35s promoters from pCSFgl1-35s with *Sfi*I and ligating it into the binary destination vector p6U which was provided by Dr. D. Becker (figure 23). The desired fragments were extracted from the gel and ligated after treatment of one vector with shrimp alkaline phosphatase (SAP, Fermentas, St Leon Roth, Germany). *B. distachyon* transformation was carried out by K. Wolff, University of Hamburg.

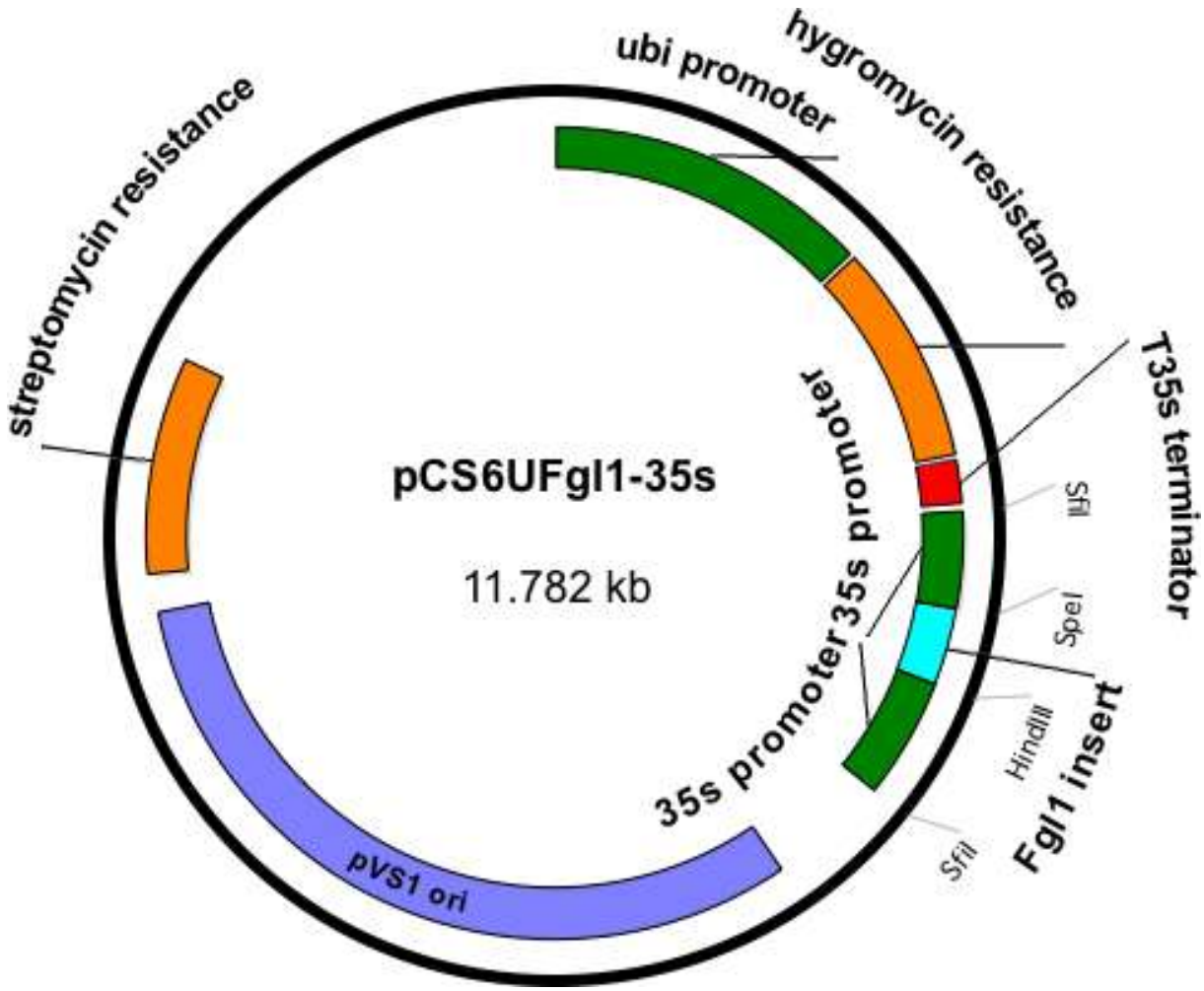


Figure 23 Vector pCS6UFgl1
This is a binary vector used for *Agrobacterium* mediated transformation of *B. distachyon* plants with an *Fgl1* RNAi construct (HIGS). It was assembled by releasing the *Fgl1* fragment flanked by the 35s promoters from pCSFgl1-35s with *Sfi*I and ligating it into the destination vector p6U which was provided by DNA Cloning Services, University of Hamburg. 35s promoter: CamV 35 s promoter T35s terminator: CamV 35 s terminator, ubi promoter: plant specific ubiquitin l promoter.

Transformation resulted in five transgenic lines (figure 24). PCR with *Fgl1* cloning primers CsFgl135s F and CSFgl135sR gave a band of the required 400 bp size (figure 25). PCR was repeated two times in order to avoid mistakes and PCR products were sequenced to verify sequence identity. Unexpectedly, a weak band was also amplified in the wild type.

Figure 24 Transgenic *B. distachyon* plants
Approximately six weeks after transplantation to soil, the T0 generation started to flower.

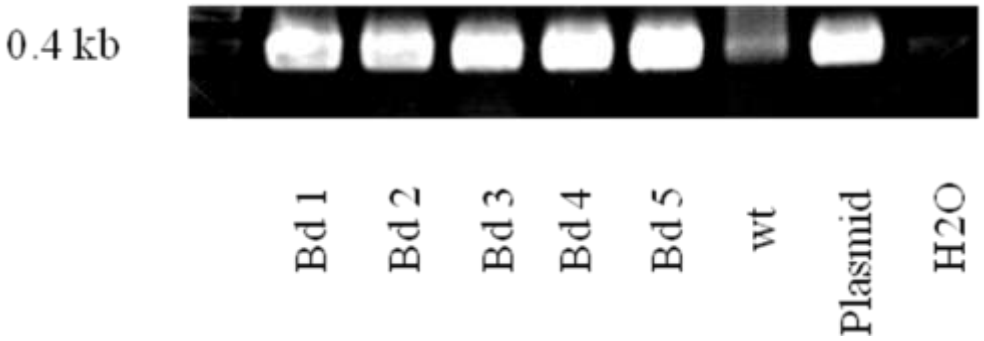


Figure 25 Control PCR results of transgenic *B. distachyon* plants, T0 generation
Using *Fgl1* cloning primers CSFgl135sF and CsFgl135sR resulted in the expected 0.4 kb band in all five plants. Unexpectedly, a weak band was also detected in the wild type. Further analysis of the T1 generation is shown in figure 25.

Transgenic *Brachypodium distachyon* plants, T1 generation

Seeds were harvested from the T0 generation and dried for 4 weeks, then replanted in a 1 : 2 sand : soil mixture, cold treated at 4 °C for 1 week and grown in the growth chamber at 24 °C at day, 18 °C at night with a 16 hours photoperiod. Line 5 did not produce enough viable seeds to continue experiments. The resulting T1 plants were analyzed for the *Fgl1* RNAi construct by PCR, Southern blot and RT-PCR. In PCR, primers LH1.2 and Pr2 that are flanking the 35S promoter were used to verify the correct insert. Three plants of four lines have been tested and all were positive. PCR was repeated 2 times in order to avoid mistakes and PCR products were sequenced to verify sequence identity. For Southern Blot, 25 mg

DNA of these plants were digested with *EcoR*I, a random cutter, and 5 mM spermidin for better DNA stability and digest results. The membrane was probed with a *Fgl1* specific probe. As shown in figure 26, 3 plants of line 1, 3 plants of line 2, 2 plants of line 3, and 2 plants of line 4 have multiple integrations of the construct.

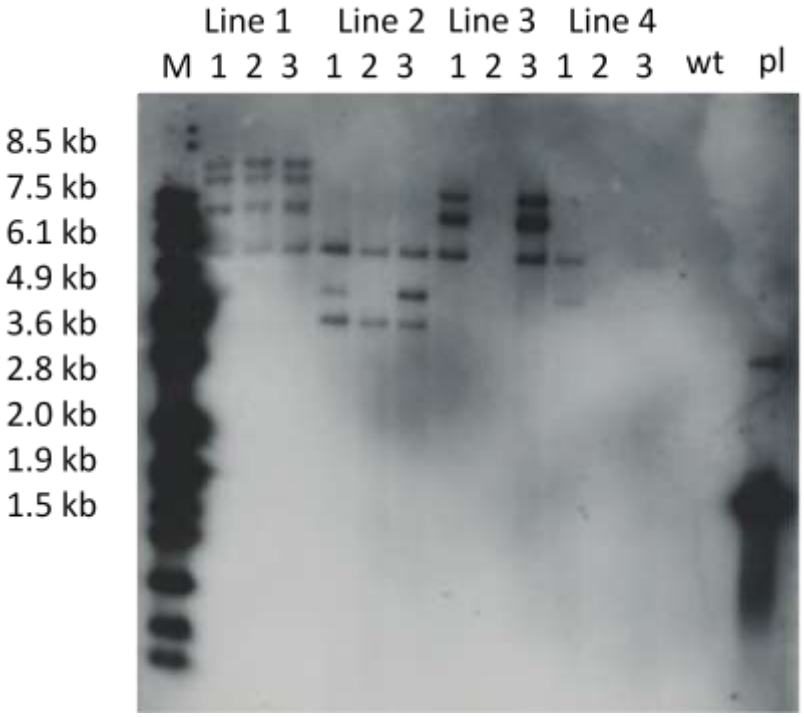


Figure 26 Southern blot of transgenic *B. distachyon* plants, T1 generation.
Three plants of four different lines were tested with a *Fgl1* specific probe. M: Marker DIG VII, wt: wild type, pl: plasmid control. Multiple bands per line are caused by multiple integration of the construct.

The same plants were used to extract RNA, and RT-PCR was performed with primers HygF and T35S2R, which amplify a part of the hygromycin resistance gene. According to the RT-PCR results, plants 1.1, 3.1, 3.3, and 4.1 expressed the construct (figure 27). PCR was repeated 2 times in order to avoid mistakes and PCR products were sequenced to verify sequence identity.

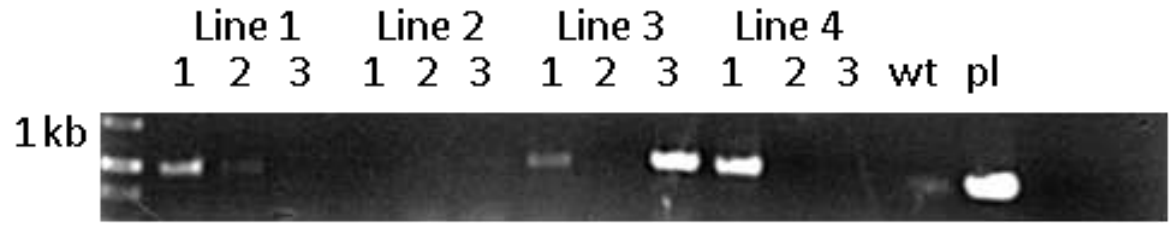


Figure 27 RT-PCR of transgenic *B. distachyon* plants, T1 generation
Using primers HygF and T35S2R, plants expressing construct pCs6UFgl135s (line 1.1, 3.1, 3.1, and 4.1) showed the expected 1 kb band which indicated that the RNAi construct was transcribed in these plants.

Infection assay with DHS RNAi transgenic *Brachypodium distachyon* lines, T2 generation

The results of this experiment are preliminary because not enough seeds were available to grow a sufficient number of plants for statistical analysis. Three plants of each line with expression of the *Fgl1* RNAi construct were infected with *F. graminearum* PH-1 conidia using 80 conidia per µl suspension and applying 1 µl on the developing flower. Plants were covered for 2.5 days with a plastic bag, then kept in a growth chamber for two weeks to monitor infection. As shown in figure 28, the symptoms were similar on all spikelets, revealing very little difference in the course of infection on wild type and transgenic plants. The experiment needs to be repeated with more plants and the percentage of infected spikelets will have to be counted for statistical evaluation. Since under the current experimental conditions not every flower develops into a seed, the fungus may have difficulties spreading through “empty” spikelets.

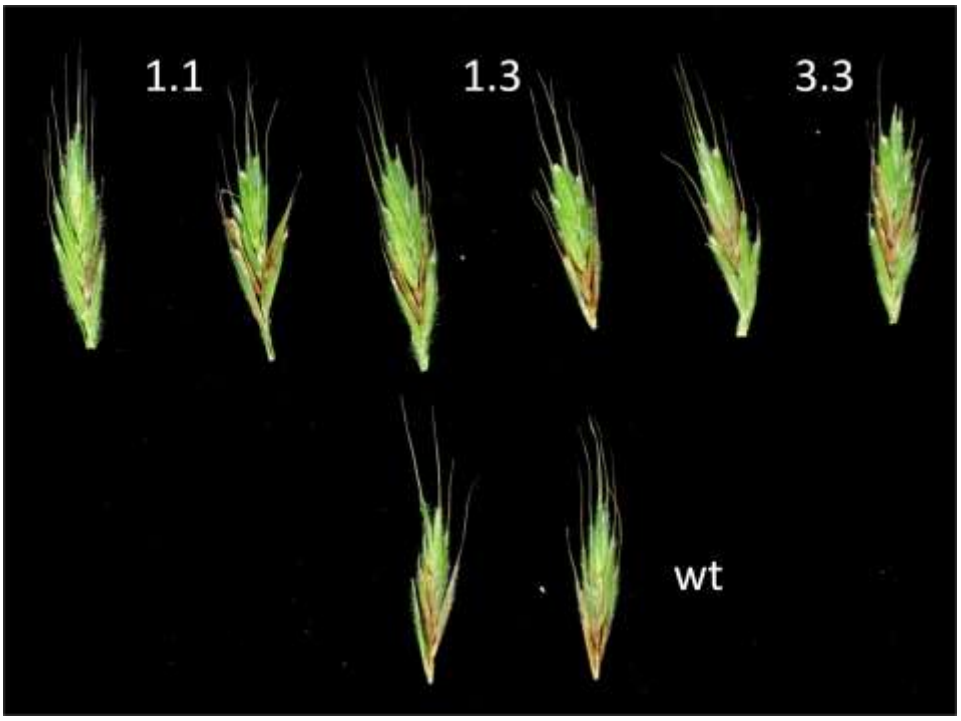


Figure 28 Infection of transgenic *B. distachyon* plants, T2 generation
Two exemplary infected heads are shown above in a longitudinal section at 14 days after infection. Lesions caused by *F. graminearum* appear brown. The transgenic lines with expression of the *Fgl1* RNAi construct (1.1, 1.3, 3.3) and the wild type plants all displayed similar symptoms. The infection had spread from the point of inoculation through the rachis and browned the tissue in the adjacent spikelet. This experiment needs to be repeated with a larger number of plants for further evaluation.

Fgl1 RNAi construct for maize

This construct was cloned in order to transform maize plants with *Agrobacterium* and generate transgenic plants expressing an RNAi construct against the fungal virulence factor *Fgl1*. It was cloned and handed over to S. Amati, University of Hamburg, for maize transformation.

The vector p7iHspeCrefgl1RNAi was constructed using pCS6UFgl135s and p7iHspCre to achieve a binary plasmid containing the *Fgl1* fragment between 35s promoters and the Basta resistance cassette, which is more efficient in maize transformation than hygromycin resistance. Both plasmids were digested with *Sfi*I, the desired fragments were extracted from the gel and ligated after treatment of one vector with shrimp alkaline phosphatase (SAP, Fermentas, St Leon Roth, Germany). The resulting plasmid is shown in figure 29.

