Cloning, expression and functional characterization of deoxyhypusine synthase from the pathogenic fungus *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae*), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.)

Dissertation

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Bestätigung

Hiermit möchte ich bestätigen, dass die Doktorarbeit von Frau Mayada Woriedh von einer Muttersprachlerin auf Richtigkeit der englischen Sprache geprüft wurde. Daher möchte ich empfehlen, dass die Dissertation in der aktuellen Form angenommen wird.

Mit freundlichen Grüßen,

Peter Witchalls

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When things get hard, let us not forget that-independent of race, colour, social situation, beliefs, or culture- everyone has experienced exactly the same. A lovely prayer written by the Egyptian Sufi master Dhu 'I-Nun (d.ad 861) neatly sumps up the attitude one needs to adopt at such times:

> O God, when I listen to the voices of the animals, to the sound of the trees, the murmur of the water, the singing of the birds, to the rushing of the wind or the rumble of the thunder, I see in them evidence of your unity; I feel that you are supreme power, supreme Knowledge, supreme wisdom, supreme justice.
>
> O God, I also recognize you in the difficulties I am experiencing now. God, let your satisfaction be my satisfaction, and let me be your joy, the joy that a Father takes in his child. And let me remember You with calmness and determination, even when it is hard for me to say: I love You.

Paulo Coelho, Brazil
Like the Flowing River, p.187

Be like the flowing river,
Silent in the night.
Be not afraid of the dark.
If there are stars in the sky, reflect them back.
If there are clouds in the sky,
Remember, clouds, like the river, are water,
So, gladly reflect them too,
In your own tranquil depths.

Manuel Bandeira

Contents

I Summary

To date, not much is known about the signals necessary for the activation taking part in fungal pathogenicity of *F. graminearum*, which causes one of the most destructive crop diseases worldwide, and about the genes that are transcribed due to the activation of this pathway. Furthermore, plants respond to fungal pathogen ingress or abiotic stress by synthesizing defence proteins that facilitate stress healing, cell death or provide protection against further pathogen attack. The accumulation of these proteins is mainly due to rapid transcriptional activation of corresponding genes, though preliminary evidence implies that translational control may also have a role in the stress response. Pathogenic signal transduction mutants are a powerful tool for studying processes essential for pathogenicity. Further characterization of expression pattern comparisons between the wild-type strain and mutants will help to solve the open questions. This will lead to a better understanding of the infection mechanisms of this important plant pathogen and could, in the long term, enable to find new targets for *F. graminearum*-specific fungicides and to produce stress-resistant cultivars.

This study focuses on the function of the eukaryotic translation initiation factor 5A (eIF5A) and its hypusination through two enzymes deoxyhypusine synthase (DHS) and deoxyhypusine hydrolase (DOHH) in the phytopathogen *Fusarium graminearum* and in mechanical abiotic stress in wheat and maize and during infection with *F. graminearum*. Recent evidence suggests that eIF5A plays a role in protein synthesis and acts as a nucleo-cytoplasmic shuttling protein that facilitates mRNA translation through selective transport from the nucleus. eIF5A, DHS and DOHH have been identified and isolated in *Fusarium graminearum* as has DHS been identified and isolated in wheat *Triticum aestivum* and maize *Zea mays*, suggesting that each distinct gene may be involved in transport of different subsets of mRNA required for a specific physiological event.

The analysis in this study revealed that eIF5A, DHS and DOHH genes are important virulence factors as well essential for cell viability in *Fusarium graminearum*. Knockout of DHS and DOHH are lethal, while overexpression of DHS in *Fusarium graminearum* appears to be involved in the signal transduction pathways that result in aggressive virulent infection following high production of mycotoxins and reactive oxygen species, cell death, and severe penetrating wound of cell walls in host plants. In contrast, overexpression of DOHH in *Fusarium graminearum* appears to be involved in contrary transduction pathways that result in a weak infection following low production of mycotoxins and reactive oxygen species, low cell death, and lacking penetration of cell walls in host plants. Although overexpression of DHS

increases the growth of *Fusarium* mutants under standard conditions and improves tolerance to high salt concentrations, it increases sensitivity to fungicide treatments. In constrast, overexpression of DOHH decreases the growth of *Fusarium* mutants, but increases tolerance to fungicide treatments. Moreover, this reduction does not restore correctly in DHS-DOHH double overexpression. DHS-DOHH overexpression appears to be balanced between DHS overexpression and DOHH overexpression, and adaptes in *Fusarium* mutants to survive under all stress conditions; oxidative stress, salt or fungicide. Also, it appears to result in an infection behavior more similar to infection patterns of *Fusarim* wild-type in host plants. Nevertheless, overexpression of these genes suppresses the growth of *Fusarium* mutants under temperature stress and produces lethal phenotypes.

Furthermore, the analysis in this study revealed that DHS in wheat and maize appears to be involved in the signal transduction pathways that result in cell death following virulent infection and systemically acquired resistance to *F. graminearum*, and systemically acquired resistance to salinity and drought stress. Transgenic wheat plants with reduced expression of DHS were developed using sense-linker-antisense T-DNA insertion by particle bombardment. In addition, transgenic maize plants with overexpression of DHS were developed too with double-stranded RNA-expressing constructs containing open reading frame of DHS introduced into maize by *Agrobacterium* mediated-transformation. Transgenic lines were isolated and propagated to be analyzed in a variety of assays. Analysis of the selectable marker expression revealed that most transgenes were transmitted faithfully. New cloning strategy was used to express the constructs by log on/off system of Cre-loxP with conditional promoters. The cloning strategy and frequencies of success of this large-scale project in wheat and maize, together with parameters for optimization at various steps, should serve as a useful framework for designing future RNAi or overexpression-based functional genomics projects in crop plants.

Finally, the capacity to effectively limit growth of various pathogens is important for the design of strategies to improve disease resistance and tolerance to abiotic conditions in crops. Development of resistant lines allows efficient crop production with reduced reliance on environmentally undesirable toxic agrichemicals. The strategy and the genes selected for testing in this study have not yet been described in wheat genome, maize genome or *Fusarium* genome.

II List of Figures and Tables

III List of Abbreviations

%	Percentage
A	Adenine
aa	Amino acid
Amp	Ampicillin
ATP	Adenosintriphosphate
bar	phosphinothricin acetyl transferase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
°C	Degree Celsius
C	Cytosine
CaMV	Cauliflower mosaic virus
cDNA	complementary Deoxyribonucleic Acid
CIMMYT	International Maize and Wheat Improvement Center
CM	Complete medium
CNI	Guanylhydrazone CNI-1493
Cre	Cre recombinase
CTAB	Cetyl trimethyl ammonium bromide
cv.	Cultivated variety; cultivar
2,4-D	2,4-Dichlorophenoxy acetic acid
DHS	Deoxyhypusine synthase
DIG	Digoxygenin
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate (s)
DOHH	Deoxyhypusine hydroxylase
DON	Deoxynivalenol
dpi	days post inoculation
DsRed	Discosoma sp. red fluorescent protein
dsRNA	double stranded RNA
dUTP	Deoxyuracil triphosphate
E. coli	Escherichia coli
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid

eIF5A	Eukaryotic initiation factor 5A
et al.	et alii = and others
Fgl	Fusarium graminearum lipase
FHB	Fusarium Head Blight
g	Gram; the metric unit of mass
G	Guanine
GC-	guanosine-cytosine content
GC7	N1-guanyl-1,7-Diaminoheptan
gDNA	genomic DNA
GFP	Green fluorescent protein
Gpmk1	Gibberella pathogenicity MAP kinase 1
GUS	Glucuronidase
hph	Hygromycin B phosphotransferase
HSP	Heat shock promoter
HSS	Homospermidin synthase
IR	Inverted repeat region
kb	kilo bases (= 1000 bp)
kDa	kilo Dalton (= 1000 Da)
L	Liter
LB	Luria-Bertani medium
M	Molar (mol/L)
MAP	Mitogen Activated Protein
MAPK	Mitogen Activated Protein Kinase
Min.	Minute
MIPS	Munich Information center for Protein Sequences
miRNA	microRNA
ml	milliliter
mM	millimolar
MOPS	3-(N-Morpholino) propane sulfonic acid
mRNA	messenger RNA
NAD+	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NIV	Nivalenol
nptII	neomycin phosphotransferase
OD	Optical Density

ORF	Open Reading Frame
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potentia Hydrogenii
PTGS	post-transcriptional gene silencing
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rpm	round per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription - polymerase chain reaction
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
SNA	Synthetic Nutrient-poor mineral Agar
sRNA	short RNA
ssRNA	single stranded RNA
T	Thymine
TBE	TRIS-Borate-EDTA
Tm	Annealing Temperature
Tri	Trichothecene synthase gene
Tris	Tris-(hydroxymethyl) aminomethane
tRNA	transfer RNA
UniProtKB	Protein knowledge base
UTR	Untranslated region
UV	Ultra violet
v	Volume
v/v	volume per volume
w/v	Weight per volume
WT	Wild-type
YPG	Yeast-extract Peptone Glucose

Units of measurements were used according to the International System of Units SI (Système International d'Unités). Chemical formulas and molecules are named after IUPAC (International Union of Pure and Applied Chemistry).

1.0 Introduction

1.1 Fusarium head blight

Fusarium head blight (FHB), also known as scab or tombstone, is a disease of wheat, barley, oats and other small cereal grains and corn (Leslie and Summerell, 2006; Walter *et al*, 2010). It is caused by several species of *Fusarium*; however, *Fusarium graminearum* is the primary species involved. This disease reduces yield and grade and may also contaminate the grain with fungal toxins (mycotoxins). Fusarium head blight also negatively affects grain quality, often resulting in lower test weights and mycotoxin contamination led a majority of the crop rejected by the industry (Jansen *et al*, 2005; Leslie and Summerell, 2006). FHB is favoured by humid conditions during flowering and early stages of kernel development (De Wolf, 2003; Jansen *et al*, 2005). Fusarium head blight (FHB) or scab of barley and wheat is considered the worst plant disease in the U.S. since the stem rust epidemics of the 1950s (Windels, 2000). The economic losses caused by scab epidemics during 1993–1998 for wheat and barley farmers in the Midwest are estimated at $3 billion. The epidemics led to barley yield decreases from 75.4 to 46.5 bushels per acre in North Dakota and from 76.2 to 60.2 bushels per acre in Minnesota. Wheat yields dropped in the two states by 48% and 39%, respectively (Jansen *et al*, 2005; McMullen *et al*, 2008).

In most areas, FHB is caused by a complex of various *Fusarium* species differing in important biological and ecological characteristics, e.g. virulence on cereals, host range, mycotoxin production, optimum growth conditions, survival on crop debris and in the soil (Ghahderijani, 2008). When cereal plants are infected with these fungi, there is a risk that grain may become contaminated with *Fusarium* mycotoxins, which may subsequently be transferred to compound feeds (Ghahderijani, 2008) The distribution and predominance of FHB pathogens differ significantly among climatic conditions, geographical zones, countries, and years (Xu and Berrie, 2005; Giraud *et al*, 2010)

In Germany, five *Fusarium* species have been reported to predominate in the FHB complex (Schollenberger *et al*, 2006; Ghahderijani, 2008): *Fusarium graminearum* Schwabe (teleomorph = *Gibberella zeae* (Schwein.) Petch), *F. culmorum* (Smith) Sacc, *F. avenaceum* (Corda ex Fr.,) (teleomorph = *G. avenaceum* Cook), *F. poae* (Peck) Wollenw, and *F. tricinctum* (Corda) Sacc) (teleomorph = *G. tricincta* El-Gholl, McRitchie, Schoult. & Ridings). *F. graminearum* is the primary species involved in FHB and the predominant species in China, the USA, Canada, some European countries and Southern Germany (Leslie and

Summerell, 2006; Ghahderijani, 2008; Becher *et al*, 2010). Environmental factors such as water activity, temperature, relative humidity, irradiation and pH value may influence the spectrum of different *Fusarium* species on wheat ears and in different areas and also their interactions (Cumagun, 2004).

1.1.1 The fungus *Fusarium graminearum*: Taxonomy, Pathology and Ecology

Gibberella zeae (Schwein.) Petch, (anamorph: *Fusarium graminearum* Schwabe) is a filamentous ascomycete that infects diverse plant species and those of economic importance include maize and small grains such as wheat, barley, rye, triticale, rice, but is also known from other annual and perennial plants (Cumagun, 2004; Cumagun and Miedaner, 2004). *Fusarium graminearum* can be a pathogen on the model plant species *Arabidopsis thaliana*, which may enable more rapid studies of host-pathogen interactions for this fungus (Leslie and Summerell, 2006). In wheat, *G. zeae* causes seedling blight, crown rot, root rot, and head blight (Cumagun, 2004). In barley and oats, *G. zeae* causes Fusarium mould and head blight (De Wolf, 2003), Also, it causes red cob in maize. Maize lines vary widely in their sensitivity to *F. graminearum* (Leslie and Summerell, 2006).

Life and disease cycle. *F. graminearum* is a haploid homothallic ascomycete. The fruiting bodies called perithecia develop on the mycelium and give rise to ascospores, which land on susceptible parts of the host plant and germinate (Figure 1). The fungus causes head blight on wheat, barley, and other grass species as well as ear rot on corn. The primary inoculum are the ascospores, sexual spores which are produced in the perithecia (Beyer and Verreet, 2005; Gilbert *et al*, 2008). Outcrossing enables the fungus to have a high genotypic diversity, which allows natural populations to adapt faster to selective pressures, such as cultivar resistance or fungicides (Jenczmionka *et al*, 2003). Spores are forcibly discharged and can germinate within six hours upon landing on the plant surface. The scab disease is monocyclic; after one cycle of infection with ascospores, the fungus produces macroconidia by asexual reproduction (Beyer *et al*, 2004). These structures overwinter in the soil or in plant debris on the field and give rise to the mycelium in the next season. The colonization of the other crops and grasses is important because the fungus survives in the crop residues that remain on the soil surface. The fungus reproduces in the crop residues and is moved by wind or rain to the developing wheat, barley or maize. Wheat is most susceptible during the flowering growth stages (Jansen *et al*, 2005), but some infection can still occur during kernel development. Temperatures between 19 and 30°C and extended periods of moisture in the form of rain or dew favor reproduction of the fungus on crop residues and also promote infection and disease development. Disease development is

favoured when cool wet weather occurs during short periods (10–20 days) from anthesis through the soft stage of kernel development in wheat and barley and during the first 21 days after silking in maize (De Wolf *et al*, 2003; Jenczmionka *et al*, 2003; Leslie and Summerell, 2006).

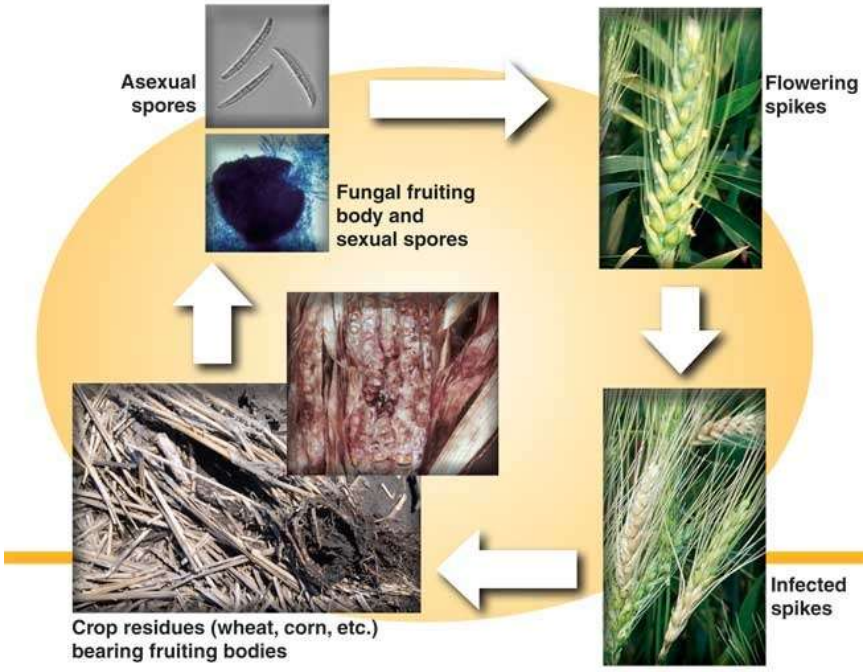

Figure 1. Fusarium head blight disease cycle. (Life cycle composition by Shaobin Zhong; NDSU Department of Plant Pathology, North Dakota State University, USA, 2008).

Formation of mycotoxins. *F. graminearum* species are responsible throughout the world for the formation of mycotoxins in infected plants and in plant products (Giraud *et al*, 2010; Lehoczki-Krsjak *et al*, 2010; Schmidt-Heydt *et al*, 2010). When cereal plants are infected with these fungi, there is a risk that grain may become contaminated with *Fusarium* mycotoxins, which may subsequently be transferred to compound food and feed (Bernhoft *et al*, 2010; Moretti *et al*, 2010). Isolates of *F. graminearum* may produce three important mycotoxins, zearalenone, nivalenol and deoxynivalenol as well as aurofusarin, culmorins, fusarin C, fusarochromanone, and steroids (Leslie and Summerell, 2006). The molecular genetics of trichothecene production is generally well understood and is regulated by the Tri6 and Tri5 gene product (Matsumoto *et al*, 1999; Brown *et al*, 2004). Production of deoxynivalenol or nivalenol is usually determined by the functioning of a single gene, Tri13, Tri7 (Lee *et al*, 2001b; Lee *et al*, 2002; Kim *et al*, 2003)(1056, 1209, 1210), while Tri14 is required for high virulence and DON production but not for DON synthesis in vitro (Dyer *et al*, 2005).

F. graminearum produces trichothecene type A toxins (HT-2 toxin, T-2 toxin) and type B toxins (deoxynivalenol, 3-acetylnivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenone-X, calonectrin) and zearalenon (Cumagun, 2004; Ghahderijani, 2008). The difference between of type A and B is the absence or presence of a keto group at C-8 of the trichothecene skeleton, respectively (Cumagun, 2004; Ghahderijani, 2008). Type B toxins could be subdivided into two major chemotypes: (1) nivalenol chemotype which produces nivalenol (NIV) and fusarenone-X and (2) deoxynivalenol chemotype which produces deoxynivalenol (DON, vomitoxin) and acetyldeoxynivalenol (ADON) (Cumagun, 2004; Ghahderijani, 2008). As a member of the sesquiterpenoid family of natural products, trichothecene toxins such as DON are potent eukaryotic protein inhibitors (Wache *et al*, 2009) causing decreased food consumption and lower weight gain in animals followed by diarrhea, vomiting, reproductive and haematological problems (Ansari *et al*, 2009). Human ingestion of contaminated grains is associated with alimentary toxic aleukia, nausea, depression of the immune system, skin necrosis and hemorrhage of lungs and gastrointestinal tract (Donmez-Altuntas *et al*, 2007; Celik *et al*, 2009; Schwerdt *et al*, 2009). Trichothecenes are also phytotoxins causing chlorosis, necrosis, and wilting (Cumagun and Miedaner, 2004). For this reason, these compounds might contribute to the pathogenicity and/or aggressiveness of the pathogen. DON producers of *F. graminearum* are considered more aggressive on wheat and rye than NIV producers (Cumagun and Miedaner, 2004).

The fungal toxin zearalenone has estrogenic properties and produces many reproductive disorders in animals. Swine are the most sensitive to the toxin, but cattle and sheep may also be affected. Zearalenone concentrations of 1–5 ppm can result in negative effects in animals and humans. Industries concerned about mycotoxins test grain prior to feeding to animals (De Wolf *et al*, 2003; Urraca *et al*, 2005). When the environmental conditions favor the fungal infection and later on development, the concentration of ZEA can dramatically increase either in field or storage conditions. Zearalenone is not degraded in common food and feed processing procedures, as it has been shown its presence in grain products like bread, locally brewed beers and processed feeds (Malekinejad *et al*, 2007; Sabater-Vilar *et al*, 2007)

1.1.2 Syptoms and infection process of *F. graminearum* on cereal crops

FHB is recognized in the field by the premature bleaching of infected spikelets and the production of orange, spore-bearing structures called sporodochia at the base of the glumes (Jansen *et al*, 2005; Leslie and Summerell, 2006). During wet weather, there may be whitish, occasionally pinkish, fluffy fungal growth on infected heads in the field. Diseased spikelets can

contain visibly affected kernels (Leslie and Summerell, 2006). The grading term given to visibly affected wheat seeds is fusarium damaged kernels (FDK), whereas in barley, it is called Fusarium mould (De Wolf, 2003; Xu and Berrie, 2005). FDK in wheat are shrunken and typically chalky white in appearance, hence the name "scab" (Cumagun, 2004), while fusarium mould on barley is an orange or black encrustation of the seed surface (De Wolf, 2003; Jansen *et al*, 2005). Much of the severely infected wheat is poorly filled and may be blown out the back of the combine due to its low kernel weight. Grain infected wheat after the flowering stage may be heavy enough to be harvested along with healthy grain, although most FDK will result from infections that occur during flowering. The fungus may eventually kill the developing seed at about the soft dough stage. Symptoms of Fusarium mould in barley or oats are usually sparse, making it difficult to tell if grain has been infected (De Wolf, 2003; Lewandowski *et al*, 2006). In maize, *F. graminearum* infects maize ear through silk channels and is most efficient when the silks are newly emergent causing ear rot (Figure 2) (Velluti *et al*, 2000; Hartmann *et al*, 2008).

Figure 2. Wheat heads and maize ears with symptoms of Fusarium head blight. From left to right: diseased spikelets become bleached or tan in appearance and may have signs or fungal reproduction; orange structure at the base of the diseased spikelet; white mold characteristic of Fusarium ear rot. Wheat photos (De Wolf, 2003). Maize photo (Department of Plant Pathology, University of Illinois, USA, 2008).

F. graminearum infects wheat spikes from anthesis through the soft dough stage of kernel development. The fungus enters the plant mostly through the flowers. However, the infection process is complex and the complete course of colonization of the host has not been described. Germ tubes seem not to be able to penetrate the hard, waxy surface of the lemma and palea which protect the flower. The fungus enters the plant through natural openings like stomates and needs soft tissue like the flowers, anthers and embryo to infect the plant (Bushnell and Leonard, 2003). From the infected floret, the fungus can grow through the rachis and cause severe damage in a short period of time under favorable conditions. Upon germination of the

spores 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). The first symptoms include a tan or brown discoloration at the base of a floret within the spikelets of the head. As the infection progresses, the diseased spikelets become light tan or bleached in appearance (Figure 2). The infection may be limited to one spikelet, but if the fungus invades the rachis the entire head will develop symptoms of the disease. The base of the infected spikelets and portions of the rachis often develop a dark brown color. When weather conditions have been favorable for pathogen reproduction, the fungus may produce small orange clusters of spores or black reproductive structures called perithecia on the surface of the glumes (De Wolf, 2003; Cumagun and Miedaner, 2004). If the heads invade extensively at early stages, kernels will fail to develop entirely.