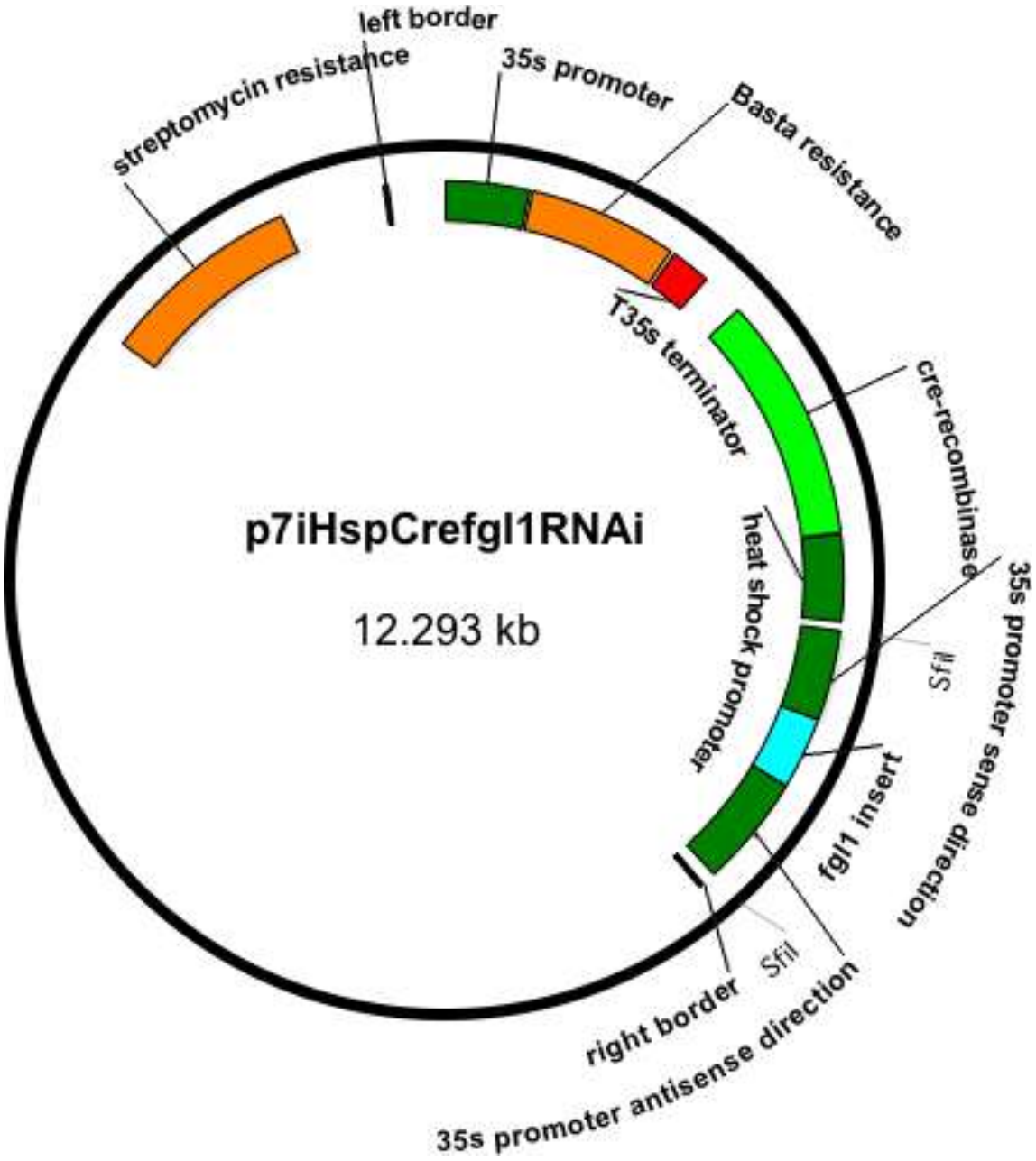


Figure 29 Vector p7iHspCreFgl1RNAi

This plasmid was designed to be transformed into maize for silencing of *Fgl1* in *F. graminearum* (HIGS). It is the result of a combination of pCS6UFgl135s which contains the *Fgl1* RNAi part and p7iHspCre carrying the Basta resistance. 35s promoter: CamV 35s promoter, T35s terminator: CamV 35 s terminator, 35s promoter: CamV 35 s promoter.

Characterization of *Homoaconitase* in *F. graminearum*

Deletion of *Homoaconitase* in *Fusarium graminearum*

Homoaconitase (FGSG_10949.3) is a 2673 bp long gene in *F. graminearum*. It includes a 231 bp iron-sulphur (Fe-S) cluster which is the essential active center of the enzyme. This gene was chosen as a target for HIGS because it is vital for the fungal metabolism. In this study, it was first tried to generate a gene disruption construct by fusion PCR utilizing the upstream and downstream flanking regions of the gene. This approach failed and sequencing of 1 kb upstream and downstream fragments revealed that the downstream part did not match the annotation (Broad Institute and Munich Information Center for Protein Sequences, MIPS). In a new approach, the 5' and 3' parts of the gene adjacent to the Fe-S cluster were used as flanking parts and fused with the hygromycin resistance cassette as described in materials and methods. The fusion PCR product (figure 30) was cloned into pGEMT and released with *Apa*I and *Not*I for transformation. Regeneration media was supplemented with 50 mM lysine to ensure the survival of lysine deficient mutants.

Figure 30 The *Homoaconitase* deletion construct
The 5' (1kb) and 3' (1.3 kb) segments of the gene were fused with the hygromycin resistance cassette in order to disrupt the gene by double homologous integration and replace the essential FeS-cluster with the resistance marker.

Several clones were used in different transformation approaches, but no transformants could be generated.

Silencing of *Homoaconitase* in *Fusarium graminearum*

Since no gene disruption mutants could be achieved, the inducible silencing construct pCSSW08Hac was generated. This vector is based on pSW08 (Barton & Prade, 2008) which is inducible by threonin with the alcohol dehydrogenase A (Alc A) promoter. The final vector pCSSW08Hac is shown in figure 31. To generate this plasmid, the *Homoaconitase* fragment was introduced by *BamH*I restriction and the hygromycin resistance cassette was introduced with *Sac*I. The desired fragments were first amplified by PCR using primers with the required restriction sites, subcloned into pGEMT and released by enzymatic digest. They were cloned into pSW08 after gel extraction. The vector was equally digested and treated with shrimp

alkaline phosphatase (SAP, Fermentas, St Leon Roth, Germany) before ligation to avoid self ligation. For transformation, the vector was linearized with *Ahd*I.

Despite several transformation attempts with different clones, no transformants could be regenerated.

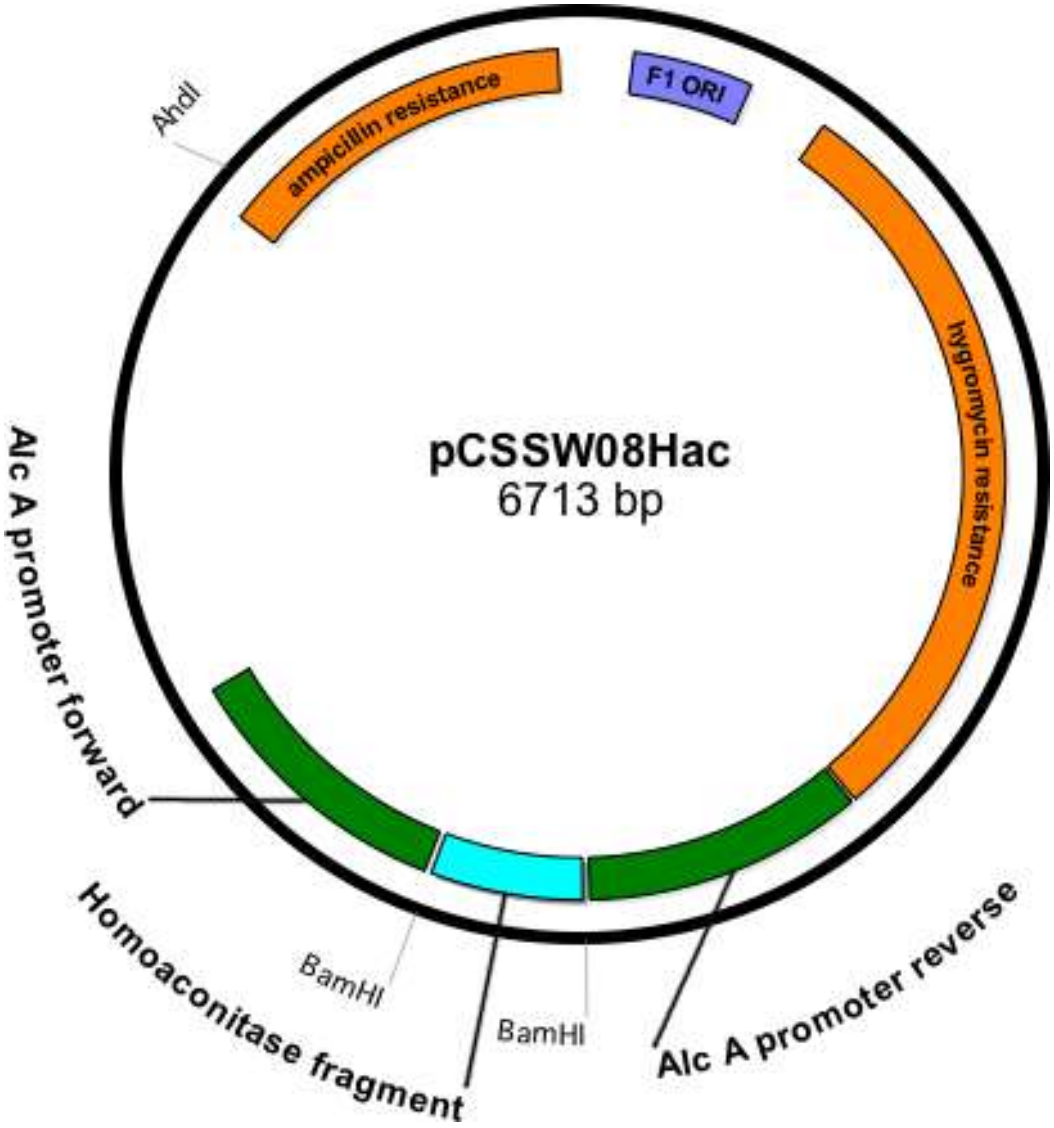


Figure 31 Vector pCSSW08Hac
This plasmid was generated to silence *Homoaconitase* in *F. graminearum* with an inducible construct. The 400 bp *Homoaconitase* fragment was introduced by *BamH*I restriction and the hygromycin resistance cassette was cloned with *Sac*I. Alc A: *A. nidulans* alcohol dehydrogenase promoter. The vector pSW08 was provided by Dr. R. Prade. The Alc A promoter is inducible with threonin.

Characterization of *Dicer 1* in *Fusarium graminearum*

Deletion of *Dicer 1* in *Fusarium graminearum*

The goal of this part of the study was to generate a null mutant of *Dicer 1* in *F. graminearum*. These strains will be valuable in the future to understand the mechanism of host induced gene silencing. It will be of great interest whether the fungal or the plant RNAi apparatus are responsible for processing dsRNA from HIGS constructs expressed in the host plant.

Three different constructs were generated: One with ca. 800 bp of the 5' and 3' flanking regions of the gene and the hygromycin resistance cassette (figure 32 A), one with ca. 800 bp of the 5' and 3' flanking regions and the geneticin resistance cassette cloned into the hygromycin cassette (figure 32 B), and one with ca. 300 bp flanking regions and the geneticin cassette (figure 32 C). Different sizes of the flanking regions were chosen because no transformants resulted from the initial construct with long flanking regions, and shorter flanking regions increase the probability of ectopic integrations. The construct with shorter flanks was supposed to facilitate the integration and produce if not deletion but at least ectopic strains. Two different resistance genes were used to increase the likelihood of transformation events. Each construct was attempted to transform in three independent experiments, resulting in no transformants. Protoplast regeneration controls with pGEMThyg and without DNA were done in parallel allowing to exclude general mistakes in the transformation procedure. Varying amounts of DNA from 20 to 40 µg and different methods of plasmid recovery with Mini and Midi plasmid preparation kits (Macherey und Nagel, Düren, Germany) did not improve the results, either.

Figure 32 The three different *Dicer 1* gene disruption constructs

A: Regular fusion PCR product of a ca. 800 bp 5' flank, hygromycin resistance, and a ca. 800 bp 3' flank.

B: Most of the hygromycin resistance cassette is replaced by the geneticin resistance cassette using the *Nde*I and *Kpn*I restriction sites. The flanking regions are 800 bp.

C: The geneticin resistance is inserted in the *Xba*I and *Bgl*II sites, leaving only short flanking regions of 300 bp for a greater likelihood of ectopic integrations. No transformants were achieved with either construct.

Silencing of *Dicer 1* in *Fusarium graminearum*

Because no gene disruption mutants could be achieved, the inducible silencing construct pCSSW08D1hyg was generated (figure 33). To clone this plasmid, the *Dicer 1* fragment was introduced by *BamH*I restriction and the geneticin resistance cassette was integrated with *Sac*I into the original pSW08 plasmid provided by Dr. R. Prade. The fragments were amplified by PCR using primers with the required restriction sites, subcloned into pGEMT and released by enzymatic digest. They were cloned into pSW08 after gel extraction. The vector was equally digested and treated with shrimp alkaline phosphatase (SAP, Fermentas, St Leon Roth, Germany) before ligation to avoid self ligation. For fungal transformation, the vector was linearized with *Afl*II. Transformation was attempted three times without any resulting transformants.

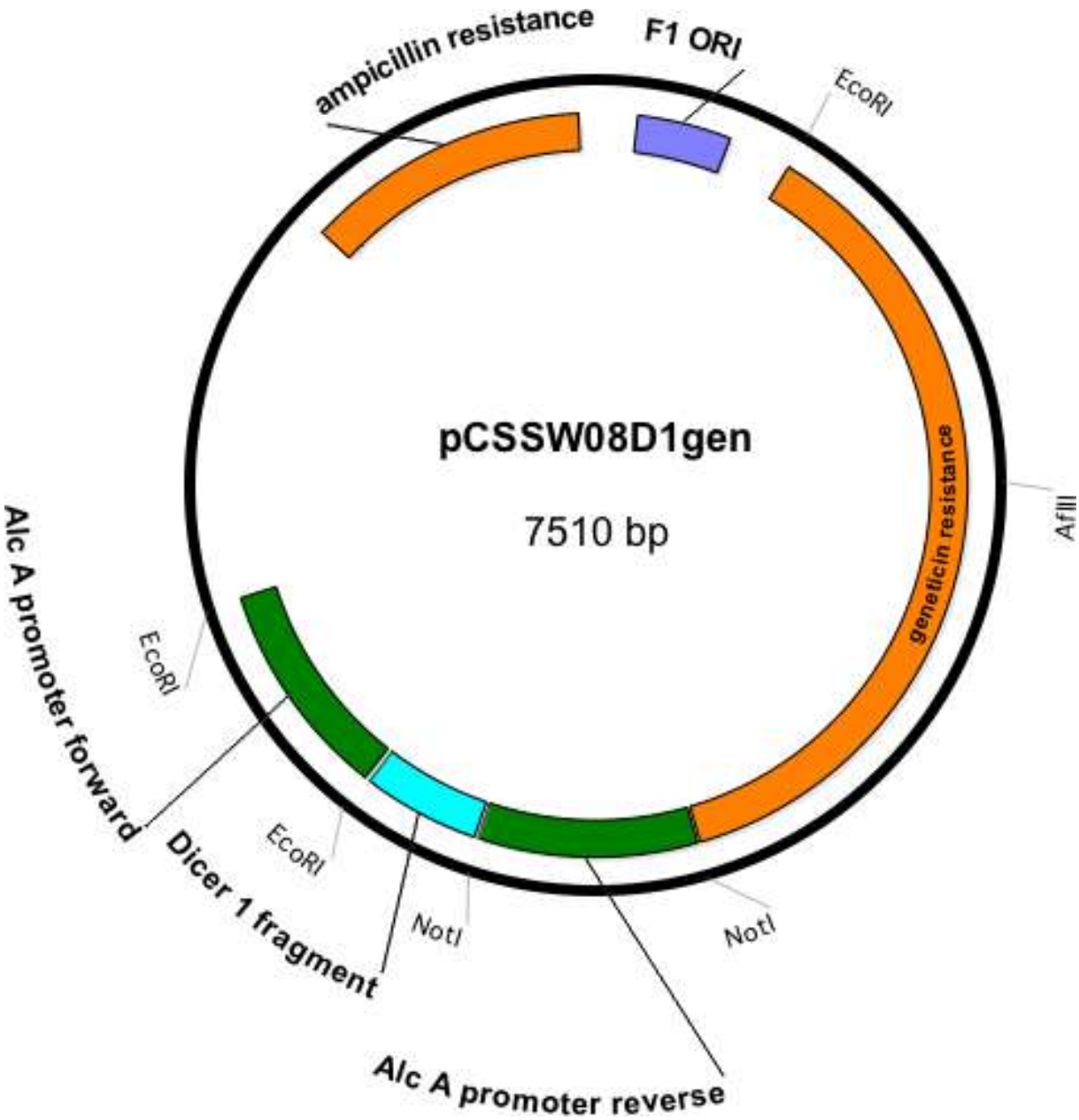


Figure 33 Vector pCSSW08D1gen

This plasmid was generated to silence *Dicer* in *F. graminearum* with an inducible construct. The 400 bp *Dicer 1* fragment was introduced by *BamH*I restriction and the geneticin resistance cassette was cloned with *Sac*I. Alc A: *A. nidulans* alcohol dehydrogenase promoter. The vector pSW08 was provided by Dr. R. Prade. The Alc A promoter is inducible with threonin.