1.1.3 Virulence factors of *F. graminearum* and plant interactions

Trichothecene production is an important character for virulence and associated with increased disease severity and for spread of the fungus within an infected wheat head (Maier *et al*, 2006). *F. graminearum* mutants with a disrupted Tri5 gene were shown to be unable to produce deoxynivalenol and to be drastically reduced in virulence (Jansen *et al*, 2005; Maier *et al*, 2006). In the absence of trichothecenes, the fungus is blocked by the development of heavy cell wall thickenings in the rachis node, a defense inhibited by the mycotoxin (Jansen *et al*, 2005). In wheat, the toxins were detected in association with cytosolic ribosomes, chloroplasts, plasmalemma, cell walls, and vacuoles. The number of enzymes examined from *F. graminearum*, other than those associated with toxin biosynthesis, is not large and includes xylanases (Belien *et al*, 2005). Toxins were transported apically in xylem and phloem of the rachis to distal uninfected florets (Kang *et al*, 2001). With the aid of antibodies reacting with cellulose, xylans, and pectin, it was shown that the cell walls of infected host cells were degraded. This finding provides evidence for the release of enzymes from the pathogen for digestion of cell walls at early stages of infection (Jansen *et al*, 2005). Therefore, these fungi rely on other penetration mechanisms, e.g. the enzymatic digestion of the plant cell wall. This makes necessary the secretion of hydrolyzing enzymes, like cutinases, cellulases, amylases and pectinases (Jenczmionka *et al*, 2003). The MAP kinase encoded by Gpmk1 regulates the expression of secreted cell-wall degrading enzymes required for pathogenicity (Leslie and Summerell, 2006) as well as the secreted lipase Fgl1, a major virulence factor of *Fusarium graminearum* (Jenczmionka *et al*, 2003). MAP kinases belong to the family of serine/threonine protein kinases. They are activated by a MAPKKK-MAPKK-MAP kinase cascade. This

cascade is conserved in eukaryotic organisms and is involved both in the transduction of a variety of extracellular signals and in the regulation of growth and differentiation process (Jenczmionka *et al*, 2003). Fungal MAP kinases have regulating functions, ranging across conidiation and conidia germination, appressorium and penetration peg formation, invasive hyphal growth and response to hyperosmotic stress and cell turgor (Jansen *et al*, 2005; Leslie and Summerell, 2006). Furthermore, MAP kinases have also been reported to play an important role during the regulation of mating (Muller *et al*, 1999).

F. graminearum can utilize a broad range of compounds as the sole nitrogen source including nitrate, ammonium, urea, and most purines and amino acids that do not contain sulfur (Leslie and Summerell, 2006). It has a fairly typical fungal cell wall composed of chitin and cross-linked galactose, mannose, arabinose and glucuronic acid (Leslie and Summerell, 2006), while it is also sensitive to the plant defense chemical apigeninidin (Leslie and Raju, 1985). Increased ferulic acid content in maize kernels is correlated with resistance to the ear rot induced by *F. graminearum* (Leslie and Summerell, 2006). Labeling of wheat 1,3-ß-glucanase and chitinase with antibodies against the tobacco enzymes indicated that these enzymes were induced during infection in wheat and barley, and considered to be partially resistant to *Fusarium* (Anand *et al*, 2003; Kong *et al*, 2005; Voigt *et al*, 2006; Shin *et al*, 2008). Also, it was suggested that FHB resistance in wheat involves defense pathways regulated by jasmonic acid and ethylene signaling, while salicylic acid–regulated systematic acquired resistance is insignificant in this process (Guangle and Yang, 2008).

In general, the first step in plant infection is perception of the host by the fungal pathogen. This is mediated by fungal receptors that recognize physical and/or chemical signals from the plant, leading to responses needed for successful infection, e.g. the development of penetration structures and/ or the secretion of cell wall-hydrolyzing enzymes. Plant perception and expression of proteins necessary for infection are linked by signal transduction pathways, which, then, are likely to be a crucial factor in disease establishment. (Jansen *et al*, 2005). After entry of a pathogen, the plant responds to infection in differnt ways. (1) If the plant is resistant, *F. graminearum* will not be able to replicate, thrive and cause disease in the plant (Hermann and Day, 2001). (2) *F. graminearum* may be able to cause a systemic infection, where the pathogen replicates and spreads from the initially infected cells to other cells of the plant showing physical symptoms and interferes with growth and development of the plants, leading to a widespread chlorosis, necrosis and bleaching (Hermann and Day, 2001). (3) An interaction can also occur between plants and pathogens when the plant actively combats the fungal pathogen through activation of specific disease resistance signaling by a hypersensitive

response and avirulence factors. In this process, the plant recognizes that it is being invaded by a foreign organism and immediately initiates a response by which the growth of the pathogen is suppressed (Hermann and Day, 2001; Sella *et al*, 2004). The cells surrounding the infection site immediately undergo programmed cell death causing necrotic lesions to localize the invading pathogen in the dead tissue and to prevent movement from cell to cell with association of the oxidative burst. The production of reactive oxygen species (ROS) is a downstream component of the hypersensitive response of the plant reaction to the oxidative burst (Morel and Dangl, 1997; Hermann and Day, 2001). Also, host cell death can occur in both susceptible (compatible) and resistant (incompatible) plant-pathogen interactions. Several studies indicate that cell death during the hypersensitive response involves activation of a plant-encoded pathway for cell death (Morel and Dangl, 1997). The cellular characteristics of the death process strongly implicate specific signals and autonomous cellular biochemical processes that execute individual cells (Gatsukovich, 2004). Apoptosis is genetically controlled cellular suicide essential for development, maintenance of cellular homeostasis, and defense against environmental insults including pathogen attack (Morel and Dangl, 1997; Gatsukovich, 2004). Some features of programmed cell death have been observed in both susceptible and resistant reactions during plant-pathogen interactions, suggesting negative regulators of apoptosis exhibited heritable resistance to several necrotrophic fungal pathogens (Hermann and Day, 2001; Gatsukovich, 2004) that overlap biochemical pathways operative in these two contrasting outcomes. Suppression of plant cell death or senescence may improve resistance to abiotic stress and necrotrophic pathogens such as *F. graminearum.*

Hence, hyphae of germinating fungal spores use different paths of infection. Effective resistance to Fusarium head blight requires expression of genes that combat these different pathways of infection. More detailed studies are required to evaluate the possibilities for targeting metabolites or the elimination of mycotoxins or for using defense genes known to be effective against various necrotrophic or biotrophic pathogens. Recently, several studies have shown that infection by *F. graminearum* induces transcript accumulation of several classes of biotic and abiotic stress-related genes in both partially resistant and susceptible cultivars (Kong *et al*, 2005). The expression of biotic and abiotic stress-related genes may result in a reduction of FHB severity in wheat, but the relationship and mode of interaction between FHB resistance and *F. graminearum* has not been clearly established. Identifying host genes differentially expressed in response to the pathogen may help illustrate cellular processes activated or repressed during the early phase of host–pathogen interactions that ultimately determine the extent of fungal colonization. At present, no highly resistant cultivars are available and not much is known about genes involved in the process of infection and plant interactions.

1.2 Hypusine biosynthesis pathway

The only hypusine-containing protein identified so far is the eukaryotic initiation factor 5A (eIF5A) (Park *et al*, 2010). Hypusine is formed in a novel posttranslational modification that involves two enzymes, deoxyhypusine synthase (DHS), and deoxyhypusine hydroxylase (DOHH). DHS (EC 2.5.1.46) catalyzes the cleavage of the polyamine spermidine and transfer of its 4-aminobutyl moiety to the ε-amino group of one specific lysine residue of the eIF5A precursor to form a deoxyhypusine intermediate. In the next step, DOHH (EC 1.14.99.29) converts the deoxyhypusine-containing intermediate to the hypusine-containing mature eIF5A. Forming of hypusine results in activation of eIF5A (Figure 3) (Park, 2006; Wolff *et al*, 2007).

Polyamines, i.e., putrescine, spermidine, and spermine, are ubiquitous in living cells and are essential for cell growth. These polyamines exist as protonated polycations at physiological pH in cells and interact with nucleic acids, DNA and RNA, acidic proteins, and phospholipids (Huang *et al*, 2007; Park *et al*, 2010). Polyamines regulate a vast array of cellular activities at the level of replication, transcription, translation, posttranslational modification, protein activation, membrane stability, and ion channelling (Park, 2006; Park *et al*, 2010). The role of polyamines in translational regulation may be most important, because a majority of polyamines are bound to RNA (Igarashi and Kashiwagi, 2009) and in that the polyamine spermidine is required for activation of the eukaryotic initiation factor 5A (eIF5A). Thus, polyamines regulate cell growth, proliferation, differentiation, transformation, and apoptosis and their cellular levels are tightly regulated at the level of biosynthesis, metabolism, and transport (Park *et al*, 2010). In eukaryotic organisms, the polyamine spermidine has an independent and specific function as the source of the 4-aminobutyl portion of hypusine [Nε-(4-amino-2-hydroxybutyl)-lysine] in the essential cellular protein eIF5A (Park, 2006; Kang *et al*, 2007; Wolff *et al*, 2007). Since the structural requirement for spermidine in the synthesis of hypusine is quite strict, this function can only be fulfilled by spermidine or by few close structural analogs. Thus, hypusine synthesis defines an absolute requirement for the polyamine spermidine in eukaryotes (Byers *et al*, 1994; Chattopadhyay *et al*, 2003; Park *et al*, 2010).

Hypusine was first discovered in bovine brain (Shiba *et al*, 1971). An 18,000-dalton protein was found to contain hypusine (Park *et al*, 1981). The 18,000-dalton protein was found to be eIF-4D (Cooper *et al*, 1983). eIF-4D was renamed as eIF-5A (1987). eIF-5A was nemaed as eIF5A (1996). eIF5A, DHS and DOHH are highly conserved proteins from archaebacteria to mammals, suggesting a vital function of eIF5A and the deoxyhypusine/hypusine modification. Deoxyhypusine and hypusine occur in archaea and eukaryotes, but not in eubacteria (Klier and

Lottspeich, 1992; Park, 2006; Park *et al*, 2010). Yet, eubacteria have an elongation factor P (Figure 3), which is a distant ortholog of eIF5A. EF-P enhances elongation by stimulating the peptidyl transferase activity of the ribosome and is an essential protein in bacteria. In plant, hypusine has been described so far in Arapidopsis, tomato, rice and tobacco (Ober and Hartmann, 1999; Wang *et al*, 2001; Wang *et al*, 2003; Wang *et al*, 2005b; Duguay *et al*, 2007). In fungi, hypusine has been described in the filamentous fungi *Neurospora crassa* and in the slime mould *Dictyostelium discoideum* (Mackintosh and Walters, 2003).

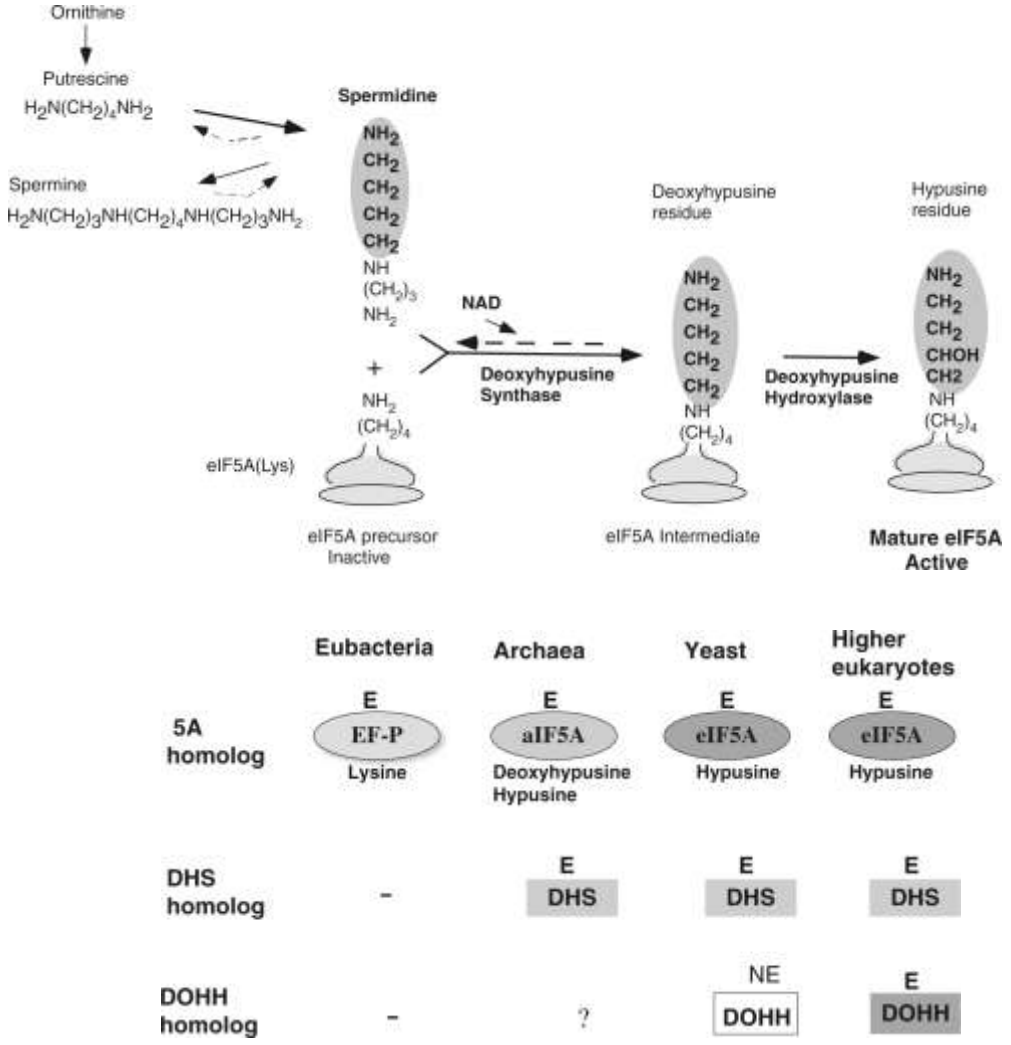

Figure 3. Evolution of hypusine biosynthesis in eIF5A. **Upper panel**, the polyamine spermidine, which is synthesized from putrescine, is the source of the aminobutyl moiety of hypusine, as indicated by shading. Hypusine synthesis occurs at one specific lysine residue of the eIF5A precursor protein, eIF5 (Lys), by two enzymatic steps, involving deoxyhypusine synthase (DHS) and deoxyhypusin hydroxylase (DOHH). Deoxyhypusine synthase (DHS) reaction is reversible, whereas the deoxyhypusine hydroxylase (DOHH) reaction is not. **Lower panel**, Evolution of eIF5A and its hypusine modification pathway. eIF5A orthologs are found in eubacteria and archaea and are essential genes in each organism. The DHS gene exists in archaea, and in all eukaryotes, but not in eubacteria (elongation factor). DOHH gene is found only in eukaryotes. E indicates essential gene, and NE indicates non-essential gene. The Figure is illustrated after (Park *et al*, 2010).

1.2.1 Eukaryotic initiation factor 5A (eIF5A)

To elucidate the biological function of eIF5A, many attempts have been made, but its exact function remains mysterious. Earlier molecular genetics and biochemical studies described eIF5A as a translation initiation factor due to its ability to stimulate the synthesis of methionyl-puromycin in vitro (Park *et al*, 1993a, 1993b). Later, depletion of eIF5A in yeast caused only a small (30%) reduction in the protein synthesis rate (Kang and Hershey, 1994). These results have been used to argue against eIF5A as a translation initiation factor for general protein synthesis. More recent evidence showed that hypusine-containing eIF5A is a nucleocytoplasmatic shuttle protein of a subset of mRNAs related to the G1/S cell cycle transition and unspliced viral mRNAs, and promoting nuclear export of specific mRNA facilitates protein synthesis and facilitates their translation. It has also been shown that eIF5A may be involved in mRNA turnover, acting downstream of decapping (Ruhl *et al*, 1993; Hanauske-Abel *et al*, 1995; Bevec *et al*, 1996; Jin *et al*, 2003; Luchessi *et al*, 2006; Schrader *et al*, 2006; Njuguna, 2009). Recently, hypusinated eIF5A contributes to the life cycle of human immunodeficiency virus by interacting with the retroviral REV protein, thereby participating in the nuclear export of unspliced and incompletely-spliced viral mRNA. Hence, human eIF5A may form part of a specific nuclear export pathway that is exploited by the virus for propagation (Liu *et al*, 1997; Hart *et al*, 2002; Hauber *et al*, 2005). In addition, immunocytochemical analyses showed differences in the distribution of non-hypusinated eIF5A precursor and the hypusine-containing mature eIF5A. While the precursor is found in both cytoplasm and nucleus, the hypusinated eIF5A is primarily localized in cytoplasm (Lee *et al*, 2009). These findings provide strong evidence that the hypusine modification of eIF5A dictates its localization in the cytoplasmic compartment where it is required for protein synthesis.

The X-ray of the crystal structure of the eIF5A (Park, 2006) reveals that this protein consists of two well-defined domains: the N-terminal domain, which contains the hypusine modification site in an exposed loop, and the C-terminal domain, which is similar to the oligonucleotide-binding domain found in several RNA-binding proteins (Figure 4). RNA binding depends on both the presence of the hypusine residue in the eIF5A protein and conserved core motifs of the target RNA (Park, 2006; Parreiras *et al*, 2007).

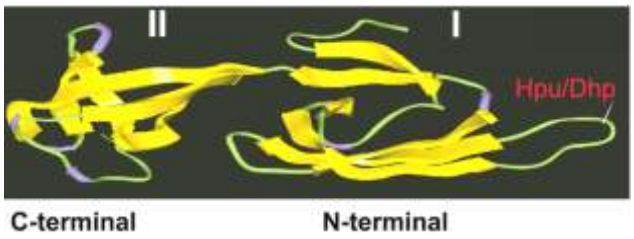

Figure 4. Crystal structures of eIF5A. X-ray structure of eIF5A precursor containing two domains: N-terminal domain (I) and C-terminal domain (II), the location of the lysine residue (in domain I) that undergoes modification to deoxyhypusine or hypusine is indicated (Park, 2006).

The most convincing evidence for the essential nature of eIF5A and its deoxyhypusine/hypusine modification was obtained from gene mutation, gene disruption or knock down studies in the yeast *S. cerevisiae* and higher eukaryotes (Schnier *et al*, 1991; Sasaki *et al*, 1996; Frigieri *et al*, 2007; Frigieri *et al*, 2008), and proved the essentiality of these genes for cell viability, cell growth, differentiation and the efficiency for proliferation (Park *et al*, 1993a, 1993b; Dias *et al*, 2008). Further studies demonstrated that yeast eIF5A is involved, too, in vesicular trafficking, cell wall integrity and reveals connections to poly(A)-binding protein, to protein kinase C signaling and to the secretory pathway (Valentini *et al*, 2002; Zanelli and Valentini, 2007; Frigieri *et al*, 2008). Currently, this gene also evidenced to promote translation elongation and ribosomal transit times and is required for the first peptide bond of protein synthesis (Cano *et al*, 2008; Frigieri *et al*, 2008; Lee *et al*, 2009; Saini *et al*, 2009).

There are two known eIF5A isoforms in humans, three in Arabidopsis, and four in lettuce (Thompson *et al*, 2003; Wang *et al*, 2003). Little is known about eIF5A in plants, although it has been cloned from alfalfa, tobacco, canola, tomato and Arabidopsis (Wang *et al*, 2001; Gatsukovich, 2004; Thompson *et al*, 2004; Lebska *et al*, 2009). It was observed that the transcript levels of eIF5A increased during natural and stress-induced senescence in tomato (Wang *et al*, 2001). In Arabidopsis, recent evidence indicates that eIF-A-1 plays a pivotal role in cell proliferation and senescence (Wang *et al*, 2003; Thompson *et al*, 2004). Separate isoforms of eIF5A appear to facilitate the translation of mRNAs required for cell division and cell death. This raises the possibility that eIF5A isoforms are elements of a biological switch that is in one position in dividing cells and in another position in dying cells. Changes in the position of this putative switch in response to physiological and environmental cues are likely to have a significant impact on plant growth and development (Thompson *et al*, 2004). Futhoremore, eIF5A-2 is involved in pathogen-induced cell death and development of disease symptoms in Arabidopsis during infection with *Pseudomonas syringae* pv. tomato (Hopkins *et*

al, 2008). Moreover, constitutive downregulation of eIF5A-1 resulted in the major suppression of xylem formation, and the antisense transgenic plants were also stunted. These data collectively indicate that modulation of eIF5A-1 expression alters xylem abundance in *Arabidopsis thaliana* (Liu *et al*, 2008).

The discovery of various isoforms of eIF5A has given rise to the distinct possibility that each isoform is responsible for transporting a unique subset of mRNA species required for a specific physiological event, such as cell division, cell death or pathogen attack. In effect, each eIF5A isoform is thought to act as a shuttle protein that initiates the translation of a developmentally required suite of genes (Parreiras *et al*, 2007).

1.2.2 Deoxyhypusine synthase (DHS)

Deoxyhypusine synthase catalyzes a complex sequence of reactions, involving two substrates, spermidine and eIF5A (Lys), and a cofactor, NAD, to convert one specific lysine residue of the eIF5A precursor to a deoxyhypusine residue. This enzyme exhibits an absolute specificity toward its protein substrate, eIF5A(Lys) (Park, 2006). DHS, like eIF5A, is well conserved through evolution and present in all eukaryotes, in certain archaea, and even several cyanobacteria (Wolff *et al*, 2007). Like the plant deoxyhypusine synthase from tobacco and *Senecio vernalis*, human DHS can accommodate putrescine as an alternative butylamine acceptor instead of eIF5A(Lys) resulting in the formation of homospermidine from spermidine (Park, 2006). However, it was demonstrated that deoxyhypusine synthesis is the preferred pathway of the DHS reaction (Park, 2006). Furthermore, all the DHS-catalyzed reactions are reversible and DHS can facilitate interconversion of spermidine, eIF5A (intermediate) and homospermidine by way of a common enzyme-imine intermediate (Park *et al*, 2010). Thus, the aminobutyl moiety of the enzyme-imine intermediate can be transferred to any one of the three acceptors, eIF5A (Lys), putrescine or 1,3-diaminopropane leading to the synthesis of deoxyhypusine, homospermidine or spermidine, respectively (Park, 2006) (Figure 3).

DHS has been found to be involved in cell proliferation via its catalytic role in the hypusination pathway of eIF5A and play a role in another independent cellular function as well (Park, 2006). There is only a single DHS gene in yeast and most eukaryotes. A haploid *S. cerevisiae* strain with disruption of the DHS gene was not viable (Wöhl *et al*, 1993; Sasaki *et al*, 1996; Park *et al*, 1998). Growth was arrested in G1 upon treatment with GC7, a potent inhibitor of DHS (Mackintosh and Walters, 2003), indicating that DHS is also essential. The inhibition of deoxyhypusine synthase by inhibitors results in suppressed growth of actively

dividing mammalian cells and demonstrates an antiproliferative potential (Park *et al*, 1994; Wolff *et al*, 2007). Screening assays of deoxyhypusine synthase inhibitors identified the guanylhydrazone CNI-1493, a novel inhibitor of DHS, with a potential to suppress replication of human immunodeficiency virus type 1 (HIV-1), which exploits activated eIF5A during the formation of progeny viruses (Sommer *et al*, 2004; Hauber *et al*, 2005). Recently, the guanylhydrazone CNI-1493 is considered being a promising drug for anti-malarial therapy, because of its dual combined action of inhibition host cell pro-inflammatory cytokine release and parasitic deoxyhypusine synthase (Specht *et al*, 2008).