Inducible silencing constructs against the endogenous gene DHS in wheat and maize

DHS encodes deoxyhypusine synthase, an enzyme that catalyzes the hypusination of translation initiation factor eIF5-α. Hypusination activates this factor which plays a role in mRNA transport and is involved in senescence and stress response (Park *et al.*, 2010).

The transgenic wheat plants transformed with a DHS RNAi construct and the maize plants transformed with an DHS overexpression construct were generated in a previous dissertation at the University of Hamburg (Woriedh, 2010) and further analyzed in this study. An inducible DHS RNAi construct for use in maize was cloned for further transformation. The constructs in this section are designed to silence or overexpress the endogenous plant DHS gene in wheat and maize.

Wheat DHS RNAi construct

This construct was used to silence the endogenous wheat DHS gene. An inducible construct was chosen to avoid lethal phenotypes by direct downregulation. The goal was to initiate expression of the RNAi construct and downregulation of wheat DHS after plants had been regenerated successfully.

The wheat DHS RNAi construct pD1ubibarDHSRNAiwheat (figure 34) was designed by Dr. D. Becker and cloned by DNA Cloning Services, University of Hamburg. The basic features of the vector are short sequences of the gene of interest, in this case the DHS from wheat, which are separated by a Gus spacer in the sense-linker-antisense fashion. This plasmid was co-transformed with the p7iHSPCreubiNptDHS (figure 35), which was also constructed by DNA Cloning Services and contains the cre-recombinase that is transcribed upon heat shock induction. The recombinase is supposed to excise the Basta resistance gene and move the ubi promoter in front of the DHS RNAi hairpin loop in vector pD1ubibarDHSRNAiwheat, so that the RNAi construct can be transcribed. Another vector, pCaNeo (Callis *et al.*, 1987), which carries the neomycin resistance cassette, was transformed along with these two vectors in order to provide an additional method of selection after removal of the Basta resistance gene.

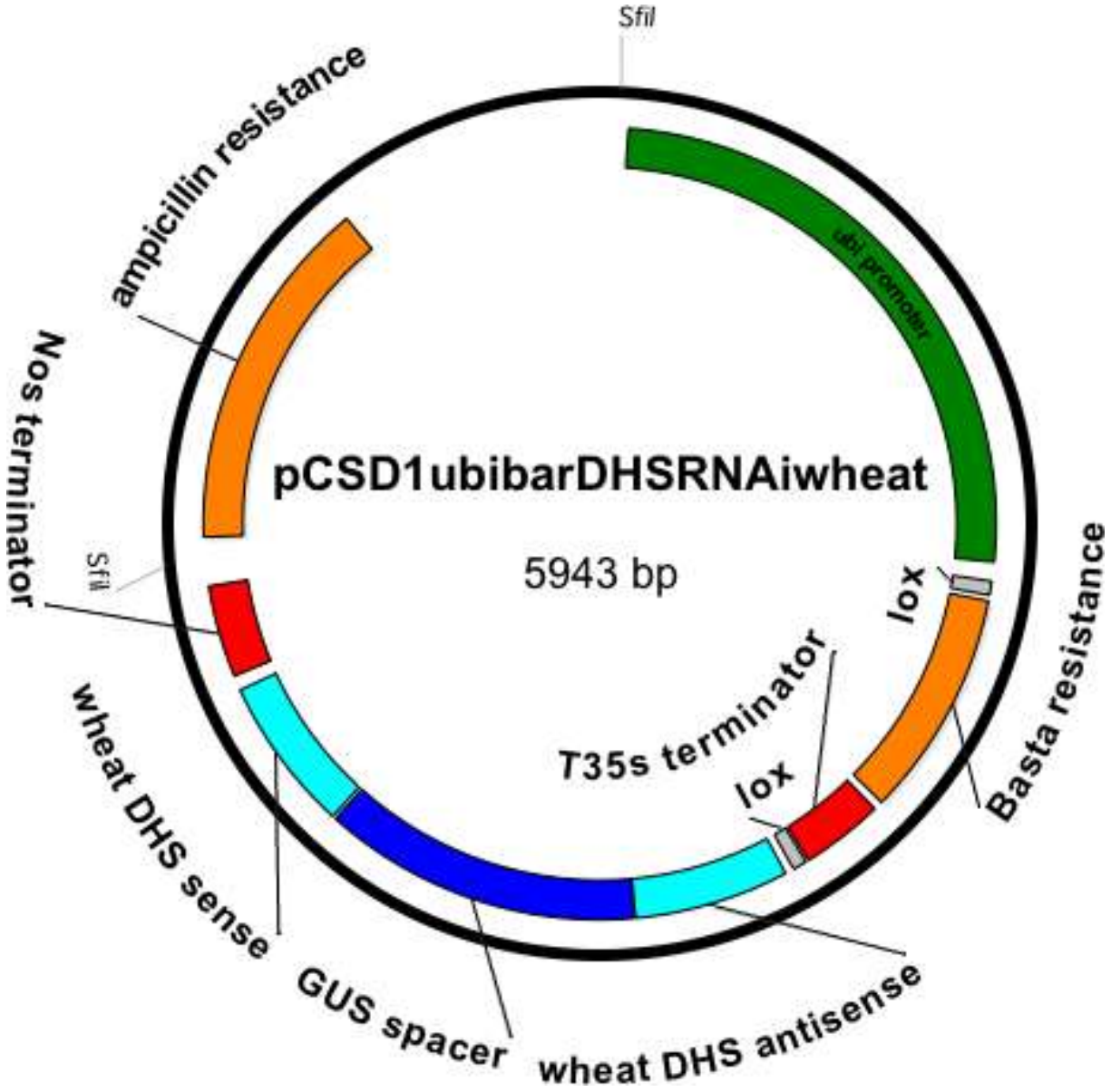


Figure 34 Vector pD1ubibarDHSRNAiwheat

This plasmid was cloned by DNA Cloning Services, University of Hamburg, and used for silencing of the endogenous DHS gene in wheat. The vector was co-transformed with p7iHSPCre (DNA Cloning Services, University of Hamburg), carrying the cre-recombinase and a heat shock promoter, and pCaNeo (Callis *et al.*, 1987), which provides a neomycin resistance cassette. Goal of the transformation was to yield plants which harbor the three plasmids. The heat shock promoter would be induced by increased temperature, activating the cre-recombinase gene. Once this enzyme was active, it should cut at the lox sites and move the ubi promoter in front of the RNAi hairpin loop, starting the RNA production and the silencing effect. Nos terminator: *Agrobacterium* nopaline synthase terminator, GUS spacer: β-glucuronidase spacer, T35s terminator: CaV 35s terminator, ubi promoter: plant specific ubiquitin promoter.

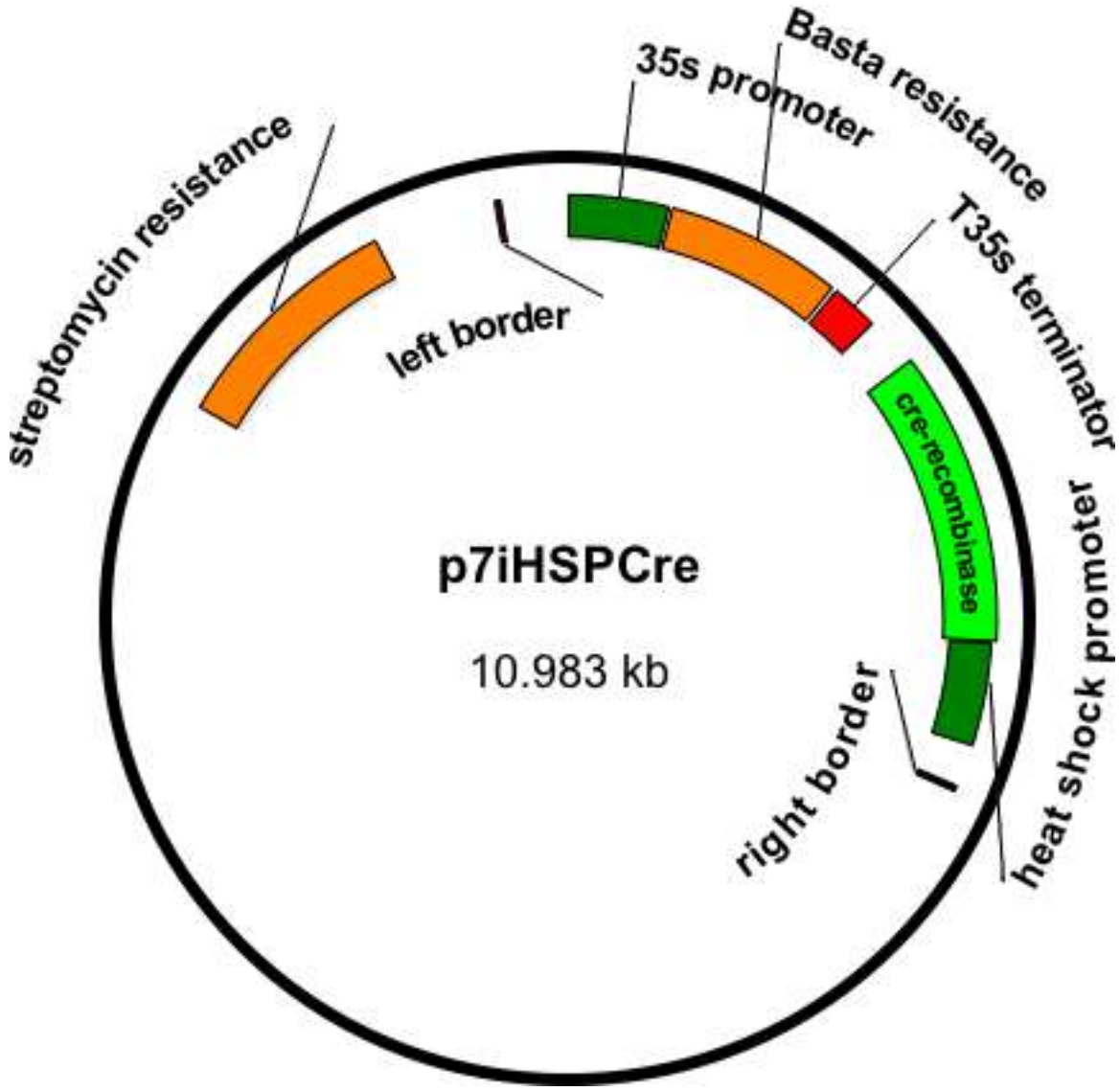


Figure 35 Vector p7iHSPCre
This plasmid was provided by DNA Cloning Services, University of Hamburg, and used for co-transformation with pD1UbibarDHSRNAiwheat. It contains the cre-recombinase and the heat shock promoter. The transcription of cre-recombinase is initiated by heat shock and the enzyme cuts vector pD1UbibarDHSRNAi at the lox sites, moving the ubi promoter in front of the silencing construct. 35s promoter: CamV 35s promoter, T35 sterminator: CamV 35S terminator.

Transgenic wheat DHS RNAi lines

Approximately 1000 wheat embryos were transformed successively by B. Hagemann and M. Woriedh, University of Hamburg, and from one transformation event, five transgenic plants were previously generated: M12-2, M12-3, M12-4, M12-5, and M12-6. These plants survived Basta selection, which means that plasmid pD1ubibarDHSRNAi carrying the DHS RNAi construct and the Basta resistance in the non-recombinant state was transformed successfully. These plants were tested by PCR for the presence of plasmid p7iHSPCre containing the

cre-recombinase and heat shock promoter using primers Cre F and Cre R. Three plants were positive for the cre-recombinase and were chosen for further analysis:

- M12-2
- M12-3
- M12-6

Heat shock and recombination of the DHS RNAi construct in wheat

Heat shock induction was used to activate the RNAi construct. To induce the heat shock promoter in wheat, ten wheat seeds per line were surface sterilized and placed between wet filter paper in Petri dishes, which were kept in a growth chamber in darkness and 22 °C for two days to allow pre-germination of the seeds. For the next 4 days, the Petri dishes were placed in a chamber with 42 °C, 80% humidity and light for 4 hours, followed by 2 hours at 37 °C, and 22 °C in the dark for the rest of the day. On the next day, the seeds were transferred to soil in the greenhouse and covered with a plastic hood for 1 week to aid germination. This seed treatment resulted in no germination. Therefore the exposure to 42 °C was reduced to 2 hours, which lead to successful germination of all seeds. After germination, DNA was analyzed by PCR to prove the recombination of the plasmid using primers CSUbiint F and CSspacerGusR. In the case of proper recombination, the expected band size was 0.4 kb, while the non-recombinant plasmid led to a 1.4 kb band (figure 36).

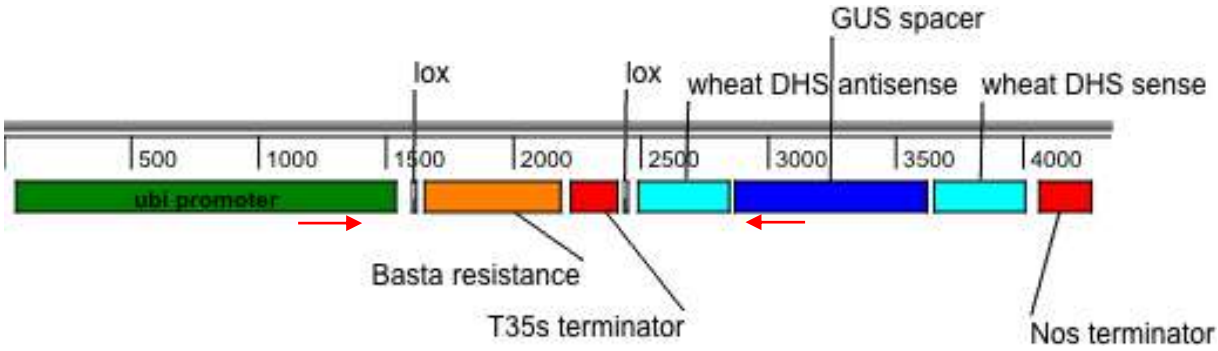


Figure 36 PCR strategy for the recombinant DHS silencing plasmid in wheat
The illustration shows the part between the *Sfi*I sites of vector pD1UbibarDHSRNAiwheat. The size of the fragment amplified by primers CSUbiintF and CsspacerGusR (red arrows) shifts according to the recombination of the plasmid from 1.4 to 0.4 kb. Ubi promoter: plant ubiquitin promoter, T35s terminator: CamV 35s terminator, GUS spacer: β-glucuronidase spacer.