Recombinant plant DHS, like its mammalian counterpart, has been shown capable of catalyzing the formation of deoxyhypusine in precursor plant eIF5A (Wang *et al*, 2001). In Arabidopsis, there is only one DHS gene, but there are multiple members of the eIF5A gene family. It has, therefore, been proposed that the single DHS enzyme activates all of the eIF5A isoforms in Arabidopsis (Duguay *et al*, 2007), and that DHS function is tightly correlated with the cellular requirement for activated eIF5A (Thompson *et al*, 2004). DHS expression is likely under tight developmental regulation, presumably by a multi-element promoter that enables its upregulation when there is a need to activate any or all of the eIF5A isoforms (Wang *et al*, 2003; Duguay *et al*, 2007). Previous studies have indicated that levels of DHS protein are upregulated during leaf senescence, whether it occurs naturally or is induced prematurely by environmental stress (Wang *et al*, 2001; Thompson *et al*, 2004; Wang *et al*, 2005a). Furthermore, and consistent with the apparent function of DHS, this increase is paralleled by a corresponding increase in one of three isoforms of eIF5A in Arabidopsis, eIF5A-1 (Wang *et al*, 2003). In a similar vein, levels of DHS and eIF5A protein have been found to increase in tomato leaves in which senescence was induced prematurely by chilling and osmotic stress (Wang *et al*, 2001). Moreover, constitutive suppression of DHS has been shown to delay the onset of senescence in leaves of Arabidopsis and canola and in tomato fruits (Wang *et al*, 2003; Wang *et al*, 2005a; Wang *et al*, 2005b). However, in each case there were additional pleiotropic effects depending on the degree of suppression. These included enhanced growth, increased tolerance to environmental stress and, in the case of strong suppression, stunted reproductive growth, reduced seed yield and male sterility (Duguay *et al*, 2007). Moreover, antisense suppression of deoxyhypusine synthase in tomato delays fruit softening, alters growth and development and is involved in the regulation of senescence, a highly regulated process of programmed cell death analogous to apoptosis. (Wang *et al*, 2005b).

1.2.3 Deoxyhypusine hydroxylase (DOHH)

The second enzyme, deoxyhypusine hydroxylase (DOHH), hydroxylates the deoxyhypusine residue and eIF5A intermediate to form the hypusine residue and mature eIF5A (Park, 2006). In contrast to the deoxyhypusine-containing protein, no reversal was observed with hypusinecontaining eIF5A, suggesting that hydroxylation at the 4-aminobutyl side chain of the deoxyhypusine residue prevents DHS-mediated reversal of the modification. Whereas the first step of hypusine synthesis is reversible, the second step, DOHH-mediated hydroxylation, locks eIF5A into an active hypusine form, thereby making the overall reaction an irreversible protein modification (Park, 2006) (Figure 3). Although deoxyhypusine hydroxylase activity could be detected in mammalian cells and tissues, the identity of this enzyme remained obscure for many years (Park *et al*, 2010). The DOHH gene was initially cloned from yeast two-hybrid screening in search of eIF5A binding proteins (Thompson *et al*, 2003). DOHH exists as a product of a single gene in all eukaryotes and has a unique superhelical structure termed "HEAT-repeat" (Kim *et al*, 2006b; Park *et al*, 2010). This enzyme has a nonheme diiron active center that activates O2 (Vu *et al*, 2009). Since a number of metalchelating inhibitors of DOHH, e.g. mimosine, caused growth inhibition and G1 cell cycle arrest in mammalian cells, DOHH has been assumed to be essential for cell growth (Abbruzzese *et al*, 1989; Hanauske-Abel *et al*, 1995). Interestingly, however, the DOHH gene is apparently not essential in the yeast *S. cerevisiae*, even though endogenous yeast eIF5A mostly exists as the fully modified hypusine form (Park *et al*, 2006). DOHH seems to be functionally more significant in the fission yeast, *Saccharomyces pombe*, since a mutation in its DOHH homolog gene caused a temperature-sensitive growth phenotype and altered mitochondrial morphology and distribution (Park *et al*, 2010). Based on this observation, a role for DOHH, or eIF5A, in micro-tubule assembly and mitochondrial function was implicated. In contrast to yeast, inactivation of DOHH is recessively lethal in multicellular eukaryotes such as *C. elegans* and *D. melanogaster* (Spradling *et al*, 1999; Patel *et al*, 2009), suggesting a role for DOHH in cell growth and proliferation.

Recently, it was found that the HEAT-like repeats present in the parasite DOHH of malaria differ in number and amino acid identity from its human ortholog and might be of considerable interest for inhibitor design (Kerscher *et al*, 2010). In plant, DOHH has not yet been described.

1.2.4 Targeting the hypusination pathway to combat biotic and abiotic stress

Polyamines are required for normal growth of microorganisms including fungi (Foster and Walters, 1993; Havis *et al*, 1994). Spermidine is known to be the predominant polyamine in fungi. The inhibition of spermidine synthase proved to be effective in reducing fungal growth in culture (Stevens and Winther, 1979). Thus, spermidine analogues were shown to possess antifungal and fungicidal activity in spite of the fact that spermidine inhibitors did not exert their antifungal effects via disruption of other cellular functions associated with polyamines (Jakus *et al*, 1993; Mackintosh and Walters, 1997a, 1997b, 2003). As the aminobutyl side chain of hypusine is derived from spermidine (Park, 2006; Wolff *et al*, 2007), spermidine inhibitors inhibit the biosynthesis of hypusine.

eIF5A, DHS, DOHH and their isoforms proved to fulfil a role in the translation of a subset of mRNAs required for specific physiological functions like proliferation, photosynthesis, early development of seedlings, osmotic stress, pathogen attack and senescence induced programmed cell death (Duguay *et al*, 2007; Hopkins *et al*, 2008). Characterization of these isoforms will help identifying their role in plant adaptation to stress, in resistance to disease and identifying potential antifungals by disruption of the posttranslational formation of hypusine.

However, five types of resistance to Fusarium head blight have been proposed (Lehoczki-Krsjak *et al*, 2010): (1) resistance to initial infection, (2) resistance to spread of infection, (3) resistance to kernel infection, (4) tolerance and (5) resistance to toxin accumulation. Only spread in the head and initial infection types of resistance can be used in the search for resistant germplasm and screening breeding lines (El-Badawy, 2001). These practices may enhance adaptation of pathogen population by selecting for aggressive strains and providing a favorable environment which results in disease outbreaks. Prospects to control FHB by chemicals are poor because no fungicides have been found so far to be effective in controlling the disease (El-Badawy, 2001). Resistance breeding is the most economical, environmentally friendly and effective way to control the disease (Jansen *et al*, 2005). Despite great efforts to find resistance genes against *F. graminearum*, no completely resistant variety is currently available. Researches are directed towards gaining insight into more details about the pathogen process and reveal spots in the life cycle in order to develop fungicides that can protect host plants from scab infection.

In most crop production regions, high levels of resistance to Fusarium head blight or abiotic stress are not currently available in varieties for planting. However, certain varieties

have moderate levels of resistance, therefore, breeding for durable resistance against this fungal disease in wheat is the most economical and effective mean of reducing yield mycotoxin contamination causes by this fungus (DeWolf, 2003). The methodology involves the identification of resistance sources and incorporates resistance into genotypes with good agronomic traits. Thus, considerable effort has been devoted to find sources of resistance that can be used in breeding programs (Dunwell, 2005).

Evaluation of barley, wheat, maize and related germplasm yielded only a few accessions with partial resistance to environmental stress (Dahleen *et al*, 2001). This resistance appears, in most cases, to be polygenic control, making the development of resistant cultivars with suitable agronomic and quality traits a challenge. The insertion of individual antifungal, antitoxin genes and defense genes via genetic transformation has the potential to aid in development of resistant crop cultivars. Among the antifungal genes targeted to combat FHB are coding sequences for proteins that degrade fungal cell walls, disorganize fungal membranes, bolster the host defense response systems, and interfere with fungal protein synthesis, pathogenesis, and/ or accumulation of DON (Dahleen *et al*, 2001).

Taken together, the biological functions studied so far in eIF5A, DHS, DOHH evaluate highly to characterize these genes in *F. graminearum*, wheat and maize as hot potential genes and may evidence whether the transgenes achieve their potential against FHB and environmental stress.

1.3 Enhanced methods to study gene expression and function in crops

Different methods have been developed to introduce foreign genes into plants. A common feature is that the transforming DNA has to bypass different membrane barriers; it first has to enter the plant cell by penetrating the plant cell wall and the plasma membrane and must then reach the nucleus and integrate into the resident chromosomes. For the majority of species gene transfer is carried out using plant material competent of regeneration to obtain complete, fertile plants. This implies the development of a tissue culture technology that becomes a special field. Although gene transfer technology has become routine in working with several plant species, in others the limiting step is not the transformation itself, but rather the lack of efficient regeneration protocols (Herrera-Estrella *et al*, 2005).

The most widely used and successful transformation methods are the nature process of gene transfer by the soil bacterium *Agrobacterium tumefaciens* and the direct uptake of DNA through particle bombardment.

The *Agrobacterium* system has several advantages over other transformation methods and it is considered as the first option to transform plants. These advantages include the following aspects: (1) In a significant percentage of the transformation events, a single copy of the T-DNA is integrated into the chromosomes of the transformed cell (Crouzet and Hohn, 2002). (2) Numerous vector systems are now available containing the T-DNA borders and various reporter and selectable marker genes, allowing researchers to choose the most appropriate combination to insert heterologous genes (Herrera-Estrella *et al*, 2005). (3) It is possible to transfer large fragments of DNA, including bacterial artificial chromosomes (Hamilton *et al*, 1996). (4) Transformation in planta, without the necessity of tissue culture, is possible in some species such as *Arabidopsis thaliana* and *Medicago trunculata* (Zhang *et al*, 2006; Li *et al*, 2009; Mrosk *et al*, 2009).

The Biolistic method has been widely used for cereal transformation. These methods rely on the acceleration of gold particles, coated with plasmid DNA, into plant cells as a method of directly introducing the DNA**.** Some advantages of the biolistic method are the following: (1) wide variety of types of explants can be used to undergo bombardment and obtain fertile plants. (2) There is no need for specialized transformation vectors. (3) This is the only reliable method for chloroplast transformation (Herrera-Estrella *et al*, 2005; Wang and Frame, 2009).

The Protoplast transformation is the direct transfer of DNA to protoplasts using polyethylene glycol (PEG), calcium phosphate, or electroporation, which has been shown to be possible in various of plants including maize. Low reproducibility and the regeneration of plants were the main problems, because these methods are often specific for certain cultivars. The microinjection technique employs immobilized cells into which the DNA is internalized individually (Bates, 1999). However, the tedious manipulation, need for sophisticated equipment, and difficulty of regeneration of plants have not permitted its wider utilization (Herrera-Estrella *et al*, 2005), while it is used as a favorite method for fungi and yeast transformation (Morgan, 1983; Turgeon *et al*, 2010).

In addition to the potential application of transgenic plants in agriculture and biotechnology they can be used to study the expression of foreign DNA, to carry out the functional analysis of plant DNA sequences, to investigate the mechanism of viral DNA replication and cell-to-cell spread, as well as to study transposition. Moreover, the versatility of the gene transfer vectors is such that they may be used to isolate genes unamenable to isolation using conventional protocols (Walden and Schell, 1991; Nagaya *et al*, 2010). However, to study a gene effectively, a good expression system is required such as:

Tracking experiments in which transgenic plants have been used extensively to study gene expression and function. For this purpose, plants are transformed with chimeric gene constructs in which a reporter gene is under the control of the regulatory sequences of the gene to be analyzed. Several reporter genes are commonly used in plants, including *ß-glucuronidase*, *luciferase*, and genes involved in anthocyanin biosynthesis (Hansen and Wright, 1999). More recently, the gene for the green fluorescent protein (GFP) has become an important in vivo reporter in plants. When expressed in plant cells and illuminated with blue light, GFP produces a stable bright green fluorescence that is easily monitored nondestructively (Schornack *et al*, 2009). Thus, it can be used as a means to visualize the fate of transformed cells over time and rapidly test the influence of various factors on gene expression. These new generations of reporter genes are easily monitored for expression, and allow rapid determination of sequences important in regulating the temporal, spatial, and environmental expression of a gene in great detail (Meier *et al*, 2008), as well as a reporter gene for the plant pathogens (Li and Chye, 2009; Riedel *et al*, 2009). Reducing or increasing the expression of the target gene by sense and antisense or cosuppression strategies can be used to study gene function. Analysis of the phenotype or changes in mRNA or metabolite profiles can provide valuable information to determine gene function.

Loss of function studies, such as in a gene knockout experiment, in which an organism is engineered to lack the activity of one or more genes. A knockout experiment involves the creation and manipulation of a DNA construct in vitro, which, in a simple knockout, consists of a copy of the desired gene, which has been altered such that it is non-functional (Cushing *et al*, 2005). Its reverse genetics approach provides a platform facilitating dramatic progress in the understanding of fundamental aspects of plant metabolism. Recent experience in Arabidopsis shows that knockouts of genes encoding enzymes of primary metabolism can produce mutants with clear and sometimes unexpected phenotypes. They can provide new information about old pathways (Thorneycroft *et al*, 2001).

Gain of function which is the logical counterpart of knockouts. These are sometimes performed in conjunction with knockout experiments to more finely establish the function of the desired gene. The process is much the same as that in knockout engineering, except that the construct is designed to increase the function of the gene, usually by providing extra copies of the gene or inducing synthesis of the protein more frequently (Sharabi-Schwager *et al*, 2010; Singh *et al*, 2010; Tuteja, 2010).

RNA interference (RNAi) in recent years has been exploited as a tool for investigating gene functions in numerous organisms (McGinnis *et al*, 2005; Travella *et al*, 2006; Travella and Keller, 2009). Gene silencing by transgene-induced RNAi is useful, because the loss or reduction of gene function is dominant, circumventing the need to generate homozygous loss-of-function mutations. This not only saves time, in which useful phenotypes can be generated for entire gene families or for multiple orthologous genes in polyploids plants (Lawrence and Pikaard, 2003) with long generation times, but also allows gene knockdown studies to be conducted in F1 hybrids that inherit the RNAi-inducing transgene from only one parent. RNAi silencing has an enormous potential as a tool in functional genomics of hexaploid wheat, a species for which other methods such as insertional mutagenesis are not available (Travella *et al*, 2006). It is a useful feature for gene discovery and functional genomics. Technical barriers for high-throughput functional genomics have recently been lowered considerably by the development of pHELLSGATE vectors that utilize the Gateway recombination system and give the possibility of making hpRNA constructs for large sets of genes (Helliwell and Waterhouse, 2003; Helliwell and Waterhouse, 2005). Tang and Galili raised the hypothesis that next generation RNAi vectors should contain characteristics of micro-RNA structures, because microRNAs do not trigger the PKR pathway, the RNA-dependent protein kinase pathway that causes nonspecific cell death in mammalian cells and could function as part of the plant stress response (Langland *et al*, 1995; Tang and Galili, 2004). RNAi has proven to be very efficient in interfering with gene expression in various plant systems such as *Petunia hybrida, Arabidopsis thalian, Papaver somniferum, Torenia hybrida, Coffea arabica,* and *Oryza sativa* (Fukusaki *et al*, 2004; Lee *et al*, 2004; Ogita *et al*, 2004; Miki *et al*, 2005; Travella *et al*, 2006). The wide use of this powerful technique reflects its ease of application and the possibilities for genome-wide reverse genetics. Gene constructs encoding intron spliced RNA with a self-complementary hairpin (hp) structure have been shown to induce posttranscriptional gene silencing with almost 100% efficiency when directed against viruses or endogenous genes and transgenes (Smith *et al*, 2000). Gene silencing has been described in both plant and animal systems as a means to suppress gene activity at the level of mRNA expression, providing a powerful tool with which to correlate genes with developmental or biochemical functions (Fire *et al*, 1998; Vaucheret and Fagard, 2001; Mello and Conte, 2004; Cigan *et al*, 2005). Post-transcriptional silencing can be triggered by utilizing sequences derived from the coding region of the target gene. When abundant dsRNAs are produced by a transgene, they are processed by a dicer-like protein into small interfering RNAs (siRNAs). The siRNAs then interact with an argonaute-like protein and other associated proteins to direct degradation of mRNAs that share sequence identity (Hamilton and Baulcombe, 1999; Baulcombe, 2004;

Meister and Tuschl, 2004; Cigan *et al*, 2005; Brodersen and Voinnet, 2006; Vaucheret, 2006; McGinnis *et al*, 2007). Both naturally occurring and transgene-mediated PTGS have been reported to either degrade or translationally repress their target transcript. The significance of naturally occurring double stranded RNA suppression has been established by its association with key developmental processes in diverse organisms such as *Caenorhabditis elegans*, Drosophila and Arabidopsis (Bartel, 2005; Jones-Rhoades *et al*, 2006; Meyers *et al*, 2008), while the incorporation of approaches involving suppression of transgenic RNA interference (RNAi) provides a powerful method for knocking out gene function at the level of expression in a variety of organisms (McManus *et al*, 2002; McGinnis *et al*, 2005; Ku and McManus, 2008). More recently, gene suppression has also been achieved by expressing double-stranded RNAs derived from promoter, rather than coding, regions of genes in plants and human cells (Matzke *et al*, 2004; Morris *et al*, 2004; Cigan *et al*, 2005; Dalakouras *et al*, 2009; Havecker *et al*, 2010). RNAi can induce gene silencing at a transcriptional or post-transcriptional level depending on the sequence contained in the dsRNA (Brodersen and Voinnet, 2006). RNAi has been used successfully to silence genes in both monocots and dicots. Many studies have focused on a small number of gene targets (Chen *et al*, 2003; Segal *et al*, 2003; Travella *et al*, 2006; Travella and Keller, 2009).

Expression studies aim to discover where and when specific proteins are produced. In these experiments, the DNA sequence before the DNA that codes for a protein, known as a gene promoter, is reintroduced into an organism with the protein coding region replaced by a reporter gene, a selectable marker, or an enzyme that catalyzes the production of a dye (Horstmann *et al*, 2004; Yoo *et al*, 2005; Kim *et al*, 2008). Thus, the time and place where a particular protein is produced can be observed (Meier *et al*, 2008). Expression studies can be taken a step further by altering the promoter to find which pieces are crucial for the proper expression of the gene and are actually bound by transcription factor proteins (Hilbricht *et al*, 2002; Girin *et al*, 2007).

Site-specific recombination systems, e.g. bacterial phage CRE-loxP and *Saccharomyces cerevisiae* FLP-FRT, function through interactions of a recombinase with its specific recognition sites (Bayley *et al*, 1992; Gilbertson, 2003). When cells have loxP/ FRT sites in their genome which expresses Cre/ FLP, a recombination event can occur between the recognation sites. The double stranded DNA is cut at both lox P or FRT sites by the Cre or FLP protein. The strands are then rejoined with DNA ligase. It is a quick and efficient process. The result of recombination depends on the orientation of the recognition sites. For instance, two lox sites on the same chromosome arm, inverted loxP sites will cause an inversion, while

a direct repeat of loxP sites will cause a deletion event. When loxP sites are on different chromosomes it is possible for translocation events to be catalysed by Cre induced recombination (Lyznik *et al*, 2003; Luo *et al*, 2007; Luo *et al*, 2008). Recently, strategies for removing marker genes or short spacer sequences from host plants using site-specific recombinases have been developed. After the loxP or FRT flanked marker gene is excised from the genome leaving one recognition site intact, the marker-free transgenic plants are segregated at the progeny level (Ebinuma *et al*, 2001; Lambert *et al*, 2007).

Wheat is clearly lagging behind compared to other major food crops such as maize (*Zea mays*), rice, and also species such as tomato (*Lycopersicon esculentum*). This is mainly due to the lack of efficient tools to study gene function in polyploid species. Hexaploid wheat has a large genome (16,000 Mb) that consists of three closely related homoeologous genomes (A, B, and D) and has a high content of repetitive DNA (80%; (Flavell *et al*, 1974)). Genes are found to be organized in gene islands or as single genes separated by large regions of nested repetitive elements (Feuillet and Keller, 2002). Due to the hexaploid nature of its genome, bread wheat has three (or a multiple of three) copies of most genes. It was found that many of these homoeologous genes are expressed (Mochida *et al*, 2003) and that there is, therefore, a high degree of functional gene redundancy in hexaploid wheat.

Insertional mutagenesis and gene silencing are efficient tools for the determination of gene function. In contrast to gain- or loss of function approaches, RNA interference (RNAi)-induced gene silencing can possibly silence multigene families and homoeologous genes in polyploids. This is of great importance for functional studies in hexaploid wheat (*Triticum aestivum*), where most of the genes are present in at least three homoeologous copies and conventional insertional mutagenesis is not effective (Travella *et al*, 2006). A major challenge in the postgenome era of plant biology is to determine the functions of all the genes in a plant genome. A straightforward approach to this problem is to reduce or knock out expression of a gene to induce a mutant phenotype that is indicative of the gene function. Insertional mutagenesis is a useful tool for this type of study and is based on transposon/ T-DNA insertions (Page and Grossniklaus, 2002; Boutros and Ahringer, 2008). However, this approach is limited by the time required to saturate a genome by lethal knockouts and is restricted to a few plant species. In addition, it is made more complicated by the problem of genetic redundancy caused by multigene families and polyploidy. In contrast to insertional mutagenesis, RNA interference (RNAi) is based on sequence-specific RNA degradation that follows the formation of double-stranded RNA (dsRNA) homologous in sequence to the targeted gene (Voinnet *et al*, 1999; Baulcombe, 2004; Herr and Baulcombe, 2004). RNAi

allows silencing one, several, or all members of a multigene family or homoeologous gene copies in polyploids by targeting sequences that are unique or shared by several genes (Lawrence and Pikaard, 2003; Miki *et al*, 2005; Travella and Keller, 2009). dsRNA is detected by the host plant genome as aberrant and is cleaved by the action of Dicer-like enzymes (Tang *et al*, 2003; Travella *et al*, 2006; Patrick *et al*, 2009; Shi *et al*, 2009) into two distinct classes of small interfering RNA (siRNA): long and short siRNAs (Hamilton *et al*, 2002; Tang *et al*, 2003). These two classes of small RNAs were proposed to have distinct RNA silencing functions: approximately 21-mers to direct posttranscriptional signaling via mRNA degradation and approximately 24-mers to trigger systemic silencing and the methylation of homologous DNA (Hamilton *et al*, 2002; Travella *et al*, 2006).

Gene transfer technology is still limited in crops by the low frequency of generation of transgenic plants. With further development and increase of the efficiency of the transformation methods, an exciting perspective will be opened up to improve tools for functional analysis of genes, with a strong impact on plant breeding (Travella *et al*, 2006).

1.4 Aim of this study

Fusarium head blight (FHB) caused by *Fusarium graminearum* and other *Fusarium* species is a major disease problem for wheat and maize production worldwide. Progress has been made to combat salinity and drought stress through standard breeding approaches, which has become a global concern too. Large-scale breeding efforts have been established to combat these problems, but the level of resistance attained is insufficient to withstand epidemic conditions. Genetic engineering provides an alternative approach to enhance the level of resistance. Many defense response genes are induced in wheat and maize during *F. graminearum* infection and environmental stress and may play a role in reducing FHB and increasing resistance to abiotic stress. The objectives of this study were (1) to characterize the hypusine pathway through analyzing of the eukaryotic initiation factor 5A functions and its catalyzed enzymes DHS and DOHH, (2) to develop *Fusarium* mutants lacking and overexpressing DHS and DOHH genes, (3) to develop transgenic maize overexpressing the defense response gene DHS, (4) to develop transgenic wheat silencing DHS gene, (5) to characterize the infection behavior of *Fusarium* mutants and (6) to test the resultant transgenic wheat and maize against the modified *Fusarium* mutants and against abiotic stress.

2.0 Experimental Procedures

2.1 Enzymes and chemicals

Restriction enzymes, DNA-modifying enzymes, and DNA and RNA markers were obtained from Fermentas (St. Leon Roth, Germany). Chemicals used in culture media and buffers were obtained from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Sigma-Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), Fluka (Buchs, Switzerlandand), and Duchefa (Haarlem, Netherlands). Chemicals used in Southern blot were obtained from Roche (Mannheim, Germany).

2.2 Oligonucleotide primers

All oligonucleotide primers used in this study were designed by using Primer Select program (DNASTAR software, USA) and LightCycler ® Probe Design Software 2.0 (Roche Applied Science, Germany). Routine PCR applications were performed by employing nonproofreading Taq DNA Polymerase (Fermentas, Germany). Platinum^{R} Taq DNA Polymerase was used for gene isolation and TA cloning (Invitrogen, Germany) and BIOTAQ^{TM} DNA Polymerase was used for TA cloning of fragments up to 5 kb (Bioline, Germany).

List of oligonucleotide primers used in this study. (F): forward primer; (R) reverse primer; (con) consensus primer; (N) nested primer. All primers are listed in 5`-3`-direction.

Primers for cloning gDNA and cDNA of *Fusarium* eIF5A, DHS and DOHH

Fg-dhs-ORF-F;	GGCCTCCAACTCTGATGCTC
Fg-dhs-ORF-R;	GATCCTCCTTCCCGATGTCC
Fg-dohh-ORF-F;	ATGTCGCCTTCTGCTGATAC
Fg-dohh-ORFR;	TCATGCAGCAGCTCCTGCG
Fg-eif5a-ORF-F;	ATGTCCGCCCCCAACGAC
Fg-eif5a-ORFR;	TTAGCCCTGGTTAGGAGCCT

Set of primers for generation of *Fusarium* DOHH disruption construct

DHH-koF;	ATGGTCAGCC TCGTGAGCTT
DHH-koR;	GACCCTTTGG TGCTGAAGCA
FG-DHH-F;	CTAGACGTAATACTCGACGATGC
FG-DHH-R;	CTGAACTGACTTTCAGGCACAC

Set of primers for generation of *Fusarium* DHS and DOHH overexpression construct

(Note: lowercase letters are recognition sites for respective enzymes; XbaI recognition site is within the amplification sequence.

FG-DHS-BamF;	AAT ggatcc ATGGCCTCCAACTCTGAT
FG-DHS-BamR;	AAT ggatcc TTATTTTTGATCCTCCTTCC
DOHH-SacI-F;	AAT gagctc GATACCCAAAATGTCGC
DOHH-XbaI-R;	CATAAATCATGCAGCAGCTCCT

Set of primers for identification of wheat DHS cDNA

cons1-F;	CCCTGGCCGCCGTNYTNAARCA
cons1-R;	CGAACCGGTCGATCATCTTGSWNGGNGTCCA
cons2-F;	CGAGGTCGTCAACCA GATGHTNGAYTGG
cons2-R;	TGTTGATGTACA CGGCGTAGTCNGCNCCRTT
cons3-F;	GGCTACCAGAAGACCACCATCTTYH TNGGNTA
cons3-R;	GCC GGAGTCGGAGCCRTCRAAYTC
cons4-F;	AACCTGCTGGTGCCGAAYGAYA AYTA
cons4-R;	GCCCCAGGACACGGCYTCRTCNGG
5`-wGSP;	CTGTATTTCGGGTCAAGTTCAGCTTC
5`-wNGSP;	CAGTCCTCGCTTGGCTTCTCA
3`-wGSP;	CCATTCATGCAAGCCCAAGGAAGA
3`-wNGSP;	GGGTCATAATTCTTGGTGGAGGC
wDHS-F;	GCGGAGGAGGCTCGTG
wDHS-R;	AAGTCACAGTTTTCCAGAG

Set of primers for identification of maize DHS cDNA

Mcons-F;	ATCGCCGGCGCCGACTTC
Mcons-R;	GCRTTGCATATATGATGCTT
DHS1-5`-GSP	CAGTAGATTGCTCCTGT
DHS1-5`-NGSP	CACAGATGCTCTATATTT
DHS1-3`-GSP	TTCCGCAATGGTGCAGAT
DHS1-3`-NGSP	CTGGAGCACACCCCGAT
DHS2-5`-GSP	GGGTGGACTGTTCCAGC
DHS2-5`-NGSP	GACGGATTCTCTGTACGC
DHS2-3`-GSP	CTGCGCGACGGAGCTGAC
DHS2-3`-NGSP	CCGGGGCGCGGCCTGAC

mDHS1-F	ATGGCCGCCGGTGCGG
mDHS2-R	TCATTTGGAGTTATGAAC
mDHS2-F	ATGGGTGCCGAGTTGG
mDHS2-R	TCAGTTTGTTGATTTAGTG

Primers used for sequencing of eIF5A, DHS and DOHH

NGPgpdAF;	TCCGAAGTAGGTAGAGCGAGT
trc-term-pan-R;	CATTGCAGATGAGCTGTATC
M13F;	GTAAAACGACGGCCAG
M13R;	CAGGAAACAGCTATGAC
Fw-gpd1-Fgl;	CTCTTACCACCTGCTCATCA
trc-term-pan-R	CATTGCAGATGAGCTGTATC
pJet1.2-F;	CGGCGTAATACGACTCACTA
pJet1.2-R;	CAGCCTGAAAATCTTGAGAGAA

primers for wheat and maize transformation

(Note: lowercase letters are recognition sites for respective enzymes)

F2-BamHI;	AAT *ggatcc* GGTTTCTAGT TCAACATCAC
R2-SpeI;	AAT *actagt*T CTCCGAGTGATCCATCAG
F2-SacI;	AAT *gagctc* GGTTTCTAGT TCAACATCAC
R2-SalI;	AAT *gtcgac* TCTCCGAGTGATCCATCAG
Cre_F;	CCATCGCTCGACCAGTTTAG
Cre_R;	TCGACCAGGTTCGTTCACTC
Bar_F;	GGTCTGCACCATCGTCAACC
Bar_R;	ACCCACGTCATGCCAGTTCC

Primers for expression analysis by RT-PCR

Beta-Tub- F;	TGCTGTTCTGGTCGATCTTG
Beta-Tub- R;	ATGAAGAAGTGAAGTCGGGG
18S rRNA-F;	CTGCCAGTAGTCATATGCTTGTCT
18S rRNA-R;	CCCCGTGTCAGGATTGG
M+WqDHS-F;	CTGCCCAGCATTGACTGA
M+WqDHS-R;	GCRTTGCATATATGATGCTT
qDHS-FG-F;	TAAACGATGAGCGATCCGTC
qDHS-FG-R;	CCAGCATCACTACCGTCGAA

qDHH-F;	AAGCTCATGGACACAAACGC
qDHH-R;	CAAAGCATACTCAGCGTCCT
qEIF5A-F;	AGCATGAGATGACCTTCGAC
qEIF5A-R;	GAGGTGAGGACAATGACGT
Fgl1-F;	GCAACTTCAAGTTCTACATCCAG
Fgl1-R;	TCTTGGATCCAGTGAAGGATG
Tri4-F	GTACCGTTATCCTGTTTGCTG
Tri4-R	CAAAGGCCATAGTGTATCCGA
Tri5-F	ATGACTACCCTCAATTCCTTCGT
Tri5-R	GAACTTCTTGGCGTCCTCTG
Tri6-F	AGACTTTGTACTCCGAAGAACCA
Tri6-R	TTGTCCTTCCTTGTCTTGCCA
Tri10-F	GACCTCATCACTCAGATTTACGCC
Tri10-R	CTCTTCTCTTCCAAGCGTCTTCC

Internal HYG primers used in Southern blot of *Fusarium*

F-hyg;	GTTGGCGACCTCGTATTGG
R-hyg;	CTTACCACCTGCTCATCACCT

Internal npt II primers used in Southern blot of *Fusarium*, wheat and maize

nptII131bp;F	TCTGGATTCATCGACTGTGG
nptII131bp;R	GGCGATACCGTAAAGCAC

2.3 *F. graminearum* cultivation, conidia induction and culture media

The wild-type strain 8/1 of *F. graminearum* used in this study was provided by Thomas Miedaner of the Landessaatzuchtanstalt Hohenheim, Germany. Induction of conidiation was carried out as described after (Jansen *et al*, 2005) on SNA-plates, which incubated for 7–14 days at 18°C under near-UV and white light (TLD 36 W-08; TL 40 W-33 RS; Philips) with a 12-h photoperiod. Conidia were then harvested from the plates with a sterile glass rod and sterile water. Macroconidia of all strains were stored as aqueous suspensions at -70°C.

The growth rate of the wild-type strain and several independent mutants were determined on SNA, YPG and on CM agar plates in the dark at 28°C. For measurements assays, cultures were cultivated in Erlenmeyer flasks that contain liquid medium as 25-40% of the total volume

of the flask liquid medium for 3 days on a rotary shaker at 175 rpm, at 28°C. The mycelium was then separated from the medium by filtration. Cultures that have had the opportunity to grow for an extended period of time, sometimes as little as 4-5 days, accumulated complex carbohydrates. Media for growing and identifying *Fusarium* strains were prepared as follows:

1. CM medium (Leach *et al*, 1982):
   -10 ml solution A (100X): 100 g/l Ca(NO3)2 x 4 H2O
   -10 ml solution B (100X): 20 g/l KH2PO4; 25 g/l MgSO4 x 7H2O; 10 g/l NaCl
   -50 ml Solution C (100X): 20% (w/v) Glucose
   -1ml suspension D (100X): 60 g/L H3BO3; 390 mg/L CuSO4 x 5H2O;13 mg/L KI; 60 mg/LMnSO4 x H2O; 51 mg/L (NH4)6Mo7O24 x 4H2O; 5.48 g/L ZnSO4 x 7H2O; 932 mg/L FeCl3 x 6 H2O; 2ml chloroform.
   Solutions A, B, C, D were sterilized by filtration.
   -Up to 1 L of autoclaved solution E: 1 g yeast extract; 0.5 g Casein, hydrolyzed by enzymatic cleavage; 0.5 g Casein, hydrolyzed by acid degradation.
2. YPG medium (Sambrook *et al*, 1989): 1% Yeast extract; 2% Peptone; 2% Glucose.
3. SNA medium (Nirenberg, 1981): 1 g KH2PO4; 1 g KNO3; 0.5 g MgSO4 x 7 H2O; 0.5 g KCl; 0.2 g Glucose; 0.2 g Saccharose for 1 L.

To prepare a solid medium, 16% granulated agar was added before autoclaving.

2.4 Growth conditions and induction of drought and salt stress in wheat and maize plants

The wheat cultivar Florida and the maize inbred line A188 plants obtained from Bioceter Klein flottbek (University of Hamburg) were subjected to osmotic stress by treatment with 5% PEG (polyetheline glycol 6000, Sigma- Germany). 2-week, 1M, and 2M old plants growing in the greenhouse were given 25 ml of 5% PEG instead of water every second day for a period of 15 days .Control plants were watered normally.

For salt stress induction, 2-week, 1M and 2M old plants were watered with 2% NaCl every second day for a period of 15 days. Plants were watered once with normal water after watering with salt water three times to wash the root regions. Control plants were watered normally. With the same conditions and plant age, drought stress was also induced through 25 ml of water given to the plants once per week. Here, the water given to the plants was controlled. Plants were grown in a greenhouse under controlled conditions with 16-h light of 24–28°C and 8-h night of 15–18°C.

2.5 gDNA and cDNA cloning of *Fusarium* eIF5A, DHS and DOHH

Cultures of *F.gramiearum* wild-type strain 8/1 for DNA and RNA extraction were started with a mass transfer of conidia and mycelia to a vigorously growing CM or YPG medium. Cultures were grown in Erlenmeyer flasks that contain liquid medium as 25-40% of the total volume of the flask and incubated at 28°C on an orbital shaker at 150-200 rpm for 2-3 days. Mycelia were harvested with a 200 µm diameter sieve and washed up.

Total RNA from fungal cultures was isolated with the Nucleo Spin RNAII Kit (Macherey Nagel, Düren, Germany) and the synthesis of cDNAs was performed with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Germany) according to the manufacturer's instructions.

F. graminearum eIF5A, DHS and DOHH open reading frames were amplified from cDNA with its specific primer pairs resulting in fragments of 495 bp, 1077 bp and 1014 bp, respectively, and cloned into pJET vector (Fermentas, Germany). Based upon the resulting sequence information, the primers were used for amplification of the whole genomic sequences. Genomic DNA, which was used as a template for amplification, was isolated by CTAB extraction buffer (1M Tris-HCl pH 7.5, 5M NaCl, 0.5 EDTA, 0.5 g CTAB) according to the *Fusarium* laboratory manual (Leslie and Summerell, 2006).

The PCR program consisted of an initial denaturation step for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C to 59°C for 45 sec, and an appropriate elongation time 90 sec at 72°C. The final elongation step was performed for 5 min at 72°C.

2.6 cDNA cloning of DHS from wheat and maize

Sequence comparison of DHS from *A. thaliana* and rice allowed the design of consensus primer pairs for RT-PCR of cons1, cons2, cons3, and cons4 revealed a motif of 738 bp. This motif has been identified by Dr. Frank Maier, Biocenter Klein Flottbek, University of Hamburg. Leaves of 4 weeks old wheat plants, cultivar- Florida, were covered with aluminium foil for 4 days at 18°C and 60% humidity to induce DHS transcription. RNA was isolated from leaves with the RNeasy Plant Mini Kit (QIAGEN). The amplified motif was cloned into pGEM-T vector (Promega, USA) and sequenced to generate specific primers to identify the complete sequence of DHS cDNA. The GeneRacer Advanced Race Kit (Invitrogen) was used to amplify 5`-end of the gene with the gene specific primer (5`-wGSP) and the gene nested specific primer (5`-wNGSP) producing a fragment of 312 bp. The 3`-end of the gene was also

amplified with the gene specific primer (3`-wGSP) and the nested gene specific primer (3`-wNGSP) producing a fragment of 578 bp. The full-length of DHS cDNA from wheat was amplified with the primer pairs (wDHS-F, wDHS-R).

Based on wheat DHS sequences, predicted sequences from maize database and rice DHS allowed designing conserved primers for RT-PCR of (Mcons-F, Mcons-R). In regard to these primers, a fragment of 796 bp was amplified from senescence-induced cDNA of maize plants, Inbred-A188, in the same procedure of wheat DHS induction. The PCR fragment was cloned into pJET vector (Fermentas, Germany) and sequenced. Based upon the resulting sequence information, two similar sequence fragments were identified with low monomorphisms. To identify the complete sequence, specific primers were generated. Primers are shown in section 2.2. PCRs with GSP and 3`- or 5`- nested primers were performed according to proposed cycling parameters of the GeneRacer Kit manual (Invitrogen). Sequence analysis of cloned PCR fragments into pJET vector (Fermentas, Germany) produced two genes of DHS in maize. The whole DHS*1* and DHS2 open reading frame was amplified with the primer pairs (mDHS1-F, mDHS1-R) and (mDHS2-F, mDHS2-R), respectively.

DHS genes from wheat and maize were amplified with an initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 sec, annealing for 45 sec at 55°C to 63°C and extension for 2 min at 72°C, the final extension step was performed at 72°C for 10 min.

2.7 Molecular cloning of *Fusarium* DOHH knockout

A ~3-kb DNA fragment encompassing the whole open reading frame (ORF) (1400 bp) of *Fusarium* deoxyhypusine hydrolase gene (DOHH), with a 700 bp upstream region and a 900 bp downstream region, was amplified from genomic DNA by PCR with the primer pairs (DHH-koF and DHH-koR). The PCR product was cloned into pJET vector (Fermentas-Germany) according to manufacture's instructions. To remove the coding region from the cloned product, a 1400 bp fragment was digested with SamI and EcoRv which flank the region naturally. A 1786 bp hygromycin cassette was then digested with SmaI enzyme and replaced with the gene coding region by a blunt ligation protocol incorporated with SmaI and EcoRv restriction sites. The pJET vector has a unique BglII restriction site on its cloning regions which can release the final resulting disruption cassette. Primers used to identify DOHH knockout lines were FG-DHH-F and FG-DHH-R.

2.8 Molecular cloning of *Fusarium* DHS and DOHH overexpression

A 1077 bp cDNA fragment encompassing the whole open reading frame (ORF) of *Fusarium* deoxyhypusine synthase gene (DHS) was amplified with primer pairs incorporated with BamHI restriction site (FG-DHS-Bam-F and FG-DHS-Bam-R). The PCR product was cloned into pJET vector (Fermentas-Germany) according to manufacture's instructions and sequenced. pANgluA-DHS (9277 bp) was constructed by inserting the PCR product within BamHI site flanked by the *gpd* promoter and *trpC* terminator. The resulting plasmid was sequenced again to orient the sense direction of the cloned fragment and linerzed with ClaI enzyme (Fermentas-Germany) which cuts DHS sequence once at position 328 for double crossover events. Plasmid pII99-DOHH (6971 bp) for overexpression of DOHH was constructed by inserting a 1014 bp cDNA fragment encompassing the whole open reading frame (ORF) of *Fusarium* DOHH gene within SacI and XbaI restriction sites flanked by the *gpd1* promoter and *trpC* terminator. The ORF of DOHH was amplified with primer pairs incorporated with SacI and XbaI restriction sites (DOHH-SacI-F and DOHH-XbaI-R), cloned, and sequenced. The resulting plasmid was linerzed with BbvII enzyme (Fermentas-Germany) which cuts DOHH sequence once at position 556 for double crossover events.

2.9 Construction of wheat DHS-RNAi and maize DHS-overexpression

To construct DHS-RNAi in wheat, vector p1U-SG containing the maize Ubi1 promoter and the nopaline synthase terminator (Dr.Becker, University of Hamburg) was used to make the self-complementary intron-containing hp RNA construct. A fragment of 376 bp corresponding to wheat DHS of senescence-induced cDNA of cultivar-Florida was isolated by RT-PCR using specific primers with incorporated restriction sites (F2-BamHI, R2-SpeI) and (F2-SacI, R2-SalI), which produced ends compatible with each other. This allowed the gene fragments to be directionally cloned in inverted orientation within the unique sites of vector p1U-SG. PCR amplification was carried out for 35 cycles of 45 s, denaturation at 94°C, 50 s annealing at 55°C, and 90 min extension at 72°C. Platinum Taq DNA polymerase used for amplification contains antibody complexes to stable the annealing temperature. The amplified fragments were then subcloned into pJET vector (Fermentas, Germany) and sequenced. The cDNA fragment recovered by SpeI and BamHI digestion was cloned in the BamHI restriction site of plasmid p1U-SG in antisense orientation. The second ligation inserts the PCR product in the SalI restriction site of plasmid p1U-SG in inverted orientation with respect to the first cloned fragment. In the second step, the PCR product experienced ligation difficulties; therefore, ligation was done following a different strategy. The cloned PCR product was digested with SacI*, (SacI blunt buffer) then with SalI, while plasmid p1U-SG was digested first with EcoRI,

blunted with DNA Blunting Enzyme (Fremants, Germany), then digested with SalI (Figure 33). The resulting plasmid was cleaved with SpeI and AflII producing a fragment of 1575 bp of DHS sense and antisense separated by an intron derived from Gus gene. This plasmid served as a template for further cloning into pD1-ubi-bar-lox vector.

To construct DHS-overexpression in maize, the cloned open reading frame of DHS1 into pJET vector (Fermentas, Germany) from senescence -induced cDNA from maize, inbred A188, as described previously, was further cloned into p7i-HSP-Cre-Ubi-Npt-Bar by DNA Cloning Service (Hamburg).

2.10 Protoplast transformation of *F. graminearum*

In general, transformation was carried out according to a previous procedure (Leach *et al*, 1982; Maier *et al*, 2005). 50 ml of CM medium were inoculated with 105 conidia and incubated for 2 days at 28°C, 140 rpm. Mycelium was homogenized in a Waring-Blender; 200 ml CM were inoculated with 10 ml hyphal suspension and incubated overnight at 24°C. Mycelium was collected on a sterile filter and washed twice with sterile water. Then, 2 g of mycelium were resuspended in 20 ml Driselase/Glucanase (Interspex Products, San Mateo, USA; 5%: 3% in 700 mM NaCl, pH 5.6), and digested 3 h at 28°C, 75 rpm. Undigested hyphal material was removed from the protoplast suspension by filtration through gauze and nylon membrane (50 µm pore size). The protoplast suspension was combined with 20 ml 700 mM NaCl and filtered again. The protoplasts were pelleted by centrifugation (1300 X g) in a swing-out rotor and washed twice by suspension in 10 ml ice-cold 700 mM NaCl and centrifugation (830·g). The washed protoplasts were suspended in STC (0.8 M sorbitol, 50 mM Tris–HCl, pH 8.0, 50 mM CaCl2) and stored on ice until transformation. For transformation, protoplasts were suspended at a concentration of 0.5–2 X 10^8 ml^{-1} in four parts STC and one part SPTC (0.8 M sorbitol, 40% polyethylene glycol 4000, 50 mM Tris–HCl, pH 8.0, 50 mM CaCl2). 35 µg transforming plasmid DNA (linear) and 5 µl heparin (5 mg /ml in STC) were added to 100 µl of the protoplast suspension. Samples were mixed and incubated on ice for 30 min; 1 ml SPTC was mixed with the suspension and incubated at room temperature for 20 min. Protoplasts were mixed gently into 200 ml regeneration medium (yeast extract 0.1%, caseinhydrolysate 0.1%, sucrose 1M, granulated agar 1.6%; at 43°C), poured in 94 mm Petri dishes (20 ml per plate) and incubated at 28°C. After 12– 24 h, the plates were overlaid with 10 ml selective agar (1.2% granulated agar in water containing hygromycin B or geneticin, 200 µg /ml) and further incubated. Transformants were obtained after 4 days post-transformation. They were transferred to fresh plates of CM medium with 100 µg/ ml hygromycin B or geneticin and incubated at 28°C. The transformants were purified by single-spore isolation.

2.11 Microparticle Bombardment of wheat

2.11.1 Growth and material of donor wheat plants

Winter wheat plants of cultivar Florida obtained from Biocenter Klein flottbek (University of Hamburg) were grown in a greenhouse for embryo transformation under controlled conditions with 16-h light of 24–28°C and 8-h night of 15–18°C. Initially all plants were top watered but once the root system reaches the base of the pot, the plants were watered using an automated flooding system. To ensure continuous production of immature embryos, seeds were planted every 2–3 days. Particular care was given to maintaining the greenhouse conditions at the same level for all the experiments and to avoid any stress to the donor plants. Pests and disease were kept to a minimum by good housekeeping practices.

2.11.2 Isolation and pre-culture of immature embryo

Fifteen days after heading or post anthesis, the immature seeds were harvested, sterilized by rinsing in 70% (v/v) aqueous ethanol for 5 min, then soaked for 15-20 min in 1% (v/v) NaOCl with gentle shaking, and washed with sterile water. A minimum of 200 immature embryos were isolated for bombardment. Immature embryos were pre-cultured on induction medium for 1-2 days in the dark at 26°C, then 50 scutella per 9 cm Petri-dish were placed on osmotic basal salt medium for 4-6 hours prior to bombardment.