The seed treatment led to only one plant, M12-3.2, with a recombinant band (figure 37).

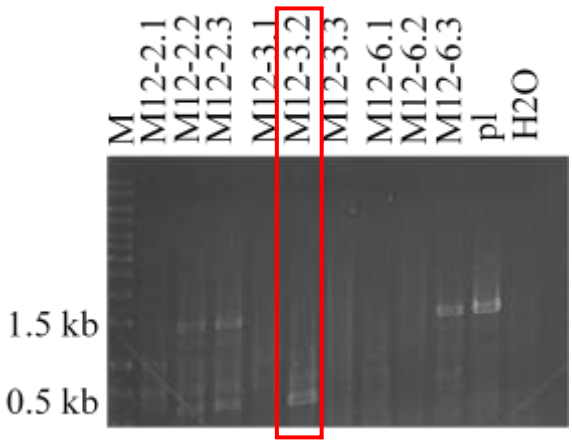


Figure 37 PCR with wheat DHS RNAi lines induced by heat shock at seed stage
Seeds were exposed to 42 °C for 2 hours on 4 consecutive days. Three plants of the three transgenic lines were tested by PCR for the recombination of the plasmid. Only one plant, M12-3.2, had the recombinant band of 0.4 kb using primers CSUbiintF and CSspacerGusR (red box). The non-recombinant size was 1.4 kb.

Another approach for construct recombination was the heat treatment of wheat seedlings one week after germination. This method was tried because seed treatment with heat shock only produced one line with recombinated plasmid. Seedlings were exposed to 42 °C, 80% humidity and light for 2 hours on 4 consecutive days. The experiment was repeated three times with ten seedlings of each line. The seedlings were then analyzed by PCR using primers CSUbiintF and CSspacerGusR, showing that two plants of one line (M12-3.1 and M12-3.3) had the recombinant band (figure 38). Due to technical difficulties with plant template in PCR some weak unspecific bands are visible. PCR was conducted with the Phire Direct Plant PCR Kit (Finnzymes, Espoo, Finland). PCR was repeated two times in order to avoid mistakes and PCR products were sequenced to verify sequence identity.

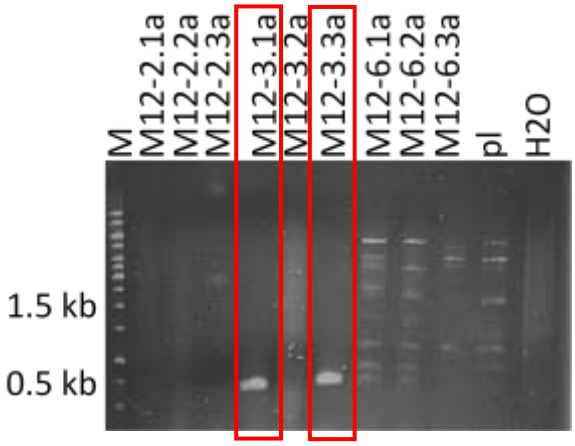


Figure 38 PCR with transgenic wheat DHS RNAi lines induced by heat shock at seedling stage

Seedlings were exposed to 42 °C for 2 hours on 4 consecutive days. Three plants of the three transgenic lines were tested by PCR for the recombination of the plasmid. Two plants, M12-3.1 and M12-3.3, had the recombinant band of 0.4 kb using primers CSUbiintF and CSspacerGusR (red boxes). The non-recombinant size was 1.4 kb. Some weaker unspecific bands result from non target amplification in the PCR.

However, when all three plants with correct recombinant plasmids obtained by the two methods of heat shock at seed and seedling stage were examined for DHS overexpression by RT-PCR, only one line, M12-3.3 from seedling treatment, proved to have the correct band of 0.4 kb using primers CSUbiintF and CSspacerGusR (figure 39). RT-PCR was conducted with 40 cycles using the Phire Direct Plant PCR Kit (Finnzymes, Espoo, Finland).

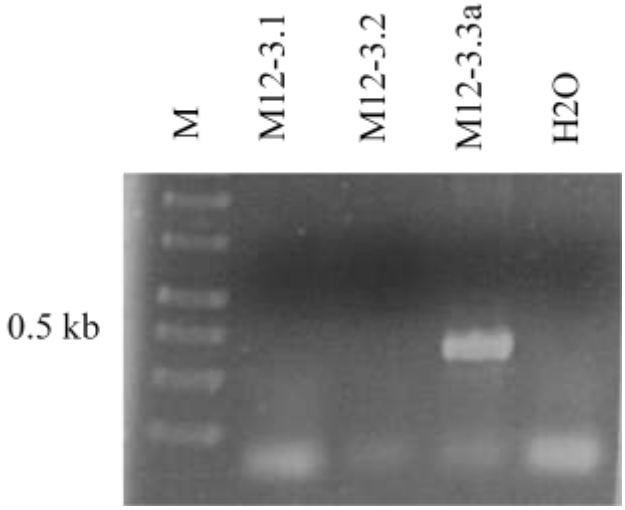


Figure 39 RT-PCR of the plants containing the recombinant wheat DHS RNAi construct

Among M12-3.1 (from seedling treatment), M12-3.2 (from seed treatment), and M12-3.3a (from seedling treatment), only M12-3.3a showed expression of the required fragment of 0.4 kb using primers CSUbiintF and CSspacerGusR.

In order to generate more lines with recombinant plasmid, the seedling treatment was adjusted to 4 consecutive days of 4 hours at 42 °C, 80% humidity and light. Plants looked wilted and damaged especially at the tip, but recovered well after being moved back to the greenhouse. RNA was isolated from five plants of each line and RT-PCR was carried out on the resulting cDNA. In this case, two plants of line M12-2 and 3 plants of line M12-3 showed the required band of 0.4 kb using primers CSUbiintF and CSspacerGusR. It is not clear why the non-recombinant band was not visible in the other plants (figure 40). RT-PCR was conducted with 40 cycles using the Phire Direct Plant PCR Kit (Finnzymes, Espoo, Finland).

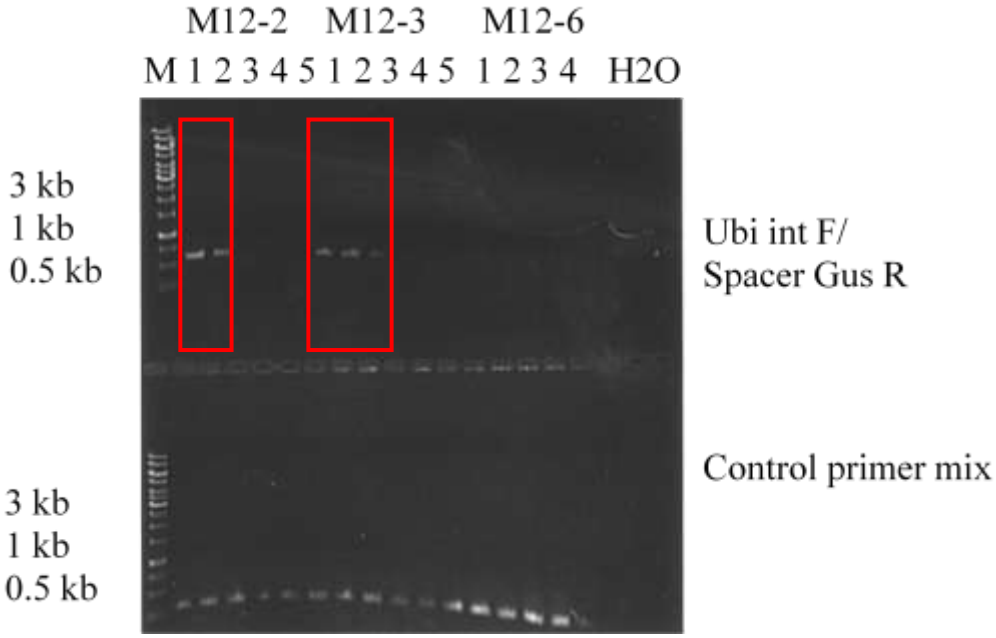


Figure 40 Second seedling heat shock experiment in wheat
Seedlings were exposed to 42 °C for 4 hours on 4 consecutive days. Five seedlings of the three lines were tested. Two plants of line M12-2 and 3 plants of line M12-3 had the recombinant band of 0.4 kb using primers CSUbiintF and CSspacerGusR in RT-PCR (red boxes). The control primer mix binds to a chloroplast gene and was taken from the Plant Phire Taq Kit (Finnzymes, Espoo, Finland).

Plants M12-2.1 and M12-2.2 as well as M12-3.1, M12-3.2, M12-3.3 and M12-3.3a, which resulted from the previous seedling heat shock experiment described above, were chosen for seed production and for infection with *F. graminearum* conidia to observe the DHS silencing effect under pathogen challenge conditions.

Relative quantification of DHS silencing in wheat, T1 generation

Q-PCR with SYBR Green master mix (Roche) was conducted using primers CSWActinqF/R and CSWDHSqF/R in order to determine the expression level of DHS in DHS RNAi wheat lines of the T1 generation. Plant material was harvested at 3 month of age. Using actin as a reference gene and the wild type cDNA as the standard, a 50% reduction of transcript was found in plant M12-3.3a. Plant M12-3.1 and M12-3.3 showed 85% reduction, and plants M12-2.1, M12-2.2 and M12-3.2 had more than 90% reduction of DHS expression (figure 41).

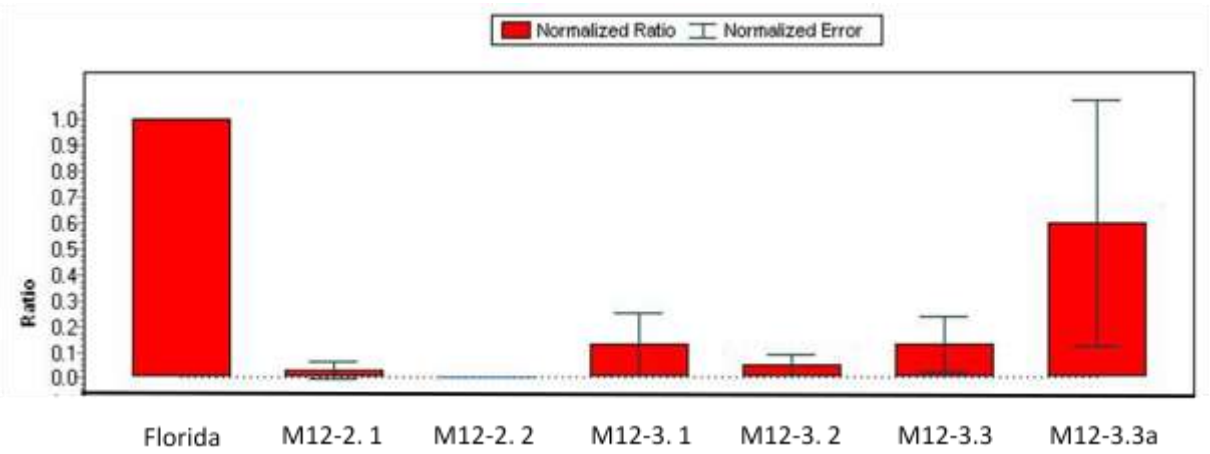


Figure 41 Relative quantification of DHS silencing by qPCR in the T1 generation
Each bar summarizes the relative decrease compared to the wild type expression level of DHS. Plant M12-2.2 has the highest decrease of almost 100%, plants M12-2.1, M12-3.1, M12-3.2, and M12-3.3 are also significantly reduced in DHS expression with 85-98% reduction, and plant M12-3.3a shows a 50% reduced expression. This diagram was compiled using the Roche Light Cycler software (Roche, Mannheim, Germany).

***F. graminearum* infection assay with DHS RNAi transgenic wheat, T1 generation**

The infection assay was carried out to determine if DHS downregulation in wheat would decrease Fusarium Head Blight symptoms. In a preliminary infection assay using 3 wheat heads of Florida wild type, M12-6.1 as control with recombinated plasmid but without gene expression, and all DHS RNAi lines could not be evaluated because even the wild type was not always infected completely. This can be explained by the fact that the cultivar Florida which was used for transformation is not as susceptible to *F. graminearum* as the cultivar Nandu which is usually used for infection assays. The infection assay needs to be repeated with a larger number of plants as soon as T1 seeds are available.

Maize DHS RNAi construct

The maize DHS RNAi construct was cloned analogous to the wheat DHS RNAi vector, replacing the wheat DHS sense and antisense fragments by parts of the maize DHS gene. The function of this vector was the inducible silencing of the endogenous maize DHS gene. The 400 bp fragments of DHS were first amplified from genomic DNA and subcloned to pGEMT, then released from pGEMT with *Afl*II and *EcoR*I and *BamH*I and *Fse*I, respectively, to be cloned successively into the destination vector. The resulting vector pCSmaizeDHSRNAi was then cut by *Sfi*I; the fragment containing the sense- linker- antisense construct was extracted from an agarose gel and ligated with the binary vector p7iHspCre, which had also been digested by *Sfi*I, treated with shrimp alkaline phosphatase (SAP, Fermentas, St. Leon-Rot, Germany) to avoid self ligation, and cleaned up over a standard column (Invitek, Germany). The resulting vector is shown in figure 42.

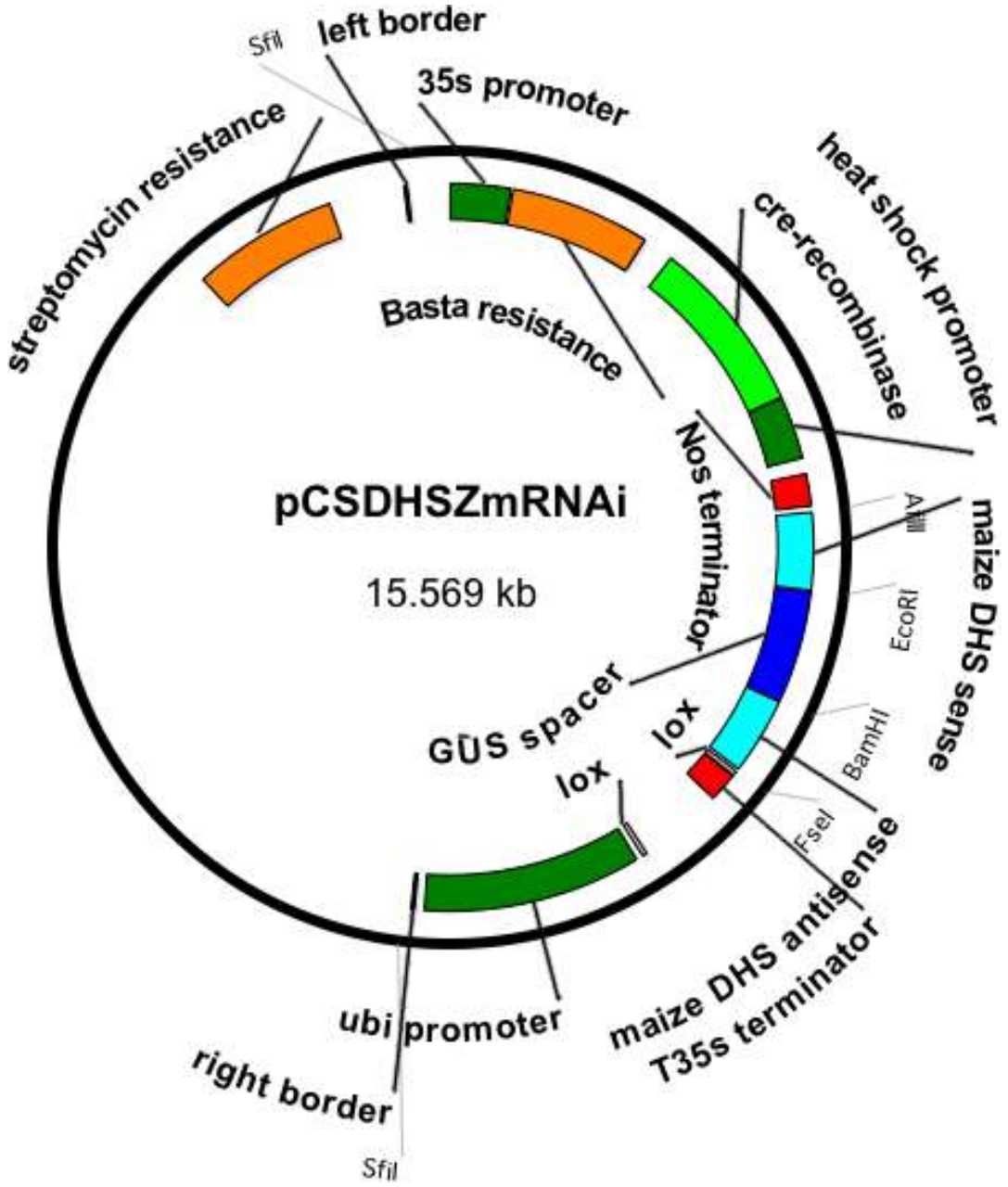


Figure 42 Vector pCSDHSZmRNAi
This is a binary plasmid for *Agrobacterium* mediated transformation which was cloned for DHS silencing in maize. The plasmid is a hybrid of p7iHspCre and pD1UbibarDHSRNAiwheat (Dr. D. Becker and DNA Cloning Services), containing the cre-recombinase with a heat shock inducible promoter, lox sites, and the maize DHS hairpin loop for inducible silencing of DHS in maize. GUS spacer: β-glucuronidase spacer, 35s promoter: CamV 35s promoter, T35s terminator: CamV 35s terminator, Nos terminator: *Agrobacterium* nopaline synthase terminator, Ubi promoter: plant specific ubiquitin promoter.