2.11.3 Preparation of DNA coated gold particles and bombardment

Five micrograms of Plasmid DNA was precipitated onto gold particles following the protocol described by (Sparks and Jones, 2009). 40 mg sub-micron gold particles (0.6 μm) from Heraeus, Germany, were briefly placed per 1ml 100% ethanol into a 1.5 ml Eppendorf tube and sonicated for 2 min, pulse span in a microfuge for 3 seconds and washed with ethanol twice, then washed with sterile water three times, and resuspended in 1 ml sterile water, then divided into 50 µl aliquots and stored at -20°C. To precipitate DNA onto gold particles, a 50 µl aliquot of prepared gold was allowed to thaw at room temperature then sonicated for 1-2 min. 5 µl DNA (2µg/µl) was added and vortexed briefly. 50 µl 2.5M $CaCl_2$ and 20 µl 0.1M spermidine were placed and vortexed into the gold + DNA solution. The DNA-coated particles were pelleted by centrifugation and the supernatant was discarded. 100% ethanol was added to wash the particles twice and resuspended. Again, the particles were resuspended fully in 240 µl 100% ethanol and maintained on ice to be used immediately for bombardment after preparation

For each bombardment, 3.5 µl of microparticle DNA were placed onto the macrocarrier. Bombardments were conducted at a distance of 5 cm from the stopping plate using a PDS 1000/He microprojectile gun with 1350 psi (1 psi = 6.895 kPa). Bombardments were carried out with a low concentration of plasmid DNA (0.4 µg plasmid DNA/1 mg microcarriers) so as to achieve lower copy number transgene insertions.

2.11.4 Tissue culture and selection of transformants

Following bombardment, treated embryos were distributed over MS medium containing 2 mg/L 2,4- dichlorophenoxyacetic acid (2,4-D), 60 g/L maltose for callus induction. Between 12 and 14 days after transfer to the induction medium, the induced embryos were scored for the embryogenic callus formation. The induced embryos were then transferred in MS medium containing 1.5-3 mg/L Basta (20% phosphinothricin, Sigma, Germany). Cultures were incubated at 26°C in darkness for 2 weeks. Embryogenic calluses were subsequently transferred to regeneration medium for shoot induction. Regenerating calluses were cultured at 26°C in the light, and subcultured onto fresh hormone medium every 2 weeks. Selection was started in either the first or second round of regeneration and continued in subsequent rounds with a photoperiod of 16 h light: 8 h dark. The light was provided by white cool florescent tubes (Solar) and the light intensity was 200 MJ/m2s. After 30 days, healthy, fully differentiated plantlets were scored and transferred to the same medium in magenta boxes, free of herbicide, for further development. Surviving green-rooted plantlets were transferred to a soil mixture and placed in the greenhouse. Herbicide resistance of the putative transgenic wheat plants was determined by spraying the leaves of plants at the fifth or sixth leaf stage three times with Basta (250 mg/L) with 7 days between applications to minimize escapes. Plants were scored as susceptible or resistant according to the degree of leaf desiccation after 7 days. The surviving plants were moved for 6 weeks to a cold chamber for induction of the reproductive stage.

2.11.5 Culture media of biolistic transformation

Media were prepared for biolistic transformation after (Day *et al*, 2005; Kikkert *et al*, 2005).

1. Murashige and Skoog macrosalts (MS-10X) (Murashige and Skoog, 1962): 16.5 g of NH4NO3, 19 g of KNO3, 4.4 g of CaCl2*2H2O (or 3.3 g of CaCl2), 3.7 g of MgSO4*7H2O, 1.7 g of KH2PO4. Salts were dissolved separately in 100 mL of distilled water, mixed, made up to 1 L, and then .stored at 4°C.

2. Murashige and Skoog microsalts (MS-1000X stock) (Murashige and Skoog, 1962): 22.30 g of MnSO4*4H2O (or 16.90 g of MnSO4*H2O), 8.60 g of ZnSO4*7H2O, 6.20 g of H3BO3, 0.83 g of KI, 0.250 g of Na2MoO4*2H2O, 0.025 g of CuSO4*5H2O, 0.025 g of CoCl2*6H2O. Dissolved in 1 L of distilled water and stored at 4°C.
3. Ferrous sulfate-chelate (500X stock): 3.73 g of Na2EDTA, 2.78 g of FeSO4*7H2O. Placed in distilled water separately, then mixed slowly, heated to dissolve, and up to 100 ml with H2O
4. MS (2X): 200 ml/L MS macrosalts, 2ml/L MS microsalts, 4 ml/L Ferrous sulphate chelate solution, 60 g maltose, pH adjusted to 5.7.
5. Osmotic medium: 200ml MS (2X), 200 ml Gelrite.
6. Induction medium: 200ml MS (2X), 200 ml Gelrite, 2 mg/L 2,4D
7. Selection medium I: 200ml MS (2X), 200 ml Gelrite, 2 mg/L 2,4D, 1.5 mg/L Basta.
8. Selection medium II: 200ml MS (2X), 200 ml Gelrite, 0.1 mg/L 2,4D, 3 mg/L Basta.
9. Regeneration medium: 100ml MS (2X), 200 ml Gelrite, 100 ml H2O

2.12 Transformation-mediated *Agrobaterium* of maize

2.12.1 Maize plant and *Agrobacterium* material

Embryos were isolated from HiIIA X HiIIB (F1) hybrids. Maize lines HiIIA and HiIIB were obtained from Biocenter Klein flottbek (University of Hamburg)**.** Plants were grown in a greenhouse for embryo transformation under controlled conditions with 16-h light of 24–28°C and 8-h night of 15–18°C. Immature zygotic embryos (1.5–2.0 mm) of the maize (*Zea mays*) were aseptically dissected from greenhouse-grown ears harvested 10 to 13 d post pollination. Ears were stored up to 3 d at 4°C before dissection. *Agrobacterium tumefaciens* strain LBA4404 was kindly provided by Dr. Dirk Becker (University of Hamburg).

2.12.2 Transformation of *A.tumefaciens*

Plasmids were introduced into *Agrobacterium* strain LBA4404 by thaw-freeze method (Jyothishwaran *et al*, 2007) and used to transform maize plants. The vector system was maintained on yeast extract beef (YEB) medium containing 100 mg /L spectinomycin (for pTF102) and 50 mg /L tetracycline (for LBA4404). Bacteria cultures for weekly experiments were initiated from stock plates that were stored for up to 1 month at 4°C before being refreshed from long-term, -70°C glycerol stocks. In all experiments, bacteria cell densities were adjusted to an optical density (OD600) between 0.35 to 0.45 using a spectrophotometer immediately before embryo infection (Frame *et al*, 2002).

2.12.3 Infection and cocultivation

Infection and cocultivation were after (Frame *et al*, 2002). *A. tumefaciens* cultures were grown for 3 d at 19°C on YEB medium amended with 100 mg/L spectinomycin and 50 mg/L tetracycline. One full loop (3 mm) of bacteria culture was scraped from the 3-d old plate and suspended in 5 ml of liquid infection medium (Inf) supplemented with 100 µM AS (Inf + AS) in a 50-mL falcon tube. The tube was fixed horizontally to a bench-top shaker or a Vortex Genie platform head and shaken on low speed (approximately 75 rpm) for 4 to 5 h at room temperature. This pre-induction step was carried out for all experiments. For infection, immature zygotic embryos (1.5–2.0 mm) were dissected to bacteria-free Inf + AS medium (1.8 ml) in 2-ml Eppendorf tubes (20–100 embryos per tube) and washed twice with this medium. The final wash was removed and 1 to 1.5 ml of *A. tumefaciens* suspension was added to the embryos. Embryo infection was accomplished by gently inverting the tube 20 times before resting it upright for 5 min with embryos submerged. Embryos were not vortexed at any time during this procedure. After infection, embryos were transferred to the surface of cocultivation medium and excess *A. tumefaciens* suspension was pipetted off the medium surface. Cocultivation medium contained 400 mg/L Cystein unless stated otherwise. In experiments in which cocultivation medium treatments were compared, embryos were washed and infected in the same tube before being distributed between media treatments.

Embryos were oriented with the embryo-axis side in contact with the medium (scutellum side up). Plates were wrapped with vent tape and incubated in the dark at 20°C or 23°C for 3 days, after which embryos were transferred to 28°C on resting medium. Cocultivated immature zygotic embryos that had initiated embryogenic Type II callus formation at their scutellum base after 4 to 7 days on resting medium were transferred to selection medium.

2.12.4 Selection and regeneration

After 4-7 days on resting medium (28°C, dark), embryos were transferred to selection medium (30 per plate) containing 10 mg/L bialaphos for 2 weeks. Putatively transformed events were identified as early as 5 weeks after infection. Regeneration of T_0 transgenic plants from Type II embryogenic callus was accomplished by a 2-3 week maturation step on Regeneration Medium I followed by germination in the light on Regeneration Medium II as described by (Frame *et al*, 2000; Frame *et al*, 2002).

2.12.5 Culture media of *Agrobaterium*-mediated transformation

Infection, cocultivation, resting, and selection media were prepared after (Zhao *et al*, 1999; Frame *et al*, 2002) except that cocultivation medium was modified to contain Cystein. All these media contained N6 salts (Chu *et al*, 1975) and vitamins, 1 mg/L 2, 4-dichlorophenoxyacetic acid, and 0.7 g/L L-Prolin in addition to the following ingredients:

1. Infection medium contained 68.4 g/L Sucrose and 36 g/L Glycin (pH 5.2) and was supplemented with 100 µm Actosyrigone (AS) prior to use.
2. Cocultivation medium contained 30 g/L Sucrose, 50 mg/L L-Gystein, 0.85 mg/L silver nitrate, 100 µm AS, and 3 g/L gelrite (pH 5.8).
3. Resting medium contained 30 g/L Sucrose, 0.85 mg/L silver nitrate, 250 mg/L cefotaxime, 50 mg/L vancomycin, and 3 g/L gelrite (pH 5.8).
4. Selection medium was identical to resting medium with the addition of 10 mg/L bialaphos.

Infection medium was filter sterilized, whereas all other media were autoclaved. AS stock solutions (100 mM) were prepared by dissolving AS in 100% (v/v) dimethyl sulfoxide (DMSO) to make a 200 mm stock which was then diluted (1:1 [v/v]) with sterile water and stored in small aliquots at 20°C. Cystein was added to cocultivation medium after autoclaving from freshly prepared, filter-sterilized stocks (100 mg/L) and cocultivation medium was used within 2 to 5 days of preparation.

5. Regeneration I medium contained Murashige and Skoog salts (MS) and vitamins (Murashige and Skoog, 1962), 60 g/L Sucrose, 100 mg/L myo-inositol, no hormones, and 3 g/L gelrite (pH 5.8) after (Armstrong and Green, 1985). Cefotaxime (250 mg/L) and bialaphos (10 mg/L) were added to this medium after autoclaving.
6. Regeneration II medium differed from medium I in that it contained 30 g/L Sucrose and no bialaphos. All media were poured into 100- X25-mL plates.

2.13 Herbicide test of wheat and maize transformants

A glufosinate leaf-spray test (L-Basta, Hoechst, Germany) was used to establish segregation ratios for expression of the *bar* gene in the progeny (Brettschneider *et al.*, 1997). The herbicide was dissolved in water (1.25 ml/L) along with 0.1% (v/v) Tween 20 for a final glufosinate concentration of 250 mg/L. Beginning 9 days after planting, seedlings were sprayed three times at 1- 2-week intervals with a freshly prepared glufosinate solution and then scored for herbicide resistance (alive) or herbicide sensitivity (dead).

2.14 Molecular analyses

2.14.1 Molecular analysis by polymerase chain reaction (PCR)

A polymerase chain reaction (PCR) was performed on genomict DNA extracted from leaves (wheat and maize plants) by DNA extraction buffer (1%. N-Lauryl Sarcosin, 100 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 100 mM NaCl). All of the Basta-resistant plants that survived the tissue culture selection were analyzed by PCR for the presence of the *bar* gene, *Cre* gene, and *neo* gene. In addition, a polymerase chain reaction (PCR) was performed on genomict DNA extracted from fungal mycelia by by CTAB extraction buffer (1M Tris-HCl pH 7.5, 5M NaCl, 0.5 EDTA, 0.5 g CTAB). All hygromycin and neomycin-resistant *Fusarium* mutants that survived the selection were analyzed by PCR for the presence of the *hph* gene or *nptII* gene. Pairs of specific primers were used. Primers are shown in section 2.2. The PCRs were carried out in a total volume of 25 µl, consisting of 150 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 3 mM MgCl2, 50% DMSO, 10 mM of each dNTP, 1 U *Taq* DNA polymerase, and 10 µmol of each primer. For PCR profile, DNA was denatured at 94°C for 3-4 min (hot start), followed by 30 amplification cycles of 94°C for 30 sec., 53°C-59°C for 54 sec, and 72°C for 2 min.

2.14.2 Expression analysis by RT-PCR and semiquantitative RT-PCR

To study the expression of the studied genes in culture and in planta, RNA was isolated with the RNeasy Plant and Fungi Kit (QIAGEN). For RT-PCR, Superscript II, RNase H-Reverse Transcriptase (Invitrogen) was used according to manufacturer's instructions. The resulting single stranded cDNA was used as a template in PCR reactions to test gene transcription in different samples. The PCR reaction was carried out with intron-spanning gene specific primers designed through LightCycler ® Probe Design Software 2.0 from Roche Applied Science. Primers are shown in section 2.2 Reaction conditions in the reverse transcription step for the expression analysis were based on the use of the housekeeping gene ß-tubulin in *Fusarium* and 18S rRNA in wheat and maize as internal controls. Semiquantitative RT-PCR was conducted from 25 to 30 PCR cycles for all tested genes before reaching the plateau phase.

For expression analysis in planta, infected spikes of wheat, abiotic stressed wheat and maize plants were harvested after different time points and frozen in liquid nitrogen for subsequent RNA isolation. To examine *Fusarium* DHS, DOHH and DHS-DOHH

overexpression on the transcriptional level, RNA was isolated from *F. graminearum* wild-type and from transformants growing in vitro culture (SNA, YPG and CM) of different time points.

All genes from wheat, maize and *Fusarium* were amplified with an initial denaturation at 94°C for 3 minutes, 29 cycles of 94°C for 30 seconds, annealing for 45 seconds at 51°C to 63°C and extension for 1 minute at 72°C, the final extension step was performed at 72°C for 5 minutes. The PCR reaction was set in 25µl of 1X PCR buffer, dNTPs (0,2 mM each), 1,5 mM $MgCl_2$, Primer mix (0,2 µM each), *Taq* DNA polymerase (1 unit), and template (20 ng).

2.14.3 Southern blot analysis

Genomic DNA from various *F. graminearum* strains was digested with restriction enzymes, separated on a 0.8 % agarose/TBE gel and blotted onto a Hybond NX membrane (Amersham Biosciences, Little Chalfont-UK). A DIG digoxygenin labeled DNA probe (Roche, Mannheim, Germany) was used for overnight hybridization at 68°C. Hybridization and washing of the blots were performed according to the manufacturer's instructions. Integration of overexpression of DHS, DOHH and DHS-DOHH, respectively, into the *F. graminearum* genome was tested by using a gene specific probe, and integration of the backbone of the cloned vectors was checked by the selective marker hygromycin B (DHS mutants), or neomycin specific probe (DOHH mutants) on the same membrane.

Detection of maize transformants was also performed in a Southern blot approach as indicated above. Integration of DHS-overexpression vector into transgenic maize plants was analyzed by using the selective marker neomycin specific probe for hybridization. Probes' sequences are shown in section 2.2. Leaf genomic DNA was prepared from 2-3 g of fresh leaf tissue from putative transgenic maize plants using DNA extraction buffer (1%. N-Lauryl Sarcosin, 100 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 100 mM NaCl). 10 µg of genomic DNA per sample was digested separately with the restriction enzyme HindIII and SfiI at 37°C overnight and separated on a 0.8% (w/v) agarose gel. DNA gel-blot analyses were conducted on DNA samples using the Dig-labeled *nptII* fragment. Detection was achieved using the DIG Luminescent Detection Kit (Roche, Germany) and the hybridization signals were visualized by exposure to Fuji X-ray film at 37°C for 40 min.

2.15 Virulence assay and pathogenicity test

The wheat cultivars Nandu (Lochow-Petkus, Bergen-Wohlde, Germany) and Amaretto (Berthold Bauer, Niedertraubling, Germany) were used for all virulence assays. Plants were grown in pots at 18-20°C with a 16-h photoperiod (8,000 lx) and 70% relative humidity.

For spike inoculation, spikes of wheat cv. Nandu and Amaretto were inoculated with WT and the overexpressing strains as described after (Jenczmionka *et al*, 2003). Briefly, wheat spikes at anthesis were point-inoculated with the *F. graminearum* wild-type strain 8/1 and all independent mutants by placing a droplet (10 µl) of conidia suspension (2×10^4 conidia/ml) or 10 µl of water within the palea and lemma of two basal florets of one spikelet in the middle of the wheat spike tested. To obtain a moist surrounding for the infection, the plants were sprayed with water before inoculation with the fungus. The inoculated spikes were enclosed in small plastic bags during the first 3 days to ensure a high humidity for infection and to prevent cross-contamination by different *F. graminearum* isolates. The inoculated plants were incubated in a growth chamber at 21 °C with a 16-h photoperiod (8,000 lx). The plant assays were evaluated 3 weeks after inoculation with the fungus.

For caryopsis inoculation, caryopses of wheat cv. Nandu were inoculated as described after (Jansen *et al*, 2005). Briefly, spikes were harvested at anthesis stage. Isolated caryopses were disinfected by incubation in 6% sodium hypochlorite for 40 min, freed from glume, lemma, and palea, and placed on 0.5% (vol_vol) phytagar (Gibco_BRL, Invitrogen Life Technologies, Germany). The dorsal side of each caryopsis was covered with 10 µl of a 0.05% Tween 20 solution, containing 2×10^4 macroconidia per ml of WT and the overexprssing strains, respectively. After inoculation, the caryopses were incubated in a growth chamber at 22°C, 16 h light period for 3 days. Three independent inoculation experiments were performed, and at least three caryopses infected with the different gynotypes were examined per experiment and time point.

2.16 Mycotoxin assay

100 mg of infected spikelets were ground to a powder under liquid nitrogen, dried for 3 h at 70°C, and then homogenized with 2 mL of distilled water. The extract was incubated for 30 min on ice and filtered. 50 µl of the supernatant was used in the test for Deoxynivalenol quantification with RIDASCREEN DON Kit (R-Biopharm, Germany) by measuring the absorbance at 450 nm against an air blank within 10 minutes with Bekman Anthos 2010-96 well microplate reader (Anthos-Mikrosysteme, Germany).

2.17 Cell death measurment

Cell death was measured spectrophotometrically by Evans blue staining, indicating loss of plasma membrane integrity as described after (Guo and Crawford, 2005). Briefly, 100 mg of fungal mycelia were submerged in a 0.1% (w/v) aqueous solution of Evans blue (Sigma-Aldrich, Germany) for 30 min followed by two 2-min cycles of vacuum. The mycelia were then washed three times with distilled water. Dye bound to dead cells was solubilized in 50% (v/v) methanol and 1% (w/v) sodium dodecyl sulphate at 60°C for 30 min and then quantified by measuring the absorbance at 600 nm using Ultrospec 3000 spectrophotometer (Phamacia Biotech, Germany).

2.18 H2O2 measurment

H2O2 was extracted as described after (Rao *et al*, 2000; Frank *et al*, 2007). Briefly, 100 mg of fungal mycelia or infected spikelets was ground to a powder under liquid nitrogen and homogenized with 1 mL of 0.2 M HClO4 in a precooled pestle and mortar. The extract was held on ice for 5 min and centrifuged at 10000g for 10 min at 4°C. The supernatant was collected and either processed immediately or quick-frozen at -80°C until further analysis. All analysis was completed within 72 h of extraction, a period in which no substantial autooxidation of H2O2 was observed. The acidic supernatant was neutralized to pH 7.0 to 8.0 with 0.2 M NH4OH, pH 9.5, and centrifuged briefly at 3000g for 2 min to sediment the insoluble material. The colored components in the extract were removed by applying the extract (0.5 mL) to a 2-mL column of AG 1X-8 resin (Bio-Rad, USA) and eluting with 3 mL of double-distilled water. H2O2 was measured using the Amplex red H2O2/peroxidase assay kit (Invitrogen, Germany) according to manufacturer's instructions. The measurment was done with 96-well black plates to prevent cross contamination and by using Fluoroskan II fluorescence 96-well microplate reader (Finstruments, Finland) with fluorescence emission detection at 590 nm and excitation in the range of 530-560 nm.

Detection of ROS was deterimined by $O2^-$ production. Detection of $O2^-$ in fungi was monitored by staining mycelia with nitro blue tetrazolium-NBT (Sigma, Germany) according to (Semighini and Harris, 2008). Briefly, after growth at 28°C for 2 days, each plate was flooded with a solution of 2.5 mM NBT in 5 mM MOPS, pH 7.6, and incubated in the dark at 28°C for 30 min. The supernatant was drained and the plates were carefully rinsed with distilled water. Plates were reincubated for 2 days in the dark at 28°C, and then images of whole colonies were made.

2.19 Total protein quantification assay

Germinating conidia on rich medium plates were scraped off a plug with1 cm^2, frozen in liquid nitrogen, and lyophilized overnight. The lyophilized pellets were ground up with equal volume of glass beads. 0.2 ml of SDS-PAGE extraction buffer (1% SDS, 10% glycerol, 25 mM Tris-HCl pH 6.8, 1mM EDTA, 0.7 M mercaptoethanol) was vigorously mixed with the lyophilized powder, boiled for 2 min, vortexed for 1 min, then boiled for another minute. The supernatants collected after microcentrifugation were then quantified with Bradford reagent (Sigma, Germany) by measuring the absorbance at 595 nm using Ultrospec 3000 spectrophotometer (Phamacia Biotech, Germany).

2.20 Microscopy

Coverslips with adherent hyphae in SNA medium were prepared for microscopy and cell walls were detected with Calcofluor (Sigma, Germany). To visualize GFP and DsRed signals, hyphae grown on coverslips in SNA medium were briefly mounted in coverslips and observed with the Zeiss fluorescence microscope (Carl Zeiss,Germany), and then images were processed using Axiovision Rel 4.7 software. Observation of fungal transformants mycelia grown on plates was made using Leica- M2FL III microscope (Leica, Germany). Images were converted from 16 to 8 bits and saved as TIF files. Additional processing was performed using CorelDraw Graphics suite X3 (USA). All assays were carried out in triplicate and repeated at least three times.

2.21 Accession numbers

Genbank accession numbers of the submitted deoxyhypusine synthase mRNA-complete Cds in wheat *Triticum aestivum* (cultivar-Florida) are / FJ376389 /, deoxyhypusine synthase (DHS1) and (DHS2) cDNA- complete Cds in maize *Zea mays* (Inbred -A188) is GU735677 and GU735678, respectively. The accession numbers of the submitted deoxyhypusine synthase (DHS), deoxyhypusine hydrolase (DOHH), and eukaryotic initiation factor 5A (eIF5A) cDNA-complete Cds in *Fusarium graminearum* (strain 8/1) are GU809212, GU735676, and GU809213, respectively.

3.0 Results

3.1 Identification of eIF5A, DHS and DOHH from *F. graminearum*

The eIF5A gene from *F. graminearum* /FGSG_01955.3 / was identified using a BlastX search against *Fusarium graminearum* Genome Database (MIPs and Broad Institute), and published in genbank under accession no. /FG01955.1/ with a length of 1350 bp and a translated sequence of 165 aa. Coding sequence has four exons: 155 bp, 267 bp, 133 bp and 402 bp.