Transgenic DHS RNAi maize lines, T0 generation

The transformation of maize plants was carried out by S. Amati, University of Hamburg. Three independent transformation events led to 2 regenerating plants each. All potentially transgenic plants were sprayed with 250 mg/l Basta on 3 consecutive days 4 weeks after

transplanting to soil. Since all plants proved to be Basta resistant, DNA was extracted and digested with *EcoR*I, which cuts the genomic DNA randomly and is a non cutter of the cre-recombinase gene. Southern blot with a probe binding specifically to the cre-recombinase, which was kindly provided by A. Hinze, University of Hamburg, showed that 4 plants had one or multiple integrations of the cre-recombinase gene (figure 43). The T1 generation will be used for heat shock experiments and phenotypical characterization in the future.

Figure 43 Southern blot of transgenic DHS RNAi maize plants, T0 generation
The DNA was digested with *EcoR*I and probed with a cre-recombinase fragment. Lines 1.1, 1.2, and 4.2 show multiple integrations of the plasmid; line 4.1 has a single integration. These plants were chosen for further analysis.

Transgenic maize DHS overexpression lines, T0 generation

In the previous PhD work concerning DHS silencing in wheat (Woriedh, 2010), a DHS overexpression construct for transformation of maize was cloned by DNA Cloning Servicess, University of Hamburg, and transformed in order to further elucidate the role of DHS in plant development. The resulting plasmid p7iHspCreUbiNptDHS (figure 44) is a binary plasmid for *Agrobacterium* mediated transformation and contains a heat shock promoter, the Basta resistance, cre-recombinase and lox sites. The construct is inducible with heat shock, which activates the cre-recombinase that cuts at the lox sites and moves the DHS gene behind the ubiquitin promoter for overexpression.

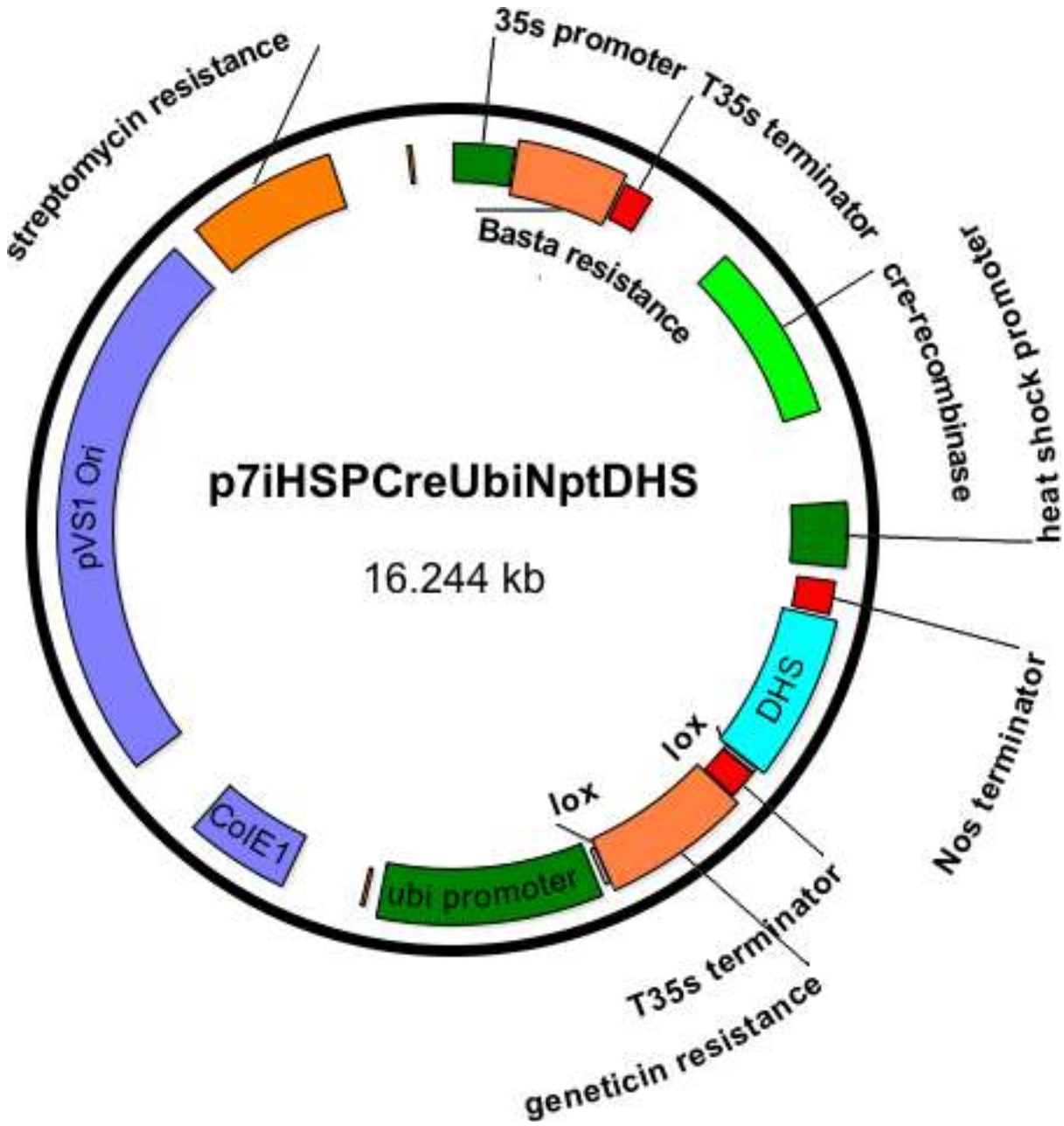


Figure 44 Vector p7iHSPCreUbiNptDHS
This plasmid was cloned by DNA Cloning Servicess and used by Dr. M Woriedh in a previous study to transform maize plants with *Agrobacterium* in order to achieve DHS overexpression lines. The plasmid contains the cre-recombiase and a heat shock promoter. The heat shock promoter can be activated by temperature increase and will drive expression of the cre-recombinase, which cuts at the lox sites and moves the maize DHS gene behind the ubi promoter, resulting in DHS overexpression. 35s promoter: CamV 35s promoter, 35s terminator: CamV 35s terminator, ubi promoter: plant specific ubiquitin pomoter.

In several transformation events, different transgenic lines had previously been produced. In this study, the T0 plants were self fertilized if possible or outcrossed with their parental wild type (HiIIA or HiIIB) if no transgenic pollen or cobs were available. The seeds were

harvested and the T1 plants re-evaluated by PCR. The following lines were selected for further studies due to sufficient seed production:

- M2-1 x A188
- M2-4
- M2-3
- M2-8e
- M2-8d
- M2-10 x HiIIA
- M4-1
- A188 x M7-1
- M10-4
- HiIIB x M10-13b
- M2-8b x Hi99

Heat shock conditions and recombination in maize

Heat shock induction was used to activate the RNAi construct. To induce the heat shock promoter in maize, the seeds were surface sterilized and placed between wet filter paper in Petri dishes, which were kept in a growth chamber in darkness and 22 °C for two days to allow pre-germination of the seeds. For the next 4 days, the Petri dishes were placed in a chamber with 42 °C, 80% humidity and light for 4 hours, followed by 2 hours at 37 °C, and 22 °C in the dark for the remaining time. On the next day, the seeds were transferred to soil in the greenhouse and covered with a plastic hood for one week to aid germination.

Transgenic maize DHS overexpression lines, T1 generation

After heat shock induction and germination, DNA was analyzed by Southern blot to prove the recombination of the plasmid (figure 45). The DNA was digested with *Sfi*I. The Southern probe binds specifically to the DHS gene and was generated by Dr. D. Becker, University of Hamburg. Recombination of the plasmid leads to a size shift from a 3.8 to a 3 kb fragment because the cre-recombinase cuts out a 0.8 kb fragment between the lox sites.

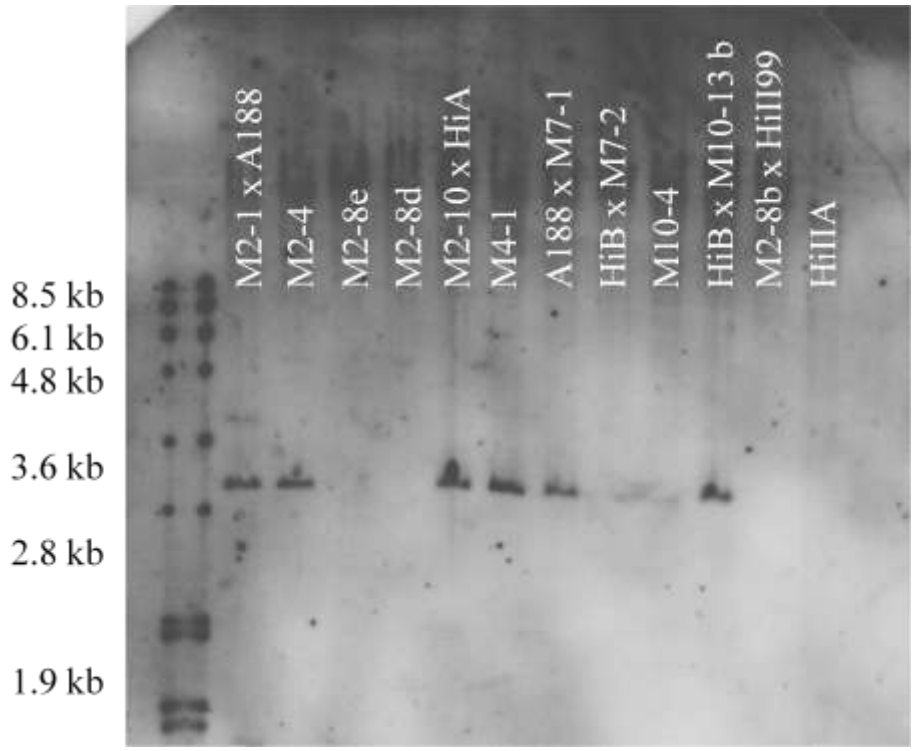


Figure 45 Southern blot of transgenic DHS overexpression maize lines, T1 generation
Twenty µg of DNA were digested with *Sfi*I. Recombination of the plasmid leads to a size shift from a 3.8 to a 3 kb fragment because the cre-recombinase cuts out a 0.8 kb fragment between the lox sites. Line M2-1 x A188 is chimeric, showing the much weaker non-recombinant and the recombinant band. Lines M2-1 x A188, M2-4, M2-10 x HiA, M4-1m A188 x M7-1, and HiB x M10-13 b all show the desired recombinated plasmid.

The following lines with recombinant bands were chosen to produce the T2 generation:

- M2-1 x A188
- M2-4
- M4-1
- A188 x M7-1
- HiIIB x M10-13b

The results from the Southern Blot were verified by RT-PCR using primers DHS2F and NosUR, which amplify a 1.4 kb band in case of non-recombination and a 0.4 kb band in case of recombination (figure 46, figure 47).

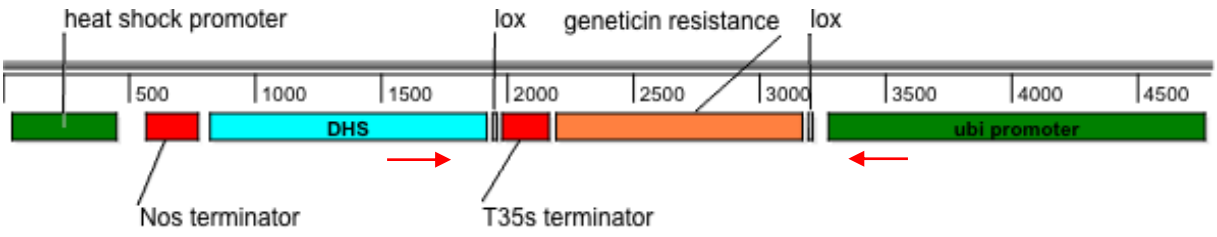


Figure 46 RT-PCR strategy for recombinant DHS overexpression plasmid in maize
If the cre-recombinase is working, it should excise the geneticin resistance gene between the lox sites and move the DHS gene in front of the ubi promoter. The size of the fragment amplified by primers CSDHS2F and NosUR (red arrows) shifts according to the recombination of the plasmid from 1.4 to 0.4 kb. Nos terminator: *Agrobacterium* nopaline synthase terminator, T35s terminator: CaV 35s terminator, ubi promoter: plant specific ubiquitin promoter.

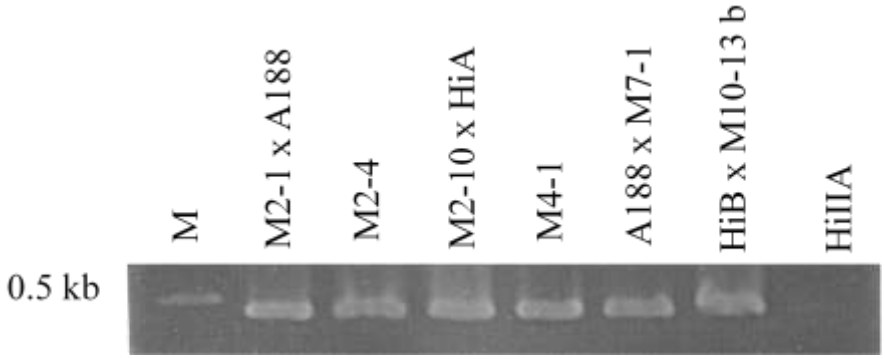


Figure 47 RT-PCR of recombinant DHS overexpression maize lines, T1 generation
The correct size of the recombinant band amplified by primers CS DHS2F and NosubiR is 0.4 kb. HiIIA: wild type maize.

M2-10 x HiIIA was not used any further because no sufficient number of seeds was obtained.

DHS overexpression in maize leads to severely increased susceptibility to salt stress

The reaction of DHS overexpression plants towards salt stress was tested because DHS is involved in stress response and has been described to increase the severity of stress related symptoms (Thompson *et al.*, 2004).

Three plants of each of the following lines were used in this experiment: M2-1 x A188, M2-4, M4-1, A188 x M7-1, HiIIB x M10-13b

When 2 month old plants were watered with 50 ml of a 2 M NaCl solution for three days, the transgenic lines showed a dramatic reaction. They collapsed and wilted completely, while the wild type remained unchanged (figure 48). This result is in unison with the hypothesis that

DHS is involved in ageing and senescence of plants and an increased amount of DHS leads to reduced stress tolerance.

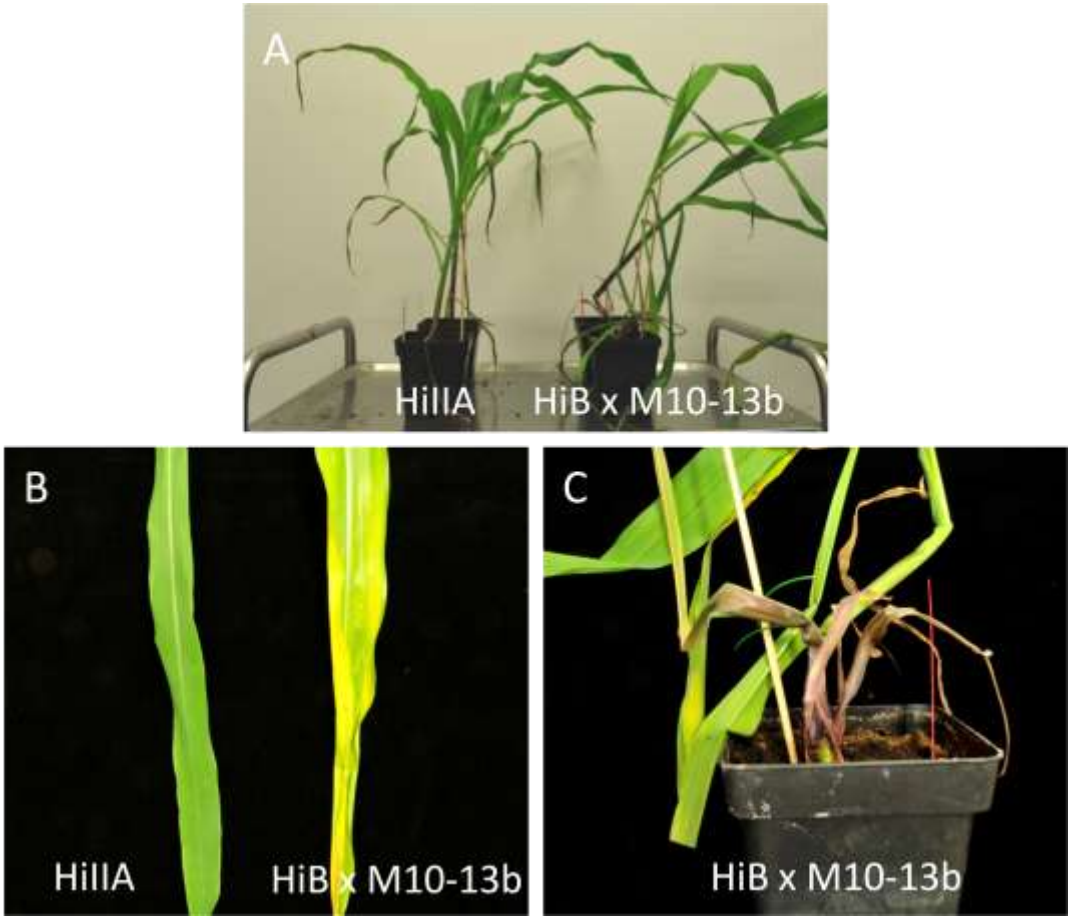


Figure 48 Salt stress reaction of an exemplary transgenic plant (M2-4)
Two month old plants were watered with 50 ml of a 2 M NaCl solution for three days. Plants wilted (A), leaves became yellow (B) and the stalks collapsed at the bottom (C). HiIIA is the wild type plant.

The induction of the heat shock promoter is stable in the seeds

The lines which showed a successful recombination event in the T1 generation were grown to maturity and self fertilized in the greenhouse. Seeds were harvested and the T2 generation was examined by PCR and RT-PCR using primers DHS2F and NosUbiR, which generate a 0.4 kb band in case of recombination, but a 1.4 kb band in case of non-recombination. It was shown that the recombination was stable in the T2 generation because all tested plants had the recombinant band (figure 49). PCR and RT-PCR were carried out with the Plant Phire Taq Kit (Finnzymes, Espoo, Finland) and 40 cycles.

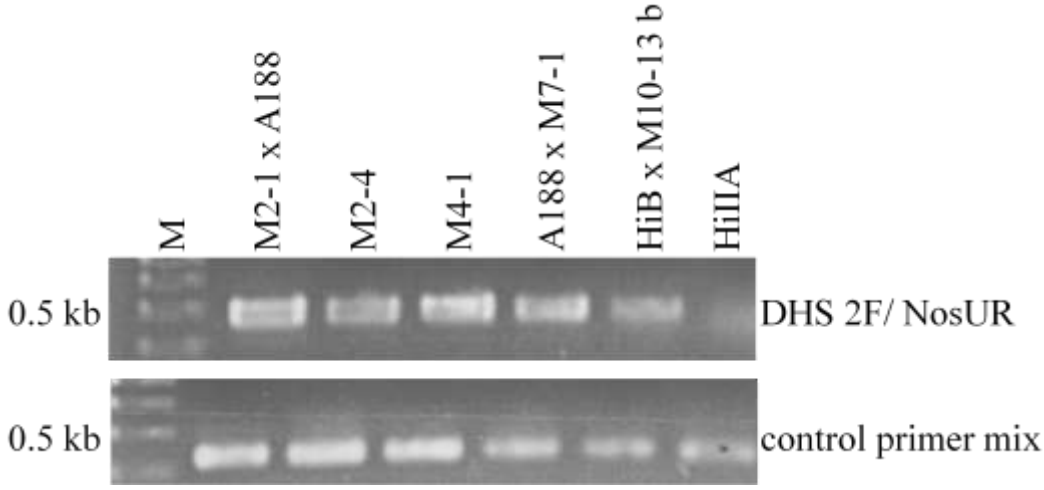


Figure 49 RT-PCR of maize DHS overexpression plants, T2 generation
All plants showed the recombinant 0.4 kb band, which proved that the construct was stably induced. The control primer mix is specific to chloroplasts and was taken from the Plant Phire Taq Kit (Finnzymes, Espoo, Finland.)

Relative quantification of DHS overexpression

Q-PCR using the SYBR Green master mix (Roche) was conducted with primers CSActinMaizeqF/R and CSMaizeDHSqF/R in order to determine the expression level of DHS in overexpressing maize lines of the T2 generation. Plant material was harvested at three month of age. Using actin as a reference gene and the wild type cDNA as the standard, a 1800 fold increase of transcript was determined for line HiB x M10-13b, a 1600 fold increase for line A188 x M7-1, and a 200 fold increase for line M4-1. Lines M2-1 x A188 and M2-4 showed no drastically increased DHS expression (figure 50).

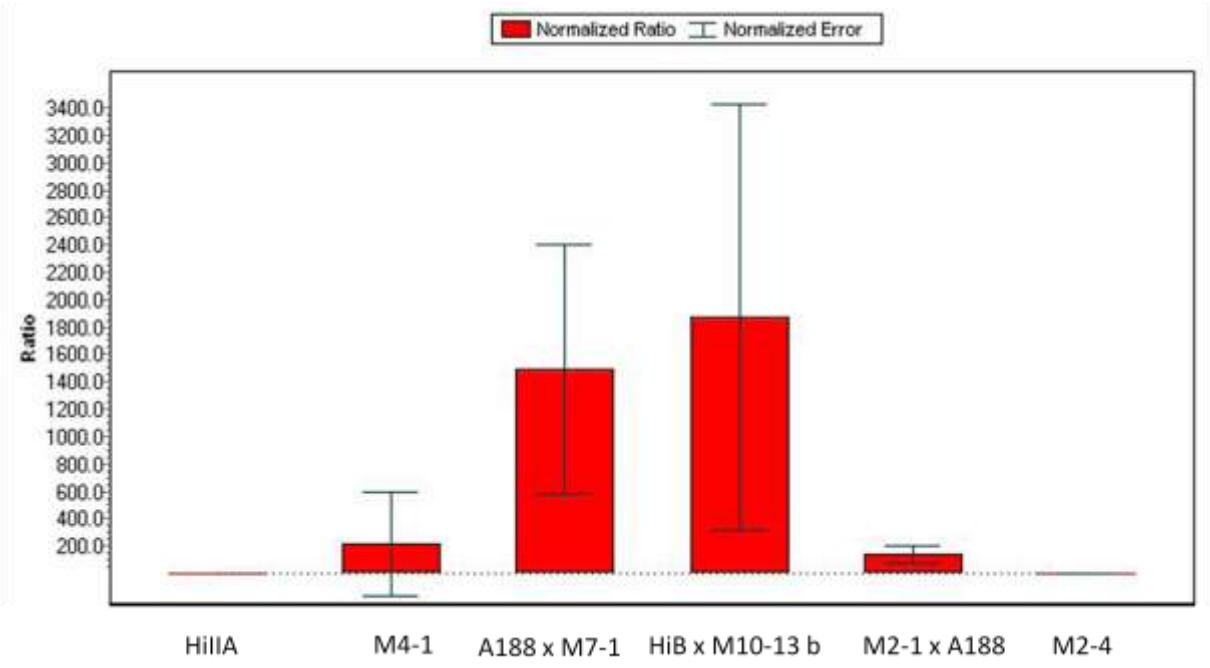


Figure 50 Relative quantification of DHS overexpression by qPCR in the T2 generation
Each bar summarizes the relative increase compared to the wild type expression level of DHS. Line HiB x M10-13b has the highest increase of 1800 fold, line A188 x M7-1 the second highest of 1600 fold, and line M4-1 the third highest of 200 fold. The diagram was compiled with the Roche Light Cycler software (Roche, Mannheim, Germany).

Maize DHS overexpressing plants (T2) are not significantly different from wild type plants in growth rate and seed set

Five plants of each transgenic line were grown in the greenhouse and observed for their growth behavior and seed set. The plant length, number of leaves, length and width of leaves as well as the stem diameter were measured every 5 days for 4 month. Cobs were pollinated and the number of seeds was counted after harvest. No significant difference could be found.

Maize DHS overexpressing plants (T2) are not significantly different in susceptibility to *Colletotrichum graminicola*, *Cochliobolus heterostrophus*, and *Setosphaeria turcica*

In this experiment, the susceptibility of maize DHS overexpression lines to three important foliar pathogens of maize was tested: *C. graminicola*, Anthracnose Leaf Blight, *C. heterostrophus*, Southern Leaf Blight, and *S. turcica*, Northern Leaf Blight. Six leaves of 6 week old plants each transgenic line were cut into 8 cm stripes, surface sterilized and placed in Petri dishes with moist filter paper. Conidia of the fungal strains were harvested with sterile water from plates, the concentration was adjusted to 50 conidia per µl, and a drop of 10 µl conidia suspension was carefully placed in the middle of the leaf section. Plates were sealed and kept at 24 °C and 16 hours light for one week. The length of the developing lesion was measured.

The experiment was repeated 2 times and no significant difference in susceptibility could be observed under the given conditions (figure 51).

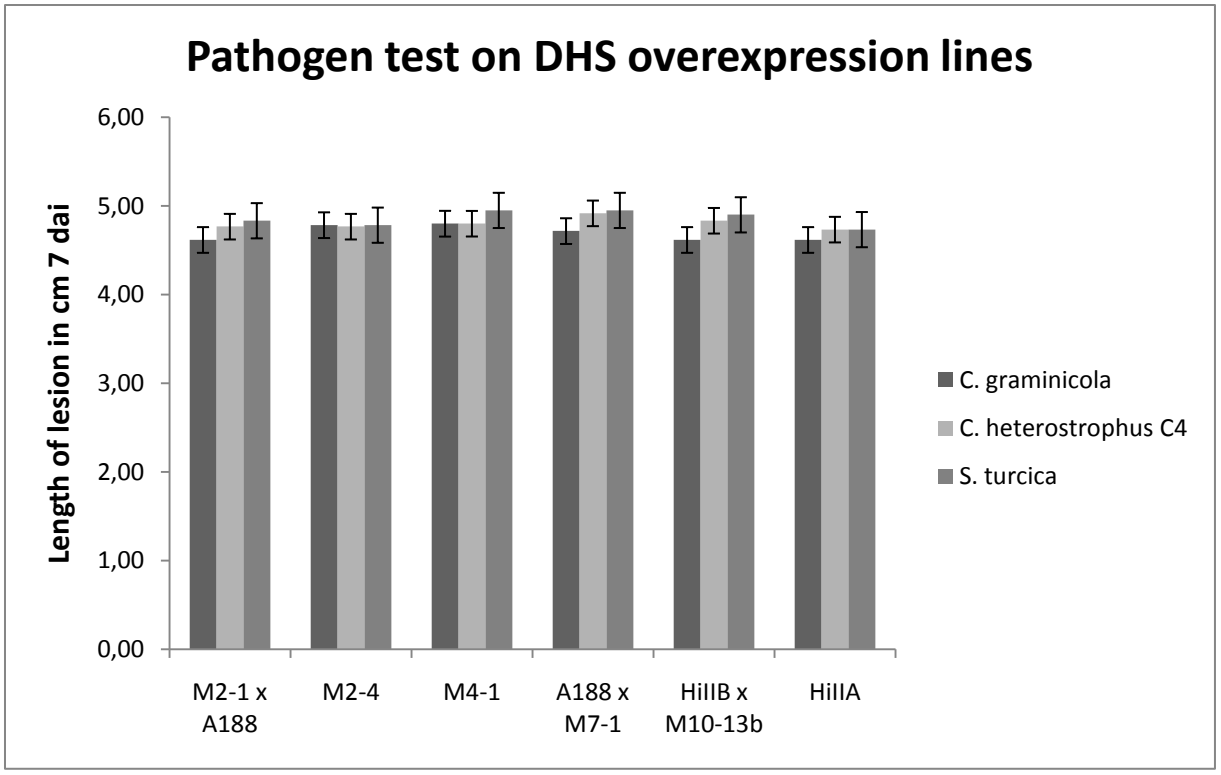


Figure 51 Pathogen test on DHS overexpression lines, T2 generation
In this assay, the pathogenicity of different fungi (*C. graminicola*, Anthracnose Leaf Blight, *C. heterostrophus*, Southern Leaf Blight, and *S. turcica*, Northern Leaf Blight) was tested on detached leaf sections of 6 week old transgenic DHS overexpression maize lines. No significant difference in the size of the lesions could be found in the transgenic lines compared to the HiIIA wild type. Dai: days after infection.

Discussion

The major aim of this study was to establish host induced gene silencing (HIGS) in the wheat- *F. graminearum* and sugar beet- *C. beticola* pathosystems. HIGS in plant- fungal pathosystems is defined as the transformation of an RNAi construct into a host plant which targets a fungal virulence or essential gene. The resulting dsRNA should downregulate the gene of interest in the fungus when it attacks the plant which should result in more resistant plants. Several subsets of experiments were conducted in this study contributing to the main goal of HIGS against *F. graminearum* and *C. beticola*.