The DHS gene from *F. graminearum* /FG00323.1/ was also identified using a BlastX search against *Fusarium graminearum* Genome Database (MIPs and Broad Institute), and published in Genbank under the same accession number with a translated sequence of 359 aa and 1157 bp gene length. Coding sequence has two exons: 213 bp and 864 bp.

The DOHH gene from *F. graminearum* /Q4HZ35 / was identified using UniProtKB – Protein knowledge base. Initially, the EC number of DOHH enzyme / 1.14.99.29/ was used to search for *Fusarium* DOHH entries in the UniProtKB database and annotated to *F. graminearum* sequences from the MIPS *Fusarium graminearum* Genome Database and Broad Institute. Enzyme classification of deoxyhypusine hydrolase was released from The Comprehensive Enzyme Information System- BRENDA. The BlastX search against *Fusarium* genome retrieved one hit / FGSG_09773.3/ with 1062 nt gene length and a protein of 338 aa. Coding sequence has two exons: 497 bp and 517 bp.

Genomic DNA and cDNA from wild-type strain 8/1 were used to amplify eIF5A, DHS and DOHH. The amplified PCR fragments were cloned and sequenced. The sequenced cDNA complete Cds of DHS, DOHH, and eIF5A from *F. graminearum* wild-type strain 8/1 were submitted to Genbank under accession numbers: GU809212, GU735676, and GU809213, respectively.

3.2 Isolation of cDNA sequence of wheat DHS

Comparison of deoxyhypusine synthase cDNAs from genbank of rice (*Oryza sativa*) and *Arabidopsis thaliana* identified conserved motifs. This motif has been identified by Dr. Frank Maier, Biocenter Klein Flottbek, University of Hamburg Previous studies reported the upregulation of DHS in leaves in response to senescence or stress induced premature senescence (Wang *et al*, 2001; 2003). Therefore, mRNA was isolated from dark stressed wheat

leaves and using PCR primers derived from the conserved DHS cDNA fragments amplified a 738 bp motif (compare Experimental Procedure). The PCR fragment of wheat was cloned, sequenced, and displayed a homology of 72% to *A. thaliana*, 91% to rice, and 98% to the motifs. This fragment was then used for designing 5`-end and 3`-end race primers. The obtained full length cDNA sequence of wheat DHS contains 1477 base pairs. It includes a 39 base pair 5` noncoding sequence, a 307 base pair 3` noncoding sequence, and an open reading frame, coding for a protein of 376 amino acids with a predicted molecular mass of 41.4 kDa, (Figure 5). DHS cDNAs have been cloned from several plant species including tomato, *Arabidopsis*, canola, tobacco, and rice (Wang *et al*, 2001; 2003; Duguay *et al*, 2007). All share a high sequence similarity to the identified DHS of wheat, which submitted to Genbank under accession number: FJ376389.

Figure 5. Nucleotide and inferred amino acid sequences of the cDNA for senescence-induced wheat DHS. Nucleotide sequence is shown as follows: lowercase letters, 5`- and 3`- noncoding cDNA sequence; uppercase letters, open reading frame; dotted underline, 738 bp motif; arrows, oligonucleotides used for 5`-end and 3`-end cDNA synthesis; astrik, stop codon. Numbers of nucleotides and amino acids are indicated.

3.3 Isolation of cDNA sequence of maize DHS

Wheat DHS was used to identify DHS gene from maize. The sequence was used as a query and searched against maize genome at DFCI- Plant Gene Indices, the computational biology and functional genomics database of Dana-Farber Cancer Institute and Harvard School of Public Health; http://compbio.dfci.harvard.edu/tgi/plant.html. The BlastX search retrieved two sequence homologues to wheat DHS: TC329871 and TC325150. The sequences were then annotated to pfam database, a large collection of protein families, which matched the deoxyhypusine synthase domain.

To avoid mismatch and polymorphisms, senescence- induced cDNA from maize leaves was synthesized using PCR primers derived from conserved sequences among rice, wheat, and the predicted sequences. The PCR fragments were served as a template for RACE-PCR. Two fragments were amplified, one from the 5`-end and the other from the 3`-end of a putative DHS. Two ORFs were deduced from the sequences of the fragments: DHS1 has a protein coding of 370 amino acids with a predicted molecular mass of 40.74 kDa and displayed a homology of 90% to wheat, 91% to rice and72% to *A. thaliana*. DHS2 has a translated coding of 379 aa with a predicted molecular mass of 41.74 kDa and a homology of 84% to wheat, 83% to rice and 67% to *A. thaliana*, respectively. DHS1 and DHS2 were submitted to Genbank under accession numbers: GU735677 and GU735678, respectively. DHS1, the highest similarity to DHS genes in other species, was further analyzed and overexpressed into maize plants. DHS1 is mentioned as maize DHS in this work.

3.4 Computational analyses of eIF5A, DHS and DOHH sequence alignment

Homologues of eIF5A, DHS and DOHH proteins were identified by BLASTP and TBLASTN searches (Altschul *et al*, 1997) in non-redundant protein and EST databases respectively, using the NCBI BLAST server. Identified EST sequences were analyzed to predict the open reading frames, and full length protein sequences were deduced by the identification of in-frame start and stop codons. Sequences were performed with the ClustalW multiple sequence alignment program (Clamp *et al*, 2004) and the alignment was analyzed with the JalView 2.07 software (Thompson *et al.*, 1994). Amino acid residues were coloured according to their percentage of identity. Dark blue: > 80% identity; blue: > 60% identity; light blue: > 40% identity; white: < 40% identity.

The human eIF5A isoform 1 (variant B) protein sequence was aligned with other human eIF5A isoform sequences and the eIF5A sequence from *F. graminearum, Saccharomyces cerevisiae, Candida albicans, Zea mays* and *Triticum aestivum* (Figure 6). Highly aligned scores were predicted in sequence regions of N-terminal domain. The region corresponding to the loop that bears the hypusination site (positions 75–86; upper numbering) is highly conserved in all amino acid sequences that were analyzed. It is, therefore, reasonable to say that the conformation adopted by this loop region of 12 nucleotides in the basic N-terminal domain is 100% conserved in these proteins, which also resulted from all species in the eukaryotic kingdom. Whereas only the N-terminal core domain is sufficient for modification by DHS and DOHH, the C-terminal domain seems to be required for the biological activity of eIF5A in cells (Cano *et al*, 2008; Park *et al*, 2010).

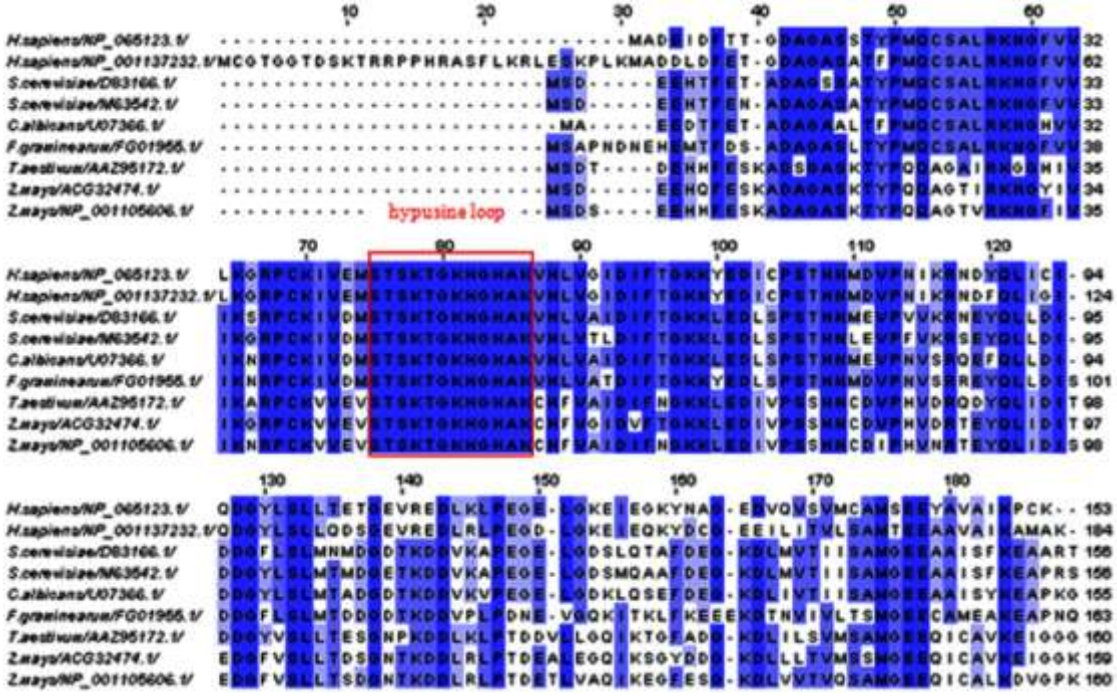

Figure 6. Sequence alignment of eIF5A genes. Nine sequences from different species are shown together. The hypusine modification site (in red box) is located at an exposed loop (hypusine loop 75-86) in the basic N-terminal domain. The GenBank codes are; NP_065123.1 human initiation factor 5A isoform I variant B; NP_001137232.1 human translation initiation factor 5A isoform 2; D83166.1 *Saccharomyces cerevisiae* translation initiation factor 5A1; M63542.1 *Saccharomyces* translation initiation factor 5A2; U07366.1 *Candida albicans* translation initiation factor 5A; GU809213 *Fusarium* translation initiation factor 5A; AAZ85172.1 wheat translation initiation factor 5A; ACG32474.1 maize translation initiation factor 5A1; NP_001105606.1 maize translation initiation factor 5A2.

BLAST searches performed in the Genbank EST database revealed that wheat deoxyhypusine synthase shares the highest similarity to DHS in rice (*Oryza sativa*) and maize (*Zea mays*). Furthermore, amino acid sequence comparisons among several different species

including *Arabidopsis thaliana, Fusarium graminearum, Saccharomyces cerevisiae, Candida glabrata* and *Homo sapiens* showed considerable conservation of sequence identity, particularly in the C-terminal active site of the enzyme, which habours an enzyme intermediate formation site (Wolff and Park, 1999; Duguay *et al*, 2007). A DHS protein alignment performed with the Clausal multiple sequence alignment program (Thompson *et al*, 1994) is shown in Figure 7.

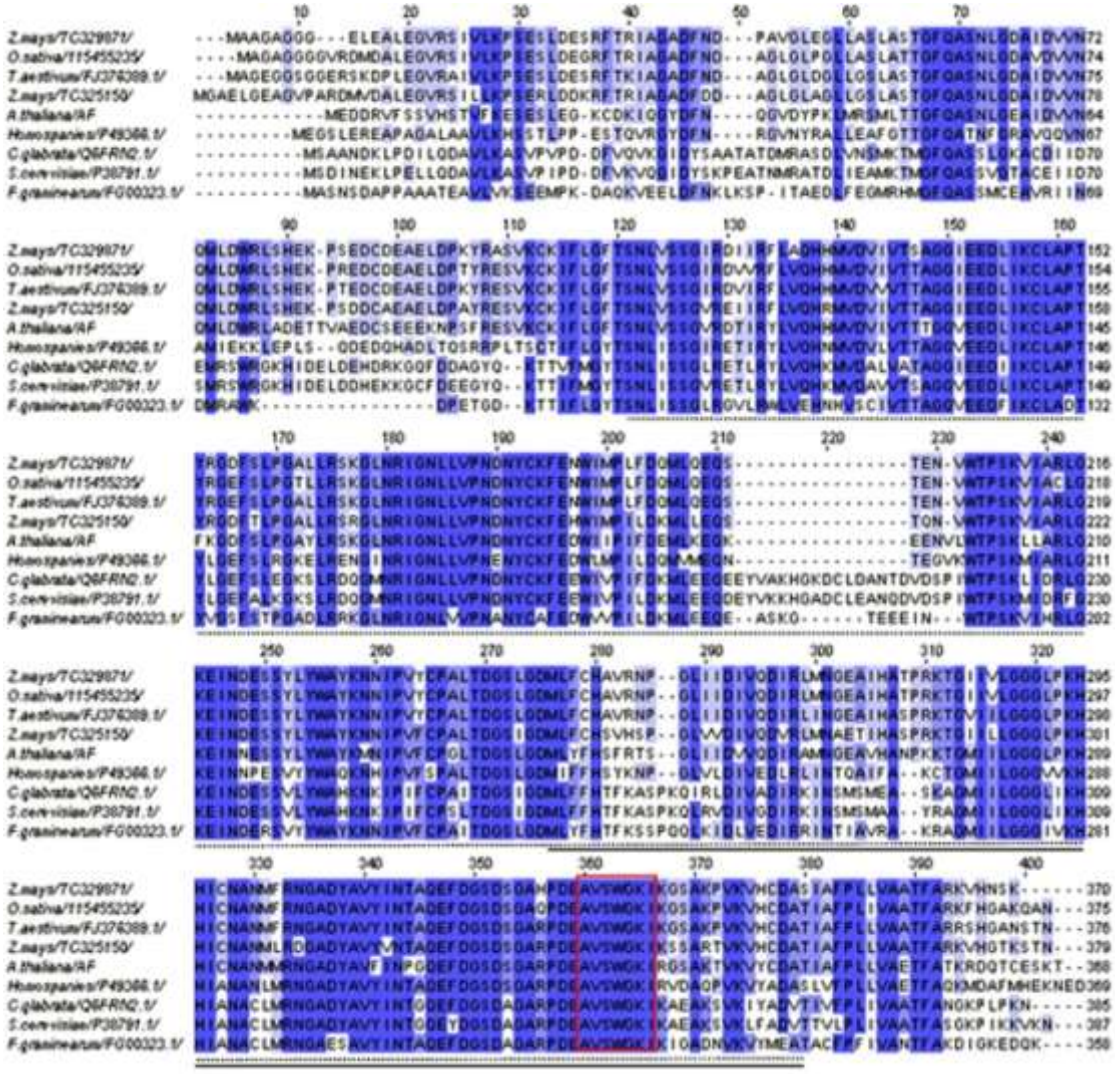

Figure 7. Amino acid alignment among putative DHS proteins. Genebank accession numbers, GU735677 DHS1 protein from maize; GU735678 DHS2 protein from maize; FJ376389.1 wheat DHS; AF296078 DHS protein from *Arabidopsis thaliana;* P49366.1 human DHS; Q6FRN2.1 *Candida glabrata*; P38791.1 *Saccharomyces cerevisiae*; GU809212 *F.graminearum.* Underlined amino acids refer to the spermidine binding site (276–379). Underdoted refer to the NAD binding site from serine 121 to aspartic acid 379. The active center of the DHS protein from glutamine 360 to lysine 366 is in red box.

There are several gaps in the amino acid stretches at positions 212, 286 in the DHS protein of wheat, maize, rice, *Arabidopsis* and human which are absent in *Fusarium* and yeast homologues. The most significant differences among DHS homologues are evident in the spermidine binding site (residues 276–379, upper numbering), although the active site is highly conserved. The active site includes a conserved region of six amino acids from Glu 360 to Lys 366 (Figure 7) (Yan *et al*, 1996; Joe *et al*, 1997). Asp 352, one of the charged amino acids at the bottom of the active site tunnel responsible for binding to spermidine, CNI-1493 and the GC7 inhibitor, is highly conserved (Umland *et al*, 2004). Moreover, in all DHS proteins, Gly 350 is within hydrogen-binding distance of the guanidinium group (Njuguna *et al*, 2006). The NAD-binding site (positions 121-379, upper numbering) is strongly conserved in all species.

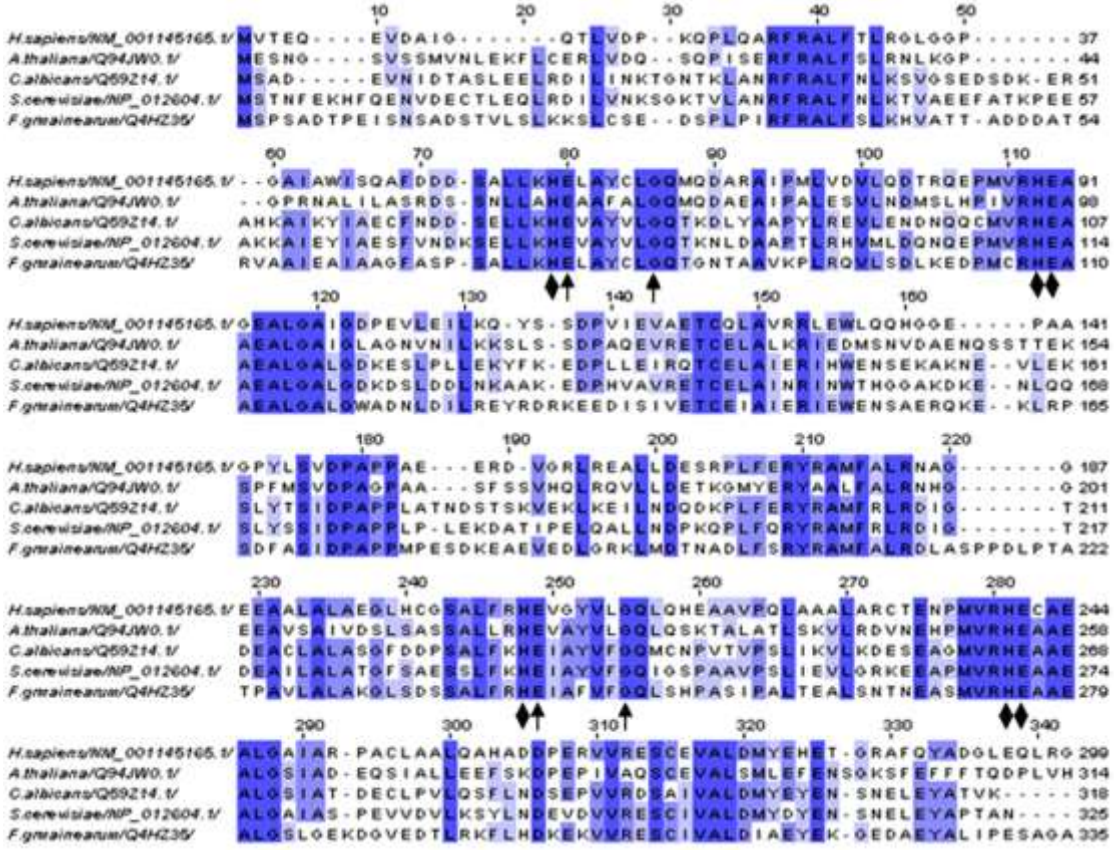

Figure 8. Amino acid sequence conservation of DOHH in eukaryotes. The diagram is based on eukaryotic DOHH sequences chosen from a range of eukaryotic species. Genbank accession No.; NM_001145165.1 human; Q94JW0.1 *Arabidopsis thaliana, Q59Z14.1 Candida albicans;* NP_012604.1 *Saccharomyces cerevisiae*; GU735676 *Fusarium graminearum*. Critical amino acids for binding of substrates are shown by symbols under the residues. The conserved HE (HisGlu) pairs are indicated.

Alignment of DOHH sequences showed that DOHH is also highly conserved in all species (Figure 8) with 44% amino sequence identity between the *Fusarium* and the human enzymes,

and 48% identity between the *Arabidopsis* and the human enzymes. A single DOHH homolog gene exists in most eukaryotes (Kim *et al*, 2006b). It is a super helical protein with eight tandem HEAT-repeats (alpha helical hairpin structure composed of ~50 amino acids) numbered 1–8 (Park *et al.*, 2006). DOHH is a metalloenzyme with two iron atoms coordinated by four strictly conserved His-Glu motifs (per enzyme monomer) (Wolff *et al*, 2007).

In Figure 8, the amino acid residues of DOHH identified to be critical for binding of iron (His79, His112, Glu113, His248, His281 and Glu282 indicated by "♦") (Kim *et al*, 2006b) and for eIF5A intermediate containing deoxyhypusine binding (Glu80, Glu249, and Gly86 and Gly255 indicated by "↑") (Kang *et al*, 2007), are strictly conserved indicating their importance in DOHH catalysis. A number of other strictly conserved amino acid residues are also noted, but how these other amino acid residues may contribute to DOHH structure or function, has not yet been determined (Kang *et al*, 2007).

3.5 DHS is induced under salt and drought stress in wheat and maize

In order to assess the effects of DHS expression on premature senescence induced by environmental stress, the temporal patterns of leaf senescence in response to drought and salinity were compared for control and stressed wheat and maize plants. Drought stress was imposed by treating the plants with 5% PEG, a treatment known to induce osmotic stress (Jacomini *et al*, 1988; Verslues *et al*, 1998; Patadea *et al*, 2009), while salt stress was imposed by treating the plants with 2% NaCl. In these experiments, 2-week old wheat and maize plants were treated every second day with 2% NaCl and 5% PEG separately for 15 days. Within 10 days of initiation of treatment, ca. 80% of the treated plants were visually dead (Figure 9A); that is, the aerial parts of the plants had turned completely brownish -yellow.

In another series of experiments, wheat and maize plants were exposed to salt and drought stress by treatment with 2% NaCl and 5% PEG beginning at 2-month and 3-month of age, when they are less sensitive to water deprivation. Within 15 days of initiation of treatment, the leaves of the treated plants were showing symptoms of stress, specifically the formation of leaf rolling, bleaching, and leaf firing (Figure.9A).

In order to obtain a more quantitative assessment of the effect of DHS expression on the response of the plants to drought, the impact of drought on plants was determined. For this purpose, the water given to the plants was controlled. 25 ml of water is given once per week for a period of 15 days beginning on day 15, 60 and 90 after planting. The transcripts of DHS were

great for treated plants, whether or not water was controlled. That is, when plants were watered normally, DHS for untreated plants was not induced (Figure.9B).

RT-PCR analyses indicated that expression of the wheat and maize DHS gene is high in osmotically stressed leaves, stems, and roots. The abundance of DHS transcript in preparations of total RNA from seedlings expressed substantially the treatment with PEG or NaCl, but showed no expression over the same period in the RNA preparations from control plants placed in water (Figure.9B). Indeed, no induction in DHS transcript level was apparent for the water control plants, indicating that the regulation of DHS expression was observed for leaves, stems, and roots of PEG and NaCl treatment.

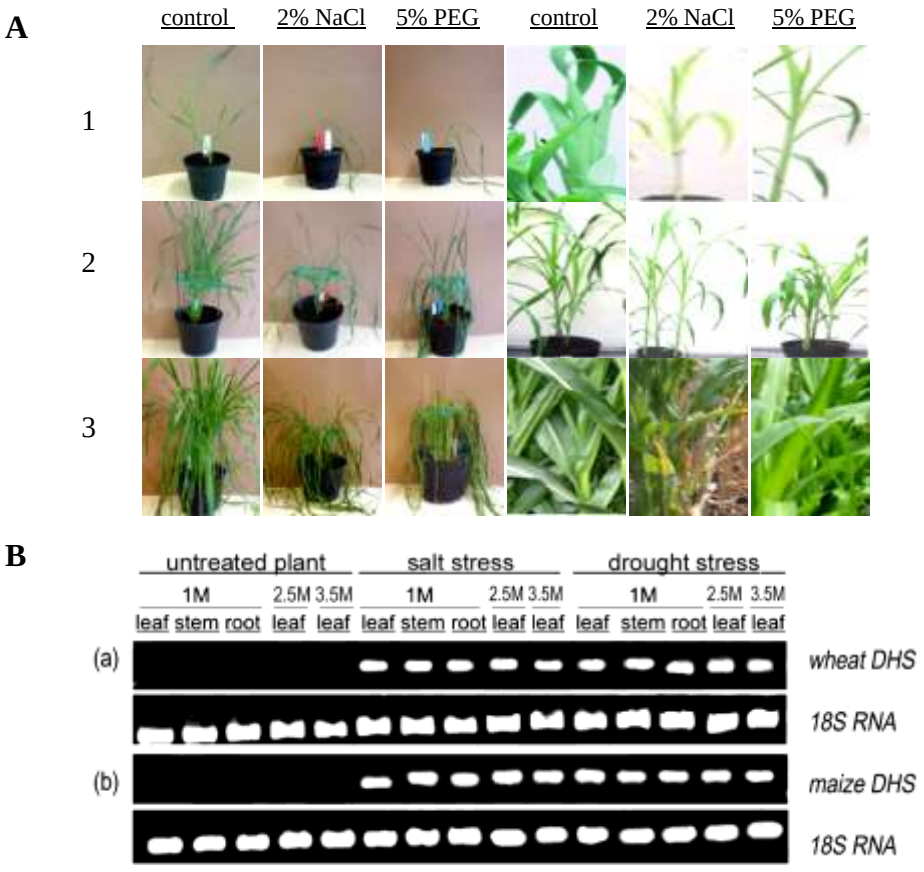

Figure 9. Stress management. A, Salt and drought stress induce leaf senescence. Lane 1, 2, and 3 show photos of leaf rolling, bleaching, and leaf firing of wheat and maize plants treated with 2% NaCl and 5% PEG every second day for a period of 15 days beginning on day 15, 60 and 90 after planting, respectively. **B**, Effect of salt and drought stress on DHS expression. Comparison of DHS transcripts between control and treated plants. 18S RNA was used as internal control of transcripts.

The same patterns of DHS expression in salt and drought-stressed leaves were obtained when RT-PCR of total RNA was analyzed with cDNA corresponding to the 2-month and 3-month old wheat and maize plants treated for 15 days (Figure.9B). These observations suggest that DHS activates an isoform of eIF5A that facilitates the nucleocytoplasmic transport of mRNAs required for natural senescence as well as premature senescence induced by environmental stress.

The upregulation of DHS expression during senescence was observed by (Wang *et al*, 2001; Wang *et al*, 2003) in tomato and Arabidopsis. When osmotic stress ensues in wheat and maize, seeds germinate but the soil dries out, so that subsequent establishment and plant stand are badly affected. This stress leads to reduced leaf > stem > root > grain expansion (in that order). Incomplete ground cover results from reduced leaf area expansion. Leaf senescence is accelerated (from the bottom of the plant first, but in conditions of high potential evapotranspiration it can occur also at the top of the plant as well) (Baenziger *et al*, 2000). That could mean that DHS is involved in regulating natural senescence as well as stress-induced senescence. However, the possibility that DHS is involved in the regulation of other physiological events is not precluded.

3.6 eIF5A, DHS and DOHH are early induced in infected spikes

To determine whether deoxyhypusine synthase sequences from wheat or *Fusarium* are transcribed during the infection of wheat spikes, the analysis of uninfected wheat spikes served as control in these experiments. Wheat spikes were inoculated at the stage of anthesis with conidia from *F. graminearum*. Expression patterns were analyzed using RNA from spikes 1, 3 and 7 days post inoculation and compared to levels of transcript in organs of uninfected plants. As shown in Figure 10A, transcripts of wheat DHS are detectable 3 days post infection and mRNA levels increased strongly at day 7 post infection. *Fusarium* DHS and DOHH gene transcripts were detectable already after 1 day post infection and apparently increased during the course of colonization, while *Fusarium* eIF5A appeared to be constitutively induced. In sharp contrast, the wheat DHS gene was not expressed in uninfected plants during normal spike development (Figure 10A). Taken together, these data demonstrated that the DHS gene is actively transcribed in wheat as well as in *Fusarium* during infection. Although *Fusarium* DHS and DOHH are upregulated during infection, *Fusarium* eIF5A is strongly transcribed at day 1 post infection.

The specificity of the primers was demonstrated in Figure 10B. *Fusarium* primers failed to amplify cDNA generated from dark stressed wheat leaves, and wheat primers also failed to amplify cDNA generated from conidia.

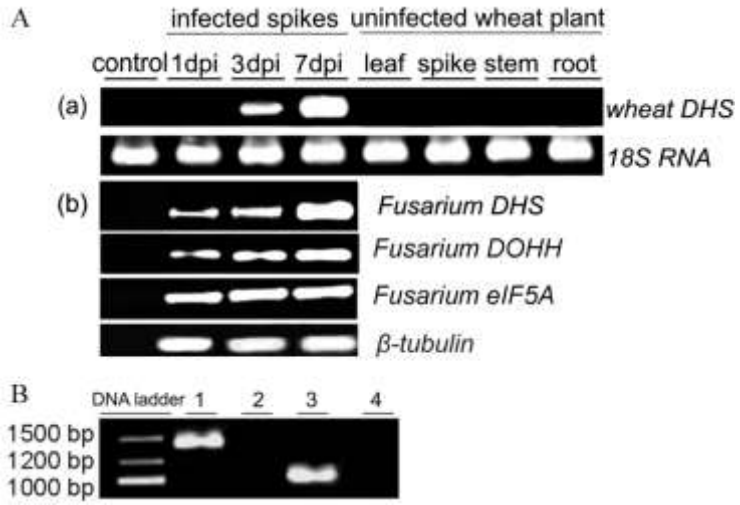

Figure 10. A, **Expression analysis of wheat DHS and *Fusarium* DHS, DOHH and eIF5A during infection of wheat spikes and non infected wheat plants by semiquantitative RT-PCR. (a)**, PCR amplification with wheat DHS specific primers. 18S rRNA wheat specific intron spanning primers were used as expression control. RNA was isolated from wheat spikes infected with *F. graminearum* and non infected spikes, as well as from non infected wheat plant organs such as leaves, stems, roots and spikes. **(b)**, PCR amplification of infected and non infected wheat spikes with *Fusarium* DHS, DOHH and eIF5A specific intron spanning primers and fungal β-tubulin intron spanning primers as expression control. Semiquantitative RT-PCR was conducted with 28 cycles for all samples. **B**, Specificity control of DHS primers in wheat and *F. graminearum.* Lane 1 and 2; cDNA generated from dark stressed wheat leaves was used as template to amplify DHS wheat with wheat DHS primers (lane 1) and with *Fusarium* DHS primers (lane 2). In lane 3 and 4 cDNA generated from germinating conidia was used as template to amplify *Fusarium* DHS with *Fusarium* DHS primers (lane 3) and with wheat DHS primers (lane 4). Marker; DNA ladder mix (Fermentas-Germany).

To determine the early induction of *Fusarium* eIF5A, DHS and DOHH compared to other *Fusarium* virulent factors during infection, expression patterns were analyzed using RNA from spikes 1 days post inoculation and compared to levels of Tri4, Tri5, Tri6, Tri10 genes, which regulate the mycotoxin trichothecene and also compared to Fgl1, lipase gene, which is nesssary for degredation of cell walls of host plants during infection. As shown in Figure 11, transcripts of *Fusarium* eIF5A, DHS and DOHH are strongly induced compared to other genes and mRNA levels of eIF5A are the highest. In contrast, *Fusarium* Tri4, Tri5, Tri6, Tri10 and Fgl1 are weakly induced. This strong early induction shows that *Fusarium* eIF5A, DHS and DOHH are essential virulence factors and required for infection of cereals.

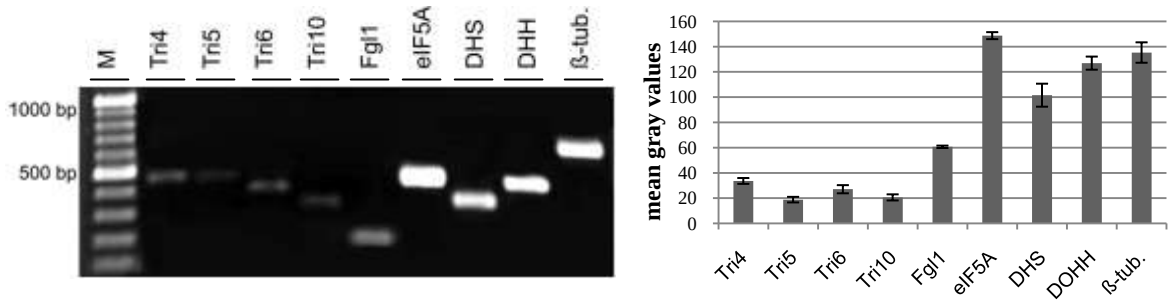

Figure 11. Expression analysis of *Fusarium* eIF5A, DHS and DOHH compared with expression of *Fusarium* Tri4, Tri5, Tri6, Tri10 and Fgl1 in 1dpi-wheat spikes by semiquantitative RT-PCR. Fungal β-tubulin was used as expression control. PCR was conducted here with 30 cycles for all samples. M; DNA ladder mix (Fermentas-Germany). Expression was measured and analysed at several PCR cycles by ImageJ 1.43q/ Java 1.6.0-20- USA.

3.7 Expression of eIF5A, DHS and DOHH in germinating conidia

Induction of *Fusarium* eIF5A, DHS and DOHH genes was analysed *in vitro* by isolating RNA from germinating conidia. Interestingly, DHS and DOHH gene transcriptions were detectable in germinating conidia 4 hours after the conidia were plated on complete medium-CM (Figure 12). Furthermore, after 2, 3, 4, 6 and 8 days *Fusarium* DOHH appeared to be strongly induced, while the DHS transcripts was strongly induced after 2, 3 and 4 days and reduced remarkably in older mycelium after 6 and 8 days. Differently, on poor medium like SNA the transcript of DHS was highly induced after 6 and 8 days similar to DOHH transcript (Figure 12). The transcription of *Fusarium* eIF5A was constitutively high induced in germinating conidia, either on rich medium or poor medium at all time points (Figure 12), suggesting a requirement of eIF5A, DHS and DOHH in germination, fungal development and fungal growth under standard and stress conditions.

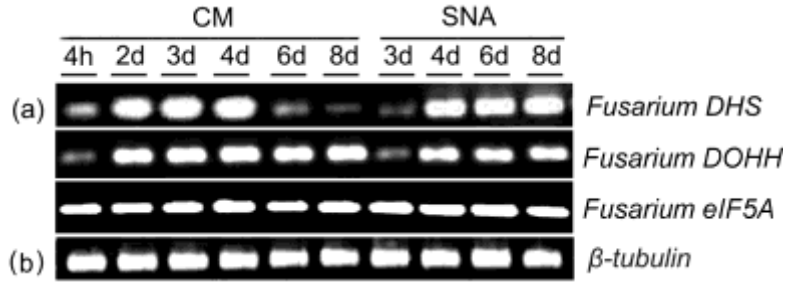

Figure 12. Expression analysis of *Fusarium* DHS, DOHH and eIF5A genes in germinating conidia by semiquantitative RT-PCR. (a), RT-PCR amplification with *Fusarium* DHS, DOHH and eIF5A specific intron spanning primers 4 hours, 2, 3, 4, 6 and 8 days after fungal conidia were plated on complete medium, and SNA medium after 3, 4, 6 and 8 days. **(b)**, RT-PCR amplification with *Fusarium* β-tubulin specific intron spanning primers as expression control 4 hours, 2, 3, 4, 6 and 8 days after fungal conidia were plated on complete medium and SNA medium after 3, 4, 6 and 8 days. PCR was conducted with several PCR cycles. Here is shown with 28 cycles for all samples before reaching the plateau.

3.8 Disruption of DHS and DOHH in *F. graminearum* is lethal

The essentiality of the DHS gene for *Fusarium* growth was examined by gene disruption and inhibition studies with the DHS inhibitor guanylhydrazone CNI-1493 (Mackintosh and Walters, 2003; Hauber *et al*, 2005; Specht *et al*, 2008). The genotype of disrupted-DHS *Fusarium* was not viable and showed a lethal phenotype (Schäfer, unpublished data), while inhibition studies showed that *in-vitro* guanylhydrazone CNI-1493 inhibited DHS enzyme activities in the fungus *F. grminearum* and stopped conidia germination completely at 10µM (Woriedh *et al*, in press), submitted article as part of this work.

To determine whether the deoxyhypusine hydrolase gene is also essential for cell viability in *Fusarium*, a functional analysis of DOHH gene disruption construct was prepared by inserting a hygromycin selection marker cassette into the DOHH genomic DNA. 100% of the ORF was deleted and replaced with the hygromycin cassette, as shown in Figure 13. Expression of *hph* is controlled by the *Aspergillus nidulans* glyceraldehyde- 3-phosphate dehydrogenase gene (*gpd*) promoter and the tryptophan C terminator (*trpC*), thereby allowing direct selection of *Fusarium* cells harboring the correctly assembled construct on medium containing the antibiotic hygromycin B.

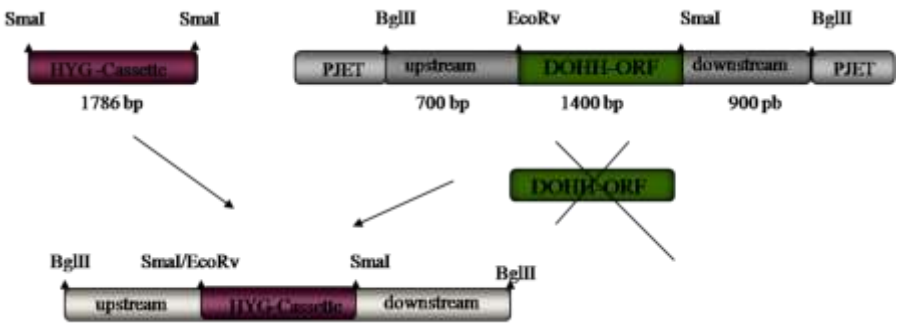

Figure 13. Strategy for creating DOHH deletion construct. 5` and 3` flank fragment containg DOHH open reading frame was amplified from genomic DNA and cloned into pJET vector (Fermentas-Germany). DOHH-ORF was cleaved with blunt enzymes; EcoRv and SmaI, and hygromycin cassette was replaced by incorporated SmaI sites. Homologous recombination created a circular construct and the final linear deletion cassette was released with BglII restriction sites.

A ~3-kb fragment with disrupted DOHH construct was used to transform *Fusarium* wild-type strain 8/1 by homologous recombination with double crossover events. Many transfections of *Fusarium* protoplasts were performed with subsequent selection on complete medium containing 100 µg/ml hygromycin B. DNA made from pooled transformants served as

a template for specific amplification of the correct deletion cassette, but all observed transformants were ectopic insertions (data not shown). Studies of null mutations can identify many roles of essential genes, but some roles of essential genes can be masked by early developmental arrest (Rogalski *et al*, 1982; Kemphues, 2005). It was within out of power to obtain loss-of-function mutations of DOHH gene in *Fusarium*, and efforts were under way to do just that. However, even with this ability to systematically knock out gene, many essential functions can remain cryptic due to genetic redundancy or to pleiotropy (Howell and Rose, 1990; Kemphues, 2005). The premise was that when they subjected to conidiation by dissection, only few conidia from each ascus grew to visible colonies, indicating that the genotype is not viable. These conidia did in fact germinate but ceased to grow after day 1 of dissection, and stopped cell division by day 2 in the same behavior as DOHH null mutants in multicellular eukaryotes, *e.g. Caenorhabditis elegans* (Sugimoto, 2004) and *Drosophila melanogaster* (Spradling *et al*, 1999; Patel *et al*, 2009). The arrest in growth of the null mutants after several cell divisions most likely results from a reduction in eIF5A below the minimum level required to support cell growth.

3.9 Characterization of DHS and DOHH overexpression in *F.graminearum*

Based on lethality of DHS and DOHH null mutants in *Fusarium grminearum*, functional analysis of these genes was approached by gene overexpression constructs of DHS and DOHH and double overexpression of DHS and DOHH.

Generations of targeted DHS overexpressing mutants were prepared by inserting the open reading frame (ORF) of DHS from cDNA into the expression pAN glu A vector (Dr.Wasmann, University of Arizona). Expression of DHS is controlled by the constitutive glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter and the tryptophan C terminator (*trpC*) from *Aspergillus nidulans*. This vector also carries the selectable marker hygromycin gene *hph*, confers the antibiotic hygromycin B, flanked by the glucoamylase (*glu*) promoter and the tryptophan C terminator (*trpC*) from *Aspergillus niger* resulting in a plasmid of 9227 bp (Figure 14).

Genertations of targeted DOHH overexpressing mutants were prepared by inserting the open reading frame of DOHH from cDNA into the expression pII99 vector (Schäfer et al, University of Hamburg). Expression of DOHH is controlled by the constitutive glyceraldehyde- 3-phosphate dehydrogenase (*gpd*) promoter and the tryptophan C terminator (*trpC*) from *Cochliobolus hetrostophus*. This vector also carries the selectable marker

neomycin phosphotransferase II gene *nptII*, confers the antibiotic geneticin, flanked by the *Cochliobolus hetrostophus* tryptophan C (*trpC*) promoter and its terminator (*trpC*) resulting in a plasmid of 6971 bp (Figure 14).

Generations of targeted DHS and DOHH overexpressing mutants in two different expression vectors with different selection marker cassettes allowed to generate double overexpressing mutants of DHS and DOHH by transferring the DOHH overexpression construct into DHS overexpressing mutants.

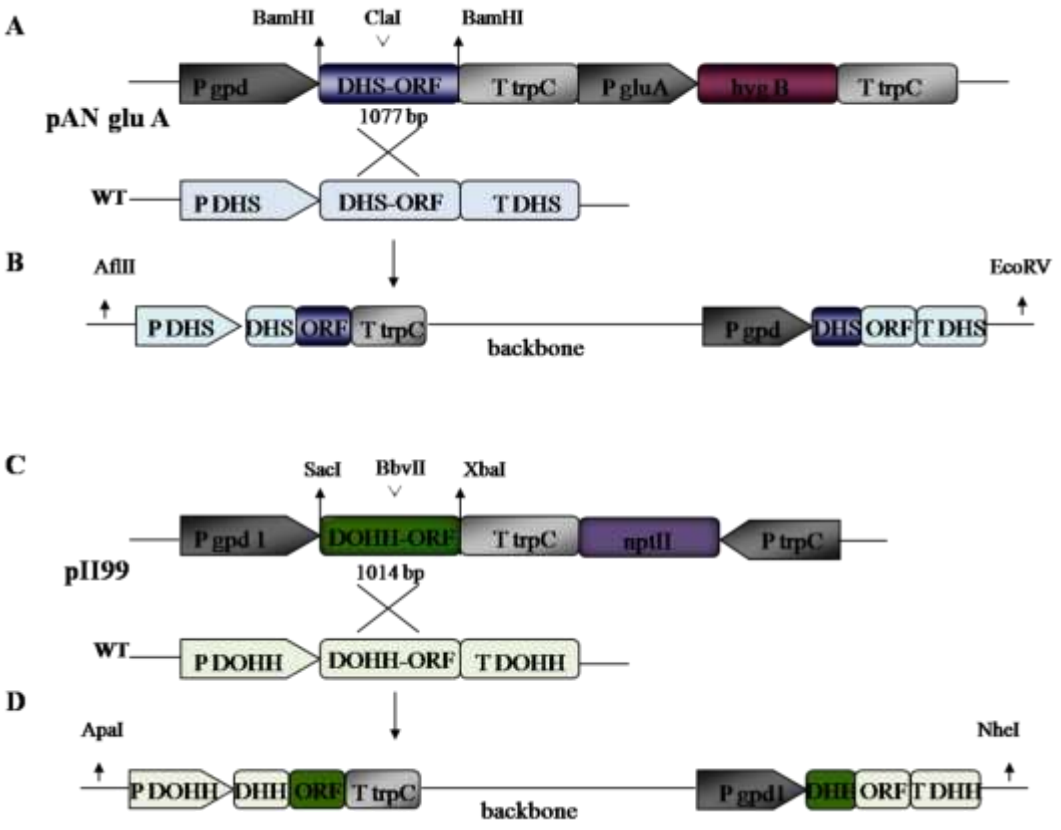

Figure 14. Scheme illustrating the generation of the DHS and DOHH overexpression constructs. A, DHS open reading frame inserted into pAN glu A vector within BamHI site. The lower panel illustrates the genomic DHS locus. **B**, Panel depicts the genomic structure of the DHS locus after integration of the DHS overexpression construct by homologous recombination. **C**, DOHH open reading frame inserted into pII99 vector within SacI and XbaI site. The lower panel illustrates the genomic DOHH locus. **D**, Panel depicts the genomic structure of the DOHH locus after integration of the DOHH overexpression construct by homologous recombination. ClaI and BbvII enzymes linerized the expression vectors for double crossover events. The enzymes that were used for molecular cloning and Southern blot of the transgenes are indicated by arrows. Dark blue, DHS cDNA fragment. Light blue, genomic DHS locus. Dark green, DOHH cDNA fragment. Light green, genomic DOHH locus.

Protoplasts of *F. graminearum* strain 8/1 were cotransformed with the expression plasmids of DHS and DOHH. Plasmids were linearized in the gene sequence to obtain efficient integration of the inactivated transgene into the genome of *F. graminearum* by homologous recombination with double crossover events (Figure 14).

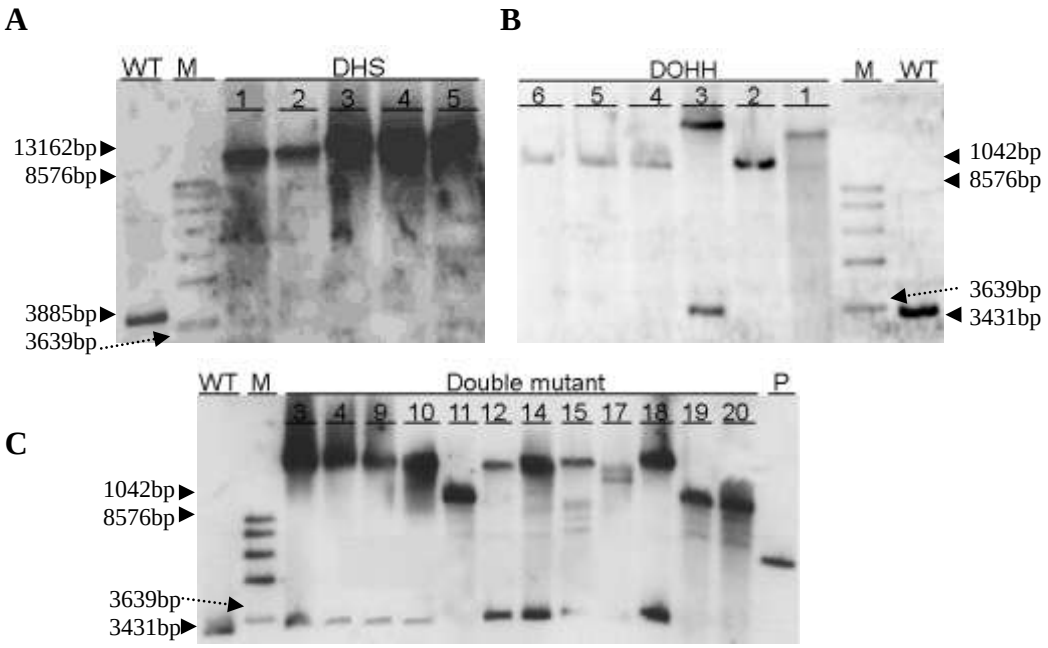

Figure 15. Southern blot analysis of the DHS, DOHH and double overexpressing mutants. A, AflII and EcoRV-digested DNA from wild-type (WT) and DHS overexpressing mutants: 1, 2, 3, 4 and 5. AflII and EcoRV do not cut the expression plasmid pAN glu A- DHS and produce a wild-type fragment of 3885 bp or 13162 bp for right integration. **B** and **C**, ApaI and NheI-digested DNA from wild-type (WT), putative DOHH and double overexpressing transformants. ApaI and NheI do not cut the expression plasmid pII99-DOHH and produce a wild-type fragment of 3431 bp or 1042 bp for right intergration. **B**, 2, 4, 5 and 6 were considered DOHH overexpressing mutants. **C**, 11, 19 and 20 were considered double overexpresing mutants. The digested DNA was separated on agarose gels, blotted, and hybridized to a DHS probe for A and to a DOHH probe for B and C. M; the dig VII DNA ladder (Roche- Germany). P; pII99-DOHH plasmid as control.