RNAi based silencing is functional in *Cercospora beticola*

One goal of this study was to proof the concept of RNAi based gene silencing in *C. beticola* after it had previously been demonstrated in *F. graminearum* (Boennighausen, 2010). By proving that gene silencing is possible in *C. beticola*, a foundation would be laid for further HIGS experiments. *C. beticola* strains with the reporter gene GFP were generated for this purpose so that silencing could be easily monitored. RNAi based silencing in phytopathogenic fungi is a prerequisite for Host Induced Gene Silencing and a widespread means in many different organisms in defense against viruses and transposons, endogenous gene regulation during growth and development, and heterochromatin formation (Brodersen & Voinnet, 2006). In plant genetic engineering, silencing has previously been used in order to improve resistance against plant viruses by the expression of virus specific sequences (Carr *et al.*, 2010). The RNAi pathway has also been described in fungi, namely in *N. crassa*. This model ascomycete has two distinct pathways: RIP (Repeat Induced Point Mutation), which leads to irreversible destruction of invasive and duplicated endogenous DNA by cytosine methylation (change in chromatin density) and G:C to A:T mutation during crosses (Selker & Stevens, 1987), and MSUD (Meiotic Silencing by Unpaired DNA), a pathway which specifically silences unpaired DNA during meiosis (Latterich, 2008). Quelling, which is similar to post transcriptional gene silencing in plants, has been described for other fungi like *P. anserina, A. fumigatus, F. oxysporum*, and *M. oryzae.* The vector pSilent-1 has been established as a high throughput tool for gene silencing in *M. oryzae* (Nakayashiki, 2005). In *F. graminearum*, the *tri6* gene of the trichothecene gene cluster has been silenced by a hairpin construct (McDonald *et al.*, 2005).

In this study, RNAi based gene silencing was successfully employed in *C. beticola* targeting a marker gene. Using GFP as the target gene greatly facilitated the analysis of transformants. RNAi has an inherent risk of off target silencing, which has been minimized by choosing a

sequence with as little similarity to non target genes as possible. The employed 400 bp have proven to be an efficient fragment size, easy to clone and sufficient for silencing. RNAi based silencing is a powerful tool in researching essential genes in filamentous fungi and can be used for the characterization of genes whose gene disruption may be lethal to the fungus. The combination of hairpin constructs with inducible promoters like the alcohol dehydrogenase promoter AlcA allows silencing at different points of development (Barton & Prade, 2008).

CTB2 is a good target candidate for Host Induced Gene Silencing in sugar beet

CTB2, a gene in the cercosporin biosynthesis pathway, was characterized by gene deletion. This gene was chosen as a potential target for later HIGS experiments because previous studies indicated that cercosporin is essential for the pathogenicity of *Cercospora ssp.* and *CTB2* is required for cercosporin biosynthesis (Chen *et al.*, 2007b).

C. beticola is a commercially important pathogen which threatens the sugar beet production worldwide. Despite all progress in breeding and genetic modification of crops, a broad and stable resistance is yet to be achieved. Cercosporin is known to be a powerful toxin with the capacity to destroy large areas of leaf surface in infected plants. Previous studies in *C. nicotiana* describe that the gene disruption of several genes involved in the toxin biosynthesis disabled the cercosporin production and rendered the fungus avirulent (Chen *et al.*, 2007a).

In the current study, we showed that the O-methyltransferase *CTB2* is essential for cercosporin biosynthesis and fungal virulence of *C. beticola.* Gene disruption mutants failed to produce cercosporin and were non pathogenic on sugar beet plants.

The gene disruption mutants generated in this study are no longer able to infect host plants, which makes *CTB2* a great target for HIGS. Future studies will show whether *CTB2* RNAi constructs can improve sugar beet resistance against *C. beticola*.

Host Induced Gene Silencing constructs were successfully generated but no wheat transformants were achieved by biolistic transformation

HIGS constructs against two genes of *F. graminearum* were cloned and transformed into wheat. The genes of choice were *Fgl1*, a secreted lipase which is a major virulence factor in *F. graminearum* (Voigt *et al.*, 2005), and *Homoaconitase*, an essential fungal gene which is necessary for the lysine biosynthesis in fungi and was previously characterized in *P. teres*

(Sonnenberger, 2002). If these genes were downregulated in the fungus during its infection of wheat plants, pathogenicity of the fungus could be reduced.

HIGS has been studied so far in the barley- *Blumeria graminis* pathosystem. In this obligate biotroph, several genes, above all two glucosyltransferases, are suitable targets for silencing by RNAi delivered by the transgenic host. Significant reduction of haustoria formation and fungal growth could be shown in both transient and stable transgenic lines (Nowara *et al.*, 2010). Moreover, HIGS could be proven in tobacco expressing a hairpin construct against the GUS gene. When inoculated with a *F. verticilloides* GUS reporter strain, the GUS gene was silenced in the fungal cells (Tinoco *et al.*, 2010).

Moreover, HIGS is applicable to other pathogens than fungi: When a root knot nematode parasitism gene, 16D10, which encodes a secretory peptide from the esophageal gland that promotes plant root growth when injected into plant root cells while feeding, is used in an RNAi silencing construct in *Arabidopsis*, nematode infection clearly diminishes (Huang *et al.*, 2006). Also, several genes encoding different proteins, including ribosomal proteins, β-tubulin homologs, and v-ATPase, were successfully used in maize plants for silencing in *Lepidoptera* and *Coleoptera* species (Baum *et al.*, 2007). Moreover, RNAi can also be translocated from host plants to parasitic plant species, as shown in the species *Cuscuta* (Westwood *et al.*, 2009).

Wheat plants were transformed biolistically with different constructs: pCSFgl1-35s, pCSHac35s, and a heat shock inducible construct, pCSActRNAiFgl135s. Although we followed an established protocol for biolistic wheat transformation and wheat has been biolistically transformed in different groups before (Jones & Sparks, 2009), no transgenic wheat plants could be generated in this study. Different controls were performed, e.g. the regeneration of plants from embryogenic callus without any bombardment and the regeneration of plants transformed with a plasmid carrying the Basta resistance only, but equally failed. As described before, embryogenic callus always develops accompanied by non embryogenic callus (Sangduen & Klamsomboon, 2001). The non embryogenic tissue is grey, soft, and watery in appearance, while the embryogenic callus is yellow, dry, and round shaped. In this study, the majority of developing callus was non embryogenic and died during the regeneration process. Although we tried to apply different amounts of 2,4-D, which promotes the shoot regeneration, and opted to isolate embryos at the perfect developmental stage, no significant improvement was achieved. We also evaluated different amounts of DNA from 5 µg to 20 µg in total and found that lower concentrations of 5 µg total DNA

worked best. This is due to the fact that high amounts of DNA promote the agglutination of gold particles to bigger pieces which can destroy the embryogenic tissue. Controls with plasmid pActI-D carrying the GFP (green fluorescent protein) gene and pMon349 carrying the GUS (β-glucuronidase) gene proved that Gus and GFP were integrated and expressed, but these vectors have no Basta resistance gene and the resulting transgenic calli were not grown longer than two month because transgenic tissue is lost without selection pressure. In further experiments it will be necessary to clone a vector with GFP and Basta resistance and use it as a control for regeneration and transformation.

So far it is not clear why it was not possible to transform wheat plants with RNAi constructs during this study. Establishing HIGS in the pathosystem wheat- *F. graminearum* will be a breakthrough for Fusarium Head Blight control. The next approach will be *Agrobacterium* mediated transformation of wheat (He *et al.*). Once the transformation is successful, HIGS constructs could be used to enhance plant resistance against different fungi at once. When targeting a gene like *Homoaconitase*, which is essential in all fungi for lysine biosynthesis, different pathogens should be equally affected by the downregulation of this gene. Moreover, constructs could be designed with several fragments of different genes of interest cloned subsequently between two promoters in sense and antisense direction. This would allow the targeting of several genes with one construct.

Attempts to delete or silence *Homoaconitase* in *F. graminearum* failed

Homoaconitase is an essential gene in the fungal lysine metabolism and was chosen as a target gene for HIGS constructs. To characterize *Homoaconitase* in *F. graminearum*, null mutation supplemented with lysine was attempted with different constructs but no mutant strains could be generated. In a previous study, *Homoaconitase* has been disrupted in the pathogen *Pyrenophora teres*, and the mutant strain was reduced in pathogenicity (Sonnenberger, 2002). These results suggest that *P. teres* is unable to utilize lysine from the host plant. Since no deletion mutants could be generated in *F. graminearum*, it may be the case that *Homoaconitase* has other functions besides lysine biosynthesis which are essential for the fungus. The construct for inducible silencing pCSSW08Hac is designed for random integration. No transformants could be generated so far with this vector. It has been previously reported that pSW08 has a low transformation rate in *F. graminearum* (Boennighausen, 2010). Transformation can be improved by directed integration of the construct, which could be achieved by cloning a fragment of a non essential gene into the vector and opening this fragment by a single cutter for homologous recombination. A good

candidate for this purpose is *Pks12* (accession number AY706311), a polyketide synthase in the *F. graminearum* aurofusarin synthesis pathway. When this gene is disrupted, the resulting transformants are white instead of the usual red color of *F. graminearum* (Maier *et al.*, 2005). So far this approach has been delayed due to the lack of suitable restriction sites. Another possibility may be the use of a GFP wild type strain for transformation and the integration of parts of the GFP gene which could be used for homologous integration in the pSW08 vector.

The role of *Dicer 1* in *F. graminearum* has to be further elucidated

Dicer is a key player in the RNAi pathway. It is a part of the RNA induced silencing complex (RISC) and processes dsRNA. In the context of HIGS, a very interesting question is whether the dsRNA expressed in the plant host is processed by the plant RNAi machinery or if and how it is exported into the fungal host and cleaved by the fungal RNAi apparatus. To answer this question we attempted to delete or silence *Dicer 1*, one of the two *Dicer* homologs in *F. graminearum*.

In fungi, many well studied organisms have two *Dicer* genes, e.g. *N. crassa*, which uses *Dicer 1* mainly for the siRNA pathway and *Dicer 2* mainly for MSUD (Meiotic Silencing of Unpaired DNA) (Alexander *et al.*, 2008). An important plant pathogen, *M. oryzae*, also contains two *Dicer* genes, but only one seems essential for silencing, while the role of the second homolog has yet to be elucidated (Kadotani *et al.*, 2004). In *Cryphonectria parasitica* and *Mucor circinelloides*, *Dicer 2* is required for gene silencing via RNAi, but the fungi are still viable after disruption of this gene (de Haro *et al.*, 2009, Segers *et al.*, 2007). In *Sacharomyces castelli*, both *Dicer* like genes are necessary for gene silencing, but neither of them is essential for survival (Drinnenberg *et al.*, 2009). However, in mice as the model species for mammals, the gene disruption of *Dicer 1* is lethal to the embryo (Maatouk *et al.*, 2008).

Like other fungi, *F. graminearum* has two *Dicer* like genes, FG04408.1 (*Dicer 1*), and FG09025.1 (*Dicer 2*). *Dicer 2* has been disrupted in a previous study, without visible effects on growth, conidiation, or virulence of the fungus (Darissa, 2010).

In this study, it was attempted with multiple techniques to delete or silence *Dicer1*, using cassettes for homologous recombination with different flanking sizes and different resistance genes as well as an inducible silencing construct, but no transformants resulted from multiple experiments. These results suggest that *Dicer1* could be essential for the survival of *F.*

graminearum and that even the slightest silencing effect caused by a leaky promoter in the inducible silencing construct is sufficient to inhibit transformant regeneration.

The next step could be the sequential gene disruption of different domains of *Dicer 1* in order to find out which part of the sequence is actually essential. A different approach would be the integration of another gene suitable for homologous recombination into the vector, e.g. *Pks12*, a polyketide synthase which leads to a clearly visible white phenotype when disrupted (Maier *et al.*, 2005). This method could facilitate integration and screening.

Agrobacterium mediated transformation in *Brachypodium distachyon* is a fast and efficient means to generate transgenic HIGS model plants with RNAi constructs

In this study, it could be shown that RNAi constructs against fungal genes can be transformed into and expressed in *B. distachyon*. Since *B. distachyon* is a valuable model plant for wheat, this is a relatively fast and efficient way to test constructs in this species before transforming wheat, which is more difficult to transform and needs longer to regenerate. We used the HIGS construct pCS6UFgl135s targeting *Fgl1*, a binary vector suitable for *Agrobacterium* mediated transformation and constitutively expressing a double promoter RNAi construct against *Fgl1*. One problem occurring in this experiment is the small number of seeds that are set on transgenic *B. distachyon* plants. Although the plants were kept in constant environmental conditions in a growth chamber, many spikelets did not produce seeds. This may be an obstacle in the infection process of *F. graminearum* because it could be difficult for the fungus to overcome "empty" spikelets. Further experiments with a greater number of plants will show if the current conditions allow a statistical evaluation of the infection.

Deoxy hypusine synthase (DHS) plays an important role in stress response in maize

In this study, DHS was examined as an example of the silencing of an endogenous gene in wheat and maize. DHS is responsible for the hypusination and activation of translation initiation factor eIF5-α (Park *et al.*, 1981). This factor is involved in the transport of mRNA specific to ageing and senescence (Lipowsky *et al.*, 2000). Previously, the function of DHS in *F. graminearum* was examined and gene disruptions were lethal (Woriedh, 2010). In this study, DHS was to be examined by downregulation in wheat and maize as well as by overexpression in maize. Transgenic plants with the silencing constructs pD1ubibarDHSRNAiwheat (cloned and transformed previously in wheat), pCSDHSZmRNAi (cloned and transformed in maize this study), and the overexpression construct

p7iHSPCreUbiNptDHS (cloned and transformed in maize previously) were analyzed in this study. All constructs are inducible by heat shock. Inducible promoters are an elegant way to start gene expression at specific time points or in certain plant organs. One of the major advantages of inducible systems is that the gene of interest is exclusively expressed at the desired locations or developmental stages. This method avoids possible side effects of the construct early during the regeneration phase as well as false positive phenotypes unrelated to the physiological trigger of the intended gene expression.

DHS is responsible for the activation of translation initiation factor eIF-5α. Studies in *A. thaliana,* which has three homologs of eIF-5α, revealed that silencing of all three eIF-5α genes resulted in deceleration of senescence and in reduced cell death in response to sublethal stress levels, which led to an increased life span, biomass, and seed yield of the plants (Thompson *et al.*, 2004). Another group found corresponding data showing that downregulation of eIF-5α-2 decreased apoptosis and led to enhanced resistance against *Pseudomonas syringae* (Hopkins *et al.*, 2008). However, it was also observed in *A. thaliana* that eIF-5α-3 overexpressing lines were more resistant towards osmotic stress and nutrient starvation (Ma *et al.*). Medical studies showed that eIF-5α is essential for stress induced apoptosis in the ER of pancreatic β-islet cells during type II diabetes, and that inhibition of DHS significantly improved glucose catabolism, insulin folding and β-islet mass (Robbins *et al.*, 2010). Additional research suggests that DHS is involved in the unfolded protein response in the ER and that its inhibition decreases translation of stress related proteins and relieves diabetes symptoms in mice (Song *et al.*, 2008).

Wheat plants expressing DHS RNAi were examined until the T1 generation. Heat shock was established successfully and a preliminary infection assay with *F. graminearum* was conducted. This assay was inconclusive because even the wild type plants were not always fully infected. This may be due to the fact that wheat cv Florida which was used for transformation is not as susceptible as cv Nandu that is normally used for *F. graminearum* infection. The infection assay will be repeated with a greater number of plants for statistical evaluation when T1 seeds are harvested. Also, the DHS expression levels should be examined at anthesis of the wheat plants both in wild type and the RNAi lines to verify whether DHS is transcribed during the infection time of *F. graminearum* and if it is downregulated by the RNAi constructs in the flowers. Further experiments could be conducted testing heat, drought, and nutrient starvation stress response of the transgenic DHS RNAi plants.

In this study, we could show for the first time that DHS overexpression in maize leads to a salt sensitive phenotype. Further experiments should include other abiotic stresses, e.g. drought or cold. We examined the effect of different maize foliar pathogens (*Cochliobolus heterostrophus, Setospahaeria turcica*, and *Colletotrichum graminicola*) on young leaves of transgenic maize DHS overexpression plants but found no obvious difference in the progress of infection. These results can be further elucidated with different experimental setups, e.g. the infection of different developmental stages of maize or the infection of whole plants.

Additional insights will be gained from transgenic DHS RNAi maize plants. As soon as T1 plants are available, it will be interesting to compare the reactive oxygen species (ROS) levels and apoptosis under stress conditions in overexpression and silencing lines.

Summary

The greater goal of this work was to establish host induced gene silencing (HIGS) in the *F. graminearum*- wheat and *C. beticola*- sugar beet pathosystem. Several approaches were taken to evaluate strategies for increased resistance against *F. graminearum* and *C. beticola* in sugar beet, wheat, and maize, as well as in the model plant *B. distachyon*. Different vectors were employed for RNA interference based silencing of fungal genes in the plant hosts.

Firstly, silencing of the reporter gene GFP was established as a proof of concept of RNAi based gene silencing in *C. beticola*, a foliar pathogen of sugar beet.

Secondly, *CTB2* was characterized as a virulence gene in *C. beticola* with great potential as a target for gene silencing. This gene, an O-methyl-transferase in the toxin cercosporin biosynthesis cluster, is essential for toxin production and pathogenicity of *C. beticola* on sugar beet. The null mutant was unable to penetrate host tissue and cause disease symptoms. *CTB2* will be a promising target for HIGS experiments in the future.

Thirdly, different silencing constructs targeting *Fgl1*, a virulence factor, and *Homoaconitase*, an essential gene in *F. graminearum*, were cloned and attempted to transform into wheat by particle bombardment, but no transgenic plants could be achieved. Transgenic *B. distachyon* plants expressing an RNAi construct targeting *Fgl1* were successfully generated by *Agrobacterium* mediated transformation in order to establish HIGS against *F. graminearum* in a model plant for wheat. An inducible silencing construct against *Fgl1* was cloned for maize transformation. It was attempted to characterize *Homoaconitase* in *F. graminearum* by gene deletion and silencing, but no transformants could be achieved.

Also, the role of *Dicer 1* was to be elucidated in *F. graminearum* to answer the question whether the fungal or the host plant *Dicer* and RNAi machinery are responsible for the dsRNA processing of HIGS constructs. So far, no gene deletion or silencing mutants could be generated.

Fourthly, previously generated transgenic wheat lines with an inducible silencing construct against an endogenous gene involved in stress response in wheat and maize, deoxy hypusine synthase (DHS), were characterized to the T1 generation. The construct was induced by heat shock and greatly reduced DHS levels were shown by qPCR. A DHS silencing construct for maize transformation was generated and transformed into maize, resulting in several

transgenic lines. Moreover, DHS overexpression was studied in maize with transgenic lines previously generated with an inducible construct, revealing a salt stress sensitive phenotype.

Zusammenfassung

Das übergeordnete Ziel dieser Arbeit war es, host induced gene silencing (HIGS) in den wechselwirkenden Organismen Weizen- *F. graminearum* und Zuckerrübe- *C. beticola* zu etablieren. Dabei wurden verschiedene Herangehensweisen gewählt um silencing von Pilzgenen über die Wirtspflanze zu erreichen.

Erstens wurde RNAi basiertes gene silencing am Beispiel des Reportergens GFP in *C. beticola* erfolgreich angewandt.

Zweitens wurde *CTB2* wurde als wichtiger Virulenzfaktor im Blattpathogen *C. beticola* und als mögliches Zielgen für RNAi vermitteltes silencing charakterisiert. *CTB2* ist eine O-Methyltransferase im Cercosporin- Biosynthese- Cluster. Eine Nullmutation bewirkt den Verlust des Toxins Cercosporin und der Virulenz des Pilzes. Es konnte histologisch gezeigt werden, dass die Penetration der Wirtspflanze durch Ausschalten von *CTB2* verhindert wurde. *CTB2* ist sehr gut für HIGS geeignet, da es ein pilzspezifisches Gen und für die Pathogenität unerlässlich ist.

Drittens wurde Weizen mit RNAi Konstrukten gegen *Fgl1* und *Homoaconitase*, wichtige Virulenz- und Fitnessfaktoren in *F. graminearum*, transformiert. Obwohl ca. 8000 Embryonen transformiert wurden und verschiedene Kontrollen positiv verliefen, konnten keine transgenen Pflanzen erzeugt werden. Daher wurde ein *Fgl1* RNAi Konstrukt in die Modellpflanze *B. distachyon* transformiert, was zu mehreren transgenen Linien führte. Dieses Experiment zeigte, dass HIGS in *B. distachyon* möglich ist und dieses Modell eine relativ schnelle und einfache Möglichkeit bietet, Vektoren zu testen, die später in Weizen angewandt werden sollen. Darüber hinaus wurde ein induzierbares RNAi Konstrukt gegen *Fgl1* für die Maistransformation kloniert.

Homoaconitase, ein Zielgen für HIGS und ein essentielles Gen in der Lysinsynthese des Pilzes, sollte in *F. graminearum* näher untersucht werden, aber es konnten keine Deletionsmutanten oder knock down Stämme erreicht werden. Auch *Dicer 1* sollte im Zuge dieser Experimente in *F. graminearum* untersucht werden, da es eine wichtige Rolle beim silencing spielt und die Frage geklärt werden muss, ob beim Host Induced Gene Silencing (HIGS) die RNAi Maschinerie der Wirtspflanze die dsRNA prozessiert, oder ob dieser Prozess innerhalb des Pilzes stattfindet. Bisher scheiterten aber sämtliche Deletions- und silencing Versuche.

Viertens wurde Deoxyhypusinsynthase (DHS), ein Gen, das bei Alterung und Stress eine wichtige Rolle spielt, in Weizen und Mais untersucht. In einer vorigen Arbeit hergestellte DH- RNAi- Weizenlinien mit einem induzierbaren Vektor wurden bis zur T1 Generation untersucht. Der Hitzeschock zur Induktion wurde erfolgreich etabliert und stark reduzierte DHS- Expression konnte mit qPCR gezeigt werden. Ein DHS- RNAi- Konstrukt gegen das endogene Mais- DHS- Gen wurde kloniert und transformiert, was in mehreren transgenen Linien resultierte. Außerdem wurden Maispflanzen mit einem induzierbaren DHS Überexpressionskonstrukt aus einer vorherigen Studie bis zur T2 untersucht, wobei ein gegenüber Salzstress sensitiver Phänotyp beobachtet werden konnte.

Declaration of Authorship

I hereby certify that the experiments in this study have been conducted by me with no other help than declared and no other sources than quoted. This work has not been submitted for any other degree.

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Appendix A Media

All recipes are given for one liter unless noted otherwise.

Fungal and bacterial growth media

YPG	g/l
yeast extract	10
peptone	20
glucose	20
agar	15

PDM	g/l
yeast extract	4.41
tryptone	7.93
glucose	10.00
$Na_2HPO_47H_2O$	12.80
KH_2PO_4	3.00
NH_4PO_4	0.50
$MgSO_4$	0.24

Autoclave $Na_2HPO_47H_2O$, KH_2PO_4, and glucose separately.

CM	g/l
A (100x)	4.41
B (100x)	7.93
glucose	10.00
$Na_2HPO_47H_2O$	12.80
KH_2PO_4	3.00
NH_4PO_4	0.50
$MgSO_4$	0.24

CM	per l
solution A	10 ml
solution B	10 ml
glucose	20 g
yeast extract	1 g
MS vitamins	1 ml
casein mix	1 g

solution A (100x)	100 g/l $Ca(NO_3)2$ x 4 H_2O.
solution B (100x)	20 g/l KH_2PO_4; 25 g/l $MgSO_4$ x $7H_2O$; 10 g/l NaCl (sterile filtration)
MS vitamins (100x)	60 g/l H_3BO_3; 390 mg/l $CuSO_4$ x $5H_2O$;13 mg/l KI; 60 mg/l; $MnSO_4$ x H_2O; 51 mg/l $(NH_4)_6Mo_7$ x $4H_2O$; 5.48 g/l $ZnSO_4$ x $7H_2O$; 932 mg/l $FeCl_3$ x 6 H_2O; 2 ml chloroform
casein mix	0.5 g casein, hydrolyzed by enzymatic cleavage; 0.5 g casein, hydrolyzed by acid degradation 16 g/l agar for plates

lactose casein hydrolysate	per l
lactose	37.5 g
$MgSO_4$ x $7H_2O$	0.5 g
casein hydrolysate	3 g
microelements	2 ml
agar	20 g

microelements 1000x	per l
$MgSO_4$ x $7H_2O$	439.8 mg
$MnSO_4$ x H_2O	203 mg
$FeCl_3$ x 6 H_2O	723.5 mg

Buffers for *C. beticola* transformation

wash buffer	
NaCl	1 M
$CaCl_2$	10 mM

osmotic buffer	
NaH_2PO_4	10 mM
$CaCl_2$	20 mM
NaCl	1.2 M

pH 6.5

enzyme mix	
Drisealase (Sigma Aldrich)	5%
Lysing enzymes (Sigma Aldrich)	5%

dissolve in osmotic buffer

STC buffer	
sorbitol	1.2 M
TrisHcl, pH 7.5	10 mM
$CaCl_2$	10 mM

PEG: 50% polyethylene glycol 4000
STC-PEG : mix 1 part of 50% PEG and 4 parts of STC buffer

regeneration media	**per l**
sucrose	342 g
yeast extract	1 g
casein, hydrolysed	1 g

Media and stock solutions for biolistic wheat transformation

MS Macrosalts (10x)	**g/l**
NH_4NO_3	16.50
KNO_3	19.00
KH_2PO_4	1.70
$CaCl_2$ x 2 H_2O	4.40
$MgSO_4$ x 7 H_2O	3.70

autoclave

MS Microsalts (1000x)	**g/100 ml**
H_3BO_3	0.6200
$MnSO_4$ x H_2O	1.1200
$ZnSO_4$ x 7 H_2O	0.5800
Na_2Mo_4 x 2 H_2O	0.0250
$CuSO_4$ x 5 H_2O	0.0025
$CoCl_2$ x 6 H_2O	0.0025
KJ	0.0800

autoclave

NaFe- EDTA (500x)	**g/100 ml**
Na_2 EDTA (Titriplex)	3.73
$FeSO_4$ x 7 H_2O	2.87

heat separately and stir gently until dissolved
pour together and keep heating and stirring until the color is dark brown and clear
cool slowly and stir overnight
filter sterilize and keep in the dark at 4°C

NaFe- EDTA (500x)	**g/100 ml**
Na_2 EDTA (Titriplex)	3.73
$FeSO_4$ x 7 H_2O	2.87

Gelrite 0.6% (2x)
2.4 g for 400 ml media

2,4 Dichlorophenoxyacetate (2,4 D) (0,1%)
0.2 g 2,4 D
dissolve in 10 ml 1 N KOH under stirring and heating
add 190 ml H_2O, filter sterilize and keep aliquots at 4 °C

Glufosinate (Basta)
20 mg/ml in water, store aliquots at -20 °C

$CaCl_2$
2.5 M solution, store 50 µl aliquots at -20 °C
Spermidin
0.1 M solution, store aliquots at -20 °C
gold particles
disperse 40 mg gold particles (0.4 – 1.2 µm) in 1 ml icecold 95% EtOH
vortex and sonicate for several minutes
spin for 1 min at 4000 rpm and wash 2 more times in EtOH
wash additional 3 times in sterile H_2O
vortex and disperse in sterile H_2O completely before making 50 µl aliquots
store at -20 °C

MS (2x)	**per l**
MS Macrosalts (10x)	200 ml
MS Microsalts (1000x)	2 ml
FeEDTA (500x)	4 ml
saccharose	60 g

pH 5.7; filter sterilize

MS osmotic	**per l**
MS Macrosalts (10x)	200 ml
MS Microsalts (1000x)	2 ml
FeEDTA (500x)	4 ml
saccharose	479.22 g

pH 5.7; filter sterilize

induction media	**per l**
MS	200 ml
Gelrite	200 ml
2,4 D	800 µl

osmotic media	**per l**
MS osmotic	200 ml
Gelrite	200 ml

selection media I	**per l**
MS	200 ml
Gelrite	200 ml
2,4-D	800 µl
Basta	30 µl

selection media II	**per l**
MS	200 ml
Gelrite	200 ml
2,4-D	40 µl
Basta	60 µl

regeneration media	**per 400 ml**
MS	100 ml
Gelrite	200 ml
Basta	60 µl

Appendix B Experimental Protocols

C. beticola transformation

Preparation of protoplasts

- start a 100 ml liquid CM culture
- grow for 3 days with gentle agitation in the dark at 20- 23 °C to avoid pigmentation
- mix in a Waring blender
- add 200 ml CM complete to 50 ml of the blended culture
- incubate overnight at 23 °C, 150 rpm
- filter through a 100 µm Wilson sieve and wash the mycelium twice with washing buffer
- dry on sterile Whatman paper
- use 20 ml enzyme mix/ g mycelium for protoplastation
- incubate at 30 °C, 90-100 rpm, 2-3 hours
- filter protoplasts through a 100 µm, then a 40 µm Wilson sieve
- add 10 ml osmotic buffer and put in a sterile centrifuge tube
- zentrifuge at 2000 rpm, 10 min, 15 °C
- repeat once
- resuspend the pellet in 1 ml osmotic buffer, count the protoplasts
- zentrifuge, 2000 rpm, 10 min, 15 °C
- resuspend the pellet in STC-PEG buffer
- adjust the protoplast concentration to 1 x 10^6 to 1 x 10^8

Transformation

- use 1 x 10^6 to 1 x 10^8 protoplasts in 100 µl
- add 20 to 40 µg DNA in a maximal volume of 30 µl in STC-PEG
- incubate on ice for 30 min
- add 1 ml 50% PEG, mix slowly
- incubate for 30 min at RT, mix in between
- add 3 ml regeneration media
- regenerate PP for 2 hours, 28 °C, gently shaking
- plate 500 µl each on regeneration media plates, using cut yellow tips
- incubate overnight at 28 °C

Overlay

- use 10 ml 1.2% H_2O agar with the appropriate antibiotic per plate

Biolistic wheat transformation

Isolation of wheat embryos

- harvest caryopses 12-14 days after pollination at soft dough stage
- stir gently with 1% NaOCl for 20 min
- wash thoroughly with sterile water 3 times
- dissect the embryos and put them on induction media plates, the scutellum side facing up
- seal the plate and preculture in the dark at 26 °C for 2 days

Particle bombardment

- place 30 embryos in the center of a small petri dish with osmotic media
- seal the plate and incubate in the dark at 26 °C overnight to increase turgor in the embryos

prepare particle aliquots as follows:

- thaw gold aliquot on ice
- add 5 µl of the plasmid carrying the gene of interest and 5 µl plasmid for Basta resistance, 5 µg DNA each
- mix gently
- mix 50 µl $CaCl_2$ and 20 µl spermidine in the lid
- close Eppendorf tube and vortex until clear
- incubate on ice for 15 min
- spin for 5 sec at 4200 rpm and remove the supernatant
- wash once with 250 µl icecold 95% EtOH
- resuspend in 240 µl icecold 95% EtOH
- disperse thoroughly

use of the gene gun

- clean equipment with 70% EtOH and allow to dry
- pipet 3.5 µl gold particle mix on the macrocarrier plate
- dry for 2 min without movement to avoid clumping
- assemble the apparatus following the manufacturer's instructions
- place the petri dish with embryos on the holder
- close the sample chamber and evacuate to 27 inch Hg
- press FIRE switch and release vacuum after the rupture disk has burst

Selection of transgenic plants

- after bombardment, move the emryos to selection media I
- culture the embryos in the dark at 26 °C for 2 weeks
- remove developing coleoptiles
- subculture on selection media I for another 2 weeks
- transfer healthy calli to selection media II
- incubate in the growth chamber with 26 °C and 16 hrs light
- remove dead calli regularly

- subculture every 2 weeks and keep on selection media II for ca. 3 month
- transplant rooted plants to Magenta boxes with regeneration media
- after 3 weeks, transplant to soil and keep under a hood for 1 week
- spray Basta (150 mg /l, 0.1% Tween) 3 times with one day break in between each spraying
- select resistant plants