The transformants were cultivated on complete medium plates containing 100 µg/ml hygromycin B for DHS overexpression and 100 µg/ml geneticin for DOHH overexpression. PCR was performed on genomic DNA of tranformants with primers spanning the selection marker cassette. Genomic DNA of those transformants, which generated a PCR product, was considered to be putative DHS or DOHH overexpressing mutants. A total of 20 transformants were screened of each expression plasmid and 24.3% failed to generate a PCR product. To exclude additional integration sites or ectopic integrations of the expression construct within the nuclear DNA, the putative mutants were subjected to genomic Southern blot analysis. A DNA fragment comprising the ORF of the overexpressed gene was used as a hybridization

probe. The resulting hybridization pattern demonstrated a single integration event in the right locus of 5 mutants of DHS transformants and 4 mutants of DOHH transformants (Figure 15). To validate the generation of gain-of-function mutants, these mutants were chosen to analyse the DHS and DOHH transcript by semiquantitative RT-PCR.

For semiquantitative RT-PCR studies, primers were used that spanned the constitutively expressed gene. As the DHS and DOHH gene was found to be poorly induced on poor medium like SNA after 3 days and highly induced after 6 and 8 days, wild-type control and the mutants were germinated on SNA for 3 days. PCR reactions using cDNA derived from the 9 mutants gave rise to a strong constitutive amplification product, whereas the control PCR with cDNA derived from *F.graminearum* wild-type produced an amplicon of the expected poorly induction (Figure 16). The house keeping gene ß-tubulin was used as control of the transcript. Several single PCR cycles were conducted before reaching the plateau.

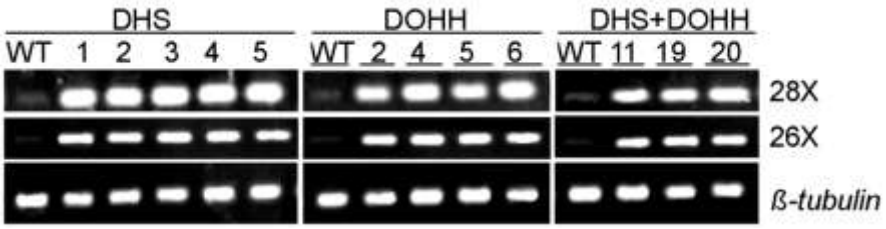

Figure 16. Confirmation by semiquantitative RT-PCR of overexpression of DHS, DOHH and double mutants. cDNA was derived from the mutants and wilde type *Fusarium* from conidia germinationg on SNA medium for 3 days at 28°C. DHS intron spanning primers were used to validate DHS overexpressing mutants, while DOHH intron spanning primers were used to validate DOHH and double overexpressing mutants. Fungal β-tubulin intron spanning primers served as control. Semiquantitative RT-PCR was conducted in this figure with 26 and 28 cycles for all samples. This experiment was repeated three times.

Additionally, protoplasts of DHS overexpressing mutants were cotransformed with the DOHH expression plasmid to generate the double overexpressing mutants. After transformation, the subsequent selection, PCR screening of the selection marker cassette geneticin, and Southernblot analysis of the putative mutants were done as described above for screening of the DOHH overexpressing mutants. 3 double overexpressing mutants resulted from Southern blot analysis (Figure 15), whereas these mutants were successfully validated for gain-of-function by semiquantitive RT-PCR, too (Figure 16). Semiquantitative RT-PCR was conducted on SNA medium in the same conditions, primers, and PCR profile of validation DOHH overexpressing mutants. The three types of resulting mutants were used for further experimental studies.

3.10 Phenotypic analysis of *Fusarium* overexpressing mutants

3.10.1 Conidial comparative structure and Anastomosis

Macroconidia (Figure 17) are the single most important cultural character in the identification of *Fusarium*. In many cases the morphology of this spore alone is sufficient to identify a culture to species (Leslie and Summerell, 2006). Synthetic nutrient poor medium-SNA (Spezieller Nährstoffarmer Agar, (Nirenberg, 1981)) is recommended for identifying macroconidia as macroconidia formed on sugar-rich media such as minimal, complete and Potato Dextrose Agar (PDA), which are often highly variable and quite different from those observed on Carnation Leaf-Piece Agar (CLA) or other nutrient poor media.

According to the *Fusarium* laboratory manual (Leslie and Summerell, 2006), macroconidia of wild-type *F. graminearum* are relatively slender, sickle shaped to almost straight, thick walled, 5- to 6-septate, with a tapered apical cell and a distinctly foot-shaped basal cell, and of medium length. Microscope slide mounts of conidia germinating in SNA liquid medium overnight at 28°C showed that the overexpressing macroconidia are significantly longer in size than the wild-type macroconidia (Figure 17A-D). The DHS overexpressing conidia are long, relatively elongate and squat with 9- to 10-septate per spore, while the DOHH overexpressing conidia are shorter, more slender, straighter and elongate with ~7 septate per spore. The double overexpressing conidia are the straightest elongate, long with 8- to 9- septate per spore.

For further characterization, the slides were also observed 3 days post germination. Normal germination tubes were shown by all types of conidia, while anastamosis tubes, an abnormal phenomenon which occurs approximately 3% among wide type conidia, showed a high average of ~65% among DHS overexpressing conidia, ~50% among double overexpressing conidia, and was absent among DOHH overexpressing conidia (Figure 17E-N). Figure 17E-N illustrate self-fusions. Here fusions occurred between conidia and/or conidial germlings of the same genotype (i.e. the same mutant).

Conidia are asexual, non-motile spores, and form germ tubes (germination tubes) and conidial anastomosis tubes (CATs) in specific conditions. Conidial anastomosis tubes are morphologically and physiologically distinct from germ tubes and are under separate genetic control (Gabriela Roca *et al*, 2005; Friesen *et al*, 2006). A colony can arise from a single spore. Under appropriate conditions, a conidium germinates to form a tip-growing germ tube that extends and successively branches to establish the fungal colony.

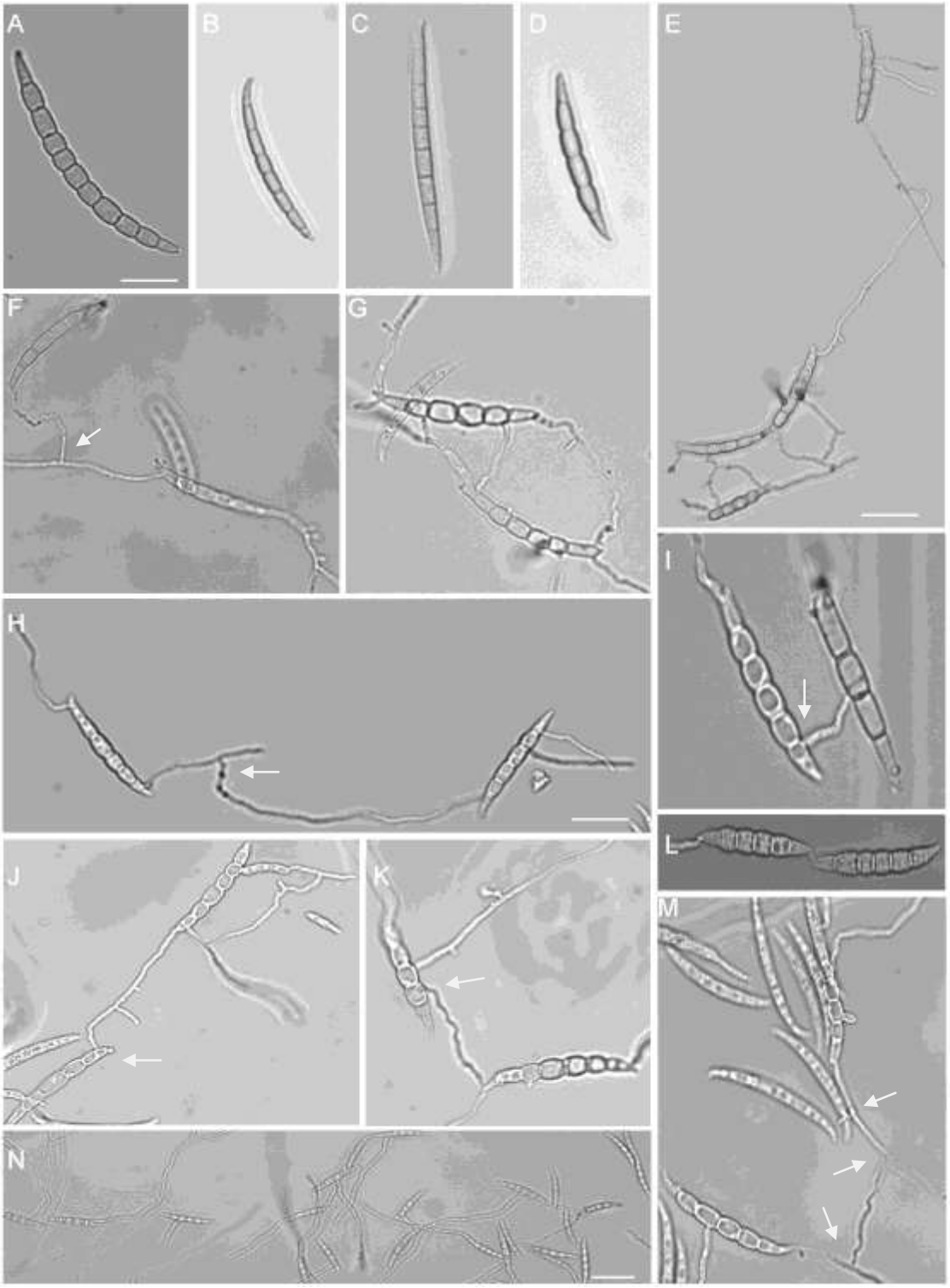

Figure 17. Morphological character and anastomosis events of *Fusarium* overexpressing mutants. **A**, Macroconidium of DHS overexpressing mutant with 9-10 septa. **B**, Macroconidium of DOHH overexpressing mutant with 7 septa**. C**, Macroconidium of double overexpressing mutant with 8-9 septa. **D**, Macroconidium of *F.graminearum* wild-type with 5-6 septa. **E**, **F**, **G**, **H**, **I**, **J**, **K**, **L** and **M**, Anastomosis events present in DHS and double overexpressing mutants and wild-type, while it is absent in DOHH overexpressing mutant (**N**). A-D, I and L scale bar = 20µm; E-M scal bar = 50µm; N scal bar = 75µm. Concentration: 2×10^4 conidia in 60 µl SNA per slide.

However, conidia and conidial germlings in close vicinity to each other commonly undergo fusion by CATs to produce an interconnected network (Figure 17E-M.). CATs are thinner, shorter, unbranched, exhibit determinate growth, and induction is dependent on conidial density. CATs home towards each other, whilst germ tubes avoid each other. After conidia are induced to form conidial anastomosis tubes, they grow toward each other, and eventually fuse. Once fusion happens, the nuclei can pass through fused CATs. These are events of fungal vegetative growth and not sexual reproduction (Gabriela Roca *et al*, 2005; Friesen *et al*, 2006).

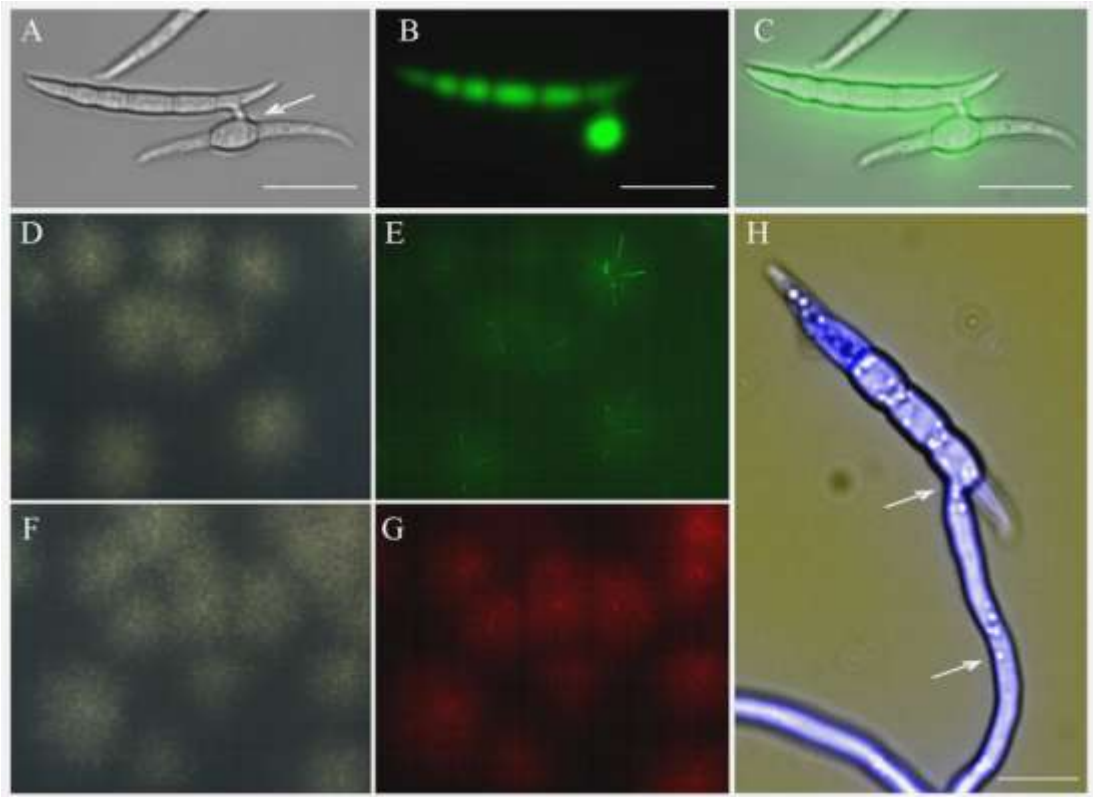

Figure 18. Fusion of conidial anastomosis tubes and nuclei transfer. A, Bright light image of conidial fusion between wild-type GFP stain and DHS overexpressing mutant. **B** and **C**, Fluorescent and overlapped image of wild-type GFP strain macroconodium and fluorescent transferred nuclei into DHS overexpressing macroconidum. A-C scale bar = 20µm. **D** and **E** show fused conidia between wild-type GFP strain and DHS overexpressing mutant growing on complete medium containing 200µg /ml of geneticin and hygromycin. **F** and **G** show fused conidia between wild-type dsRed strain and DHS overexpressing mutant growing on complete medium containing 200µg /ml of geneticin and hygromycin. **H**, Nuclei transfer of a calcofluor white stained DHS overexpressing conidium; scale bar = 10 µm. Arrows show nuclei transfer.

To show that non-self fusions with gene transfer can occur between different strains (and thus different genotypes) in *F. graminearum*, conidia of DHS overexpressing mutant carrying the hygromycin selectable marker were mixed with conidia of wild-type GFP and wild-type dsRed carrying the geneticin selectable marker. The two conidial mixtures were germinated in

a 1.5 ml tube in SNA liquid medium overnight at 28°C for 3 days, and then transferred into CM plates containing 200µg/ml of geneticin and hygromycin B. The surviving growing colonies were checked by a fluorescent microscope.

Figure 18A-C show a conidial anastomosis tube transferring a nucleus from wilde-type GFP conidium into DHS overexpressing conidium, while Figure 18D-G show GFP fluorescence and dsRed fluorescence from the resulting colonies of the fused conidia on CM plates containing the antibiotic geneticin and hygromycin. Furthermore, calcofluor white stain, a nonspecific fluorochrome stain that binds to cellulose and chitin in cell walls of fungi, was used to detect gene transfer in interconnected network of germinating DHS overexpressing conidia (Figure 18H). These results showed that fusion can occur in *F. graminearum* within the same genotype and between genotypes. Fusion between these cells seems to be important during early stages of colony establishment. CATs may improve the chances of colony establishment by allowing heterogeneously distributed nutrients or water within the environment to be shared between different germlings, and may provide a mechanism for gene transfer in the absence of sexual reproduction under separate genetic control. CATs are not present in DOHH overexpressing mutants and occur more frequently in DHS and double overexpressing mutants than wild-type strain. The biology of conidial anastomosis tubes is not completely understood and more experiments must be run in this direction.

3.10.2 Growth behavior under different conditions: Nutrition, Temperature, Salt stress and Fungicide

A number of secondary characters were also used in the identification of *Fusarium* overexpressing mutants: DHS, DOHH and double overexpressing mutants and wild-type as control. The most prominent of these secondary characters is pigmentation. Culture conditions and media are critical for the production of comparable pigments. The plant pathogenic fungus *Fusarium graminearum* produces large mycotoxic quantities of the red pigment aurofusarin and the orange-red pigment rubrofusarin. These dimeric polyketides are produced under both natural growth conditions and in artificial environments (on media). Vegetative growth rate is another commonly used secondary character. There can be some variation in this trait. Measurements were made on culture plates inoculated with a drop of 2000 conidia in 10 µl in the center of the plates and grown for 5 days at different temperatures. The colony diameter was measured, and several replicate plates were made to insure an accurate measurement at a constant temperature in complete darkness. The data were based on radial expansion of the

colony, while measurements were taken after 1, 2, 3, 4 and 5 days of growth. Two measurements were taken at 90° angles (perpendicular) per plate.

Phenotyping medium was developed to distinguish the mutations that affect different portions of the metabolism pathway. At least some of this variation is in response to environmental differences, including differences in media, temperature, salt and fungicide. For accurate use of the descriptions in this study, morphological identifications were based on characters observed as follows:

Characteristics on rich medium-CM. Plates were incubated at different temperatures of 4°C, 8°C, 25°C, 28°C, 30°C, 32°C and 35°C. WT, DHS and double overexpressing strains were sensitive to low temperature. They were not able to produce colonies at 4°C and 8°C. Conidia of all strains germinated a little and stopped growing. Microscope Images showed that mycelial density produced by DHS conidia was the highest, then WT, then double overexpressing mutant, and then DOHH mutant (Figure 19).

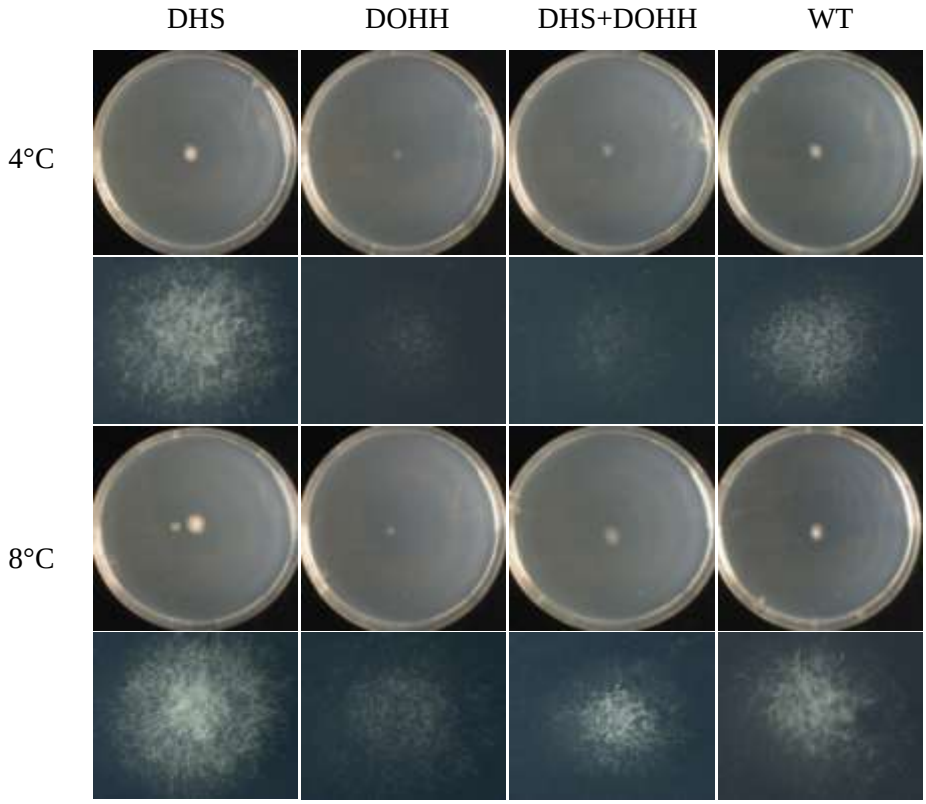

Figure 19. Growth behavior of DHS, DOHH and double overexpressing mutants cultivated on CM at low temperatures. 92 mm Petri plates were inoculated with a drop of 2000 conidia in 10µl for 7 days at 4°C and 8°C. *F.graminearum* wild-type stain 8/1 was served as control. Experiments were repeated three times with three repetition of each with similar results. Pictures were taken by a digital camera (Canon PowerShot S40).

At 25°C and 28°C, colonies of WT, DHS and double overexpressing strains grew rapidly and produced relatively large amounts of dense mycelia that vary from white to pale orange to yellow in color. Red to orange colour was produced slowly in the center (> 4 days). Cultures formed light red pigments in the agar (Figure 20A). The three strains were approximately similar in their morphology. Growth rate of DHS mutants were a little higher and growth rate of DOHH mutants at 28°C were relatively low (Figure 20B-C). DOHH overexpressing mutant was surprisely different in its morphology and growth rate. DOHH overexpressing colonies grow in a sunflower shape in which they produced relatively large amounts of orange dense aerial mycelial tips and a flat center that lacked aerial mycelium that varies from pale red, red brown to yellow at 25°C, and vary from pale red, red brown, green to yellow at 28°C. Cultures formed abundant dark red pigments in the agar (Figure 20A). The growth rate of DOHH strain was visibly lower than other strains and the colony diameter was one third lower of their colony diameters at both degrees. Also, DOHH strain showed temperature sensitive. It grew slower at 25°C than at 28°C (Figure 20).

At higher temperature all strains showed a temperature sensitivity and grew slower. The growth rate at 30°C was shrunk to half. DOHH strain had also lower growth rate than other strains. WT, DHS and double overexpressing strains formed less red pigments in the agar, while the mycelia had a white tip and a light red center. DOHH strain formed orange pigments in the agar and the mycelia had a pale orange tip and light green in the middle to orange center, while its growth rate was lower than other strains, too.

At 32°C, growth rate of all strains was shrunk to a fourth and formed small colonies. Although growth rate of WT, DHS and DOHH strains was similar at 32°C, it was slightly less than of double overexpressing strain (Figure 20). DOHH strain lost in this stage its sunflowery aerial mycelia form. At 35°C, conidia of DOHH strain did not germinate, while conidia of DHS and double overexpressing strain germinated a little and stopped. WT showed its resistance to high temperature. Conidia of WT produced a small colony of white mycelium, but the cultures did not form pigments in the agar and the growth rate was very low (Figure 20).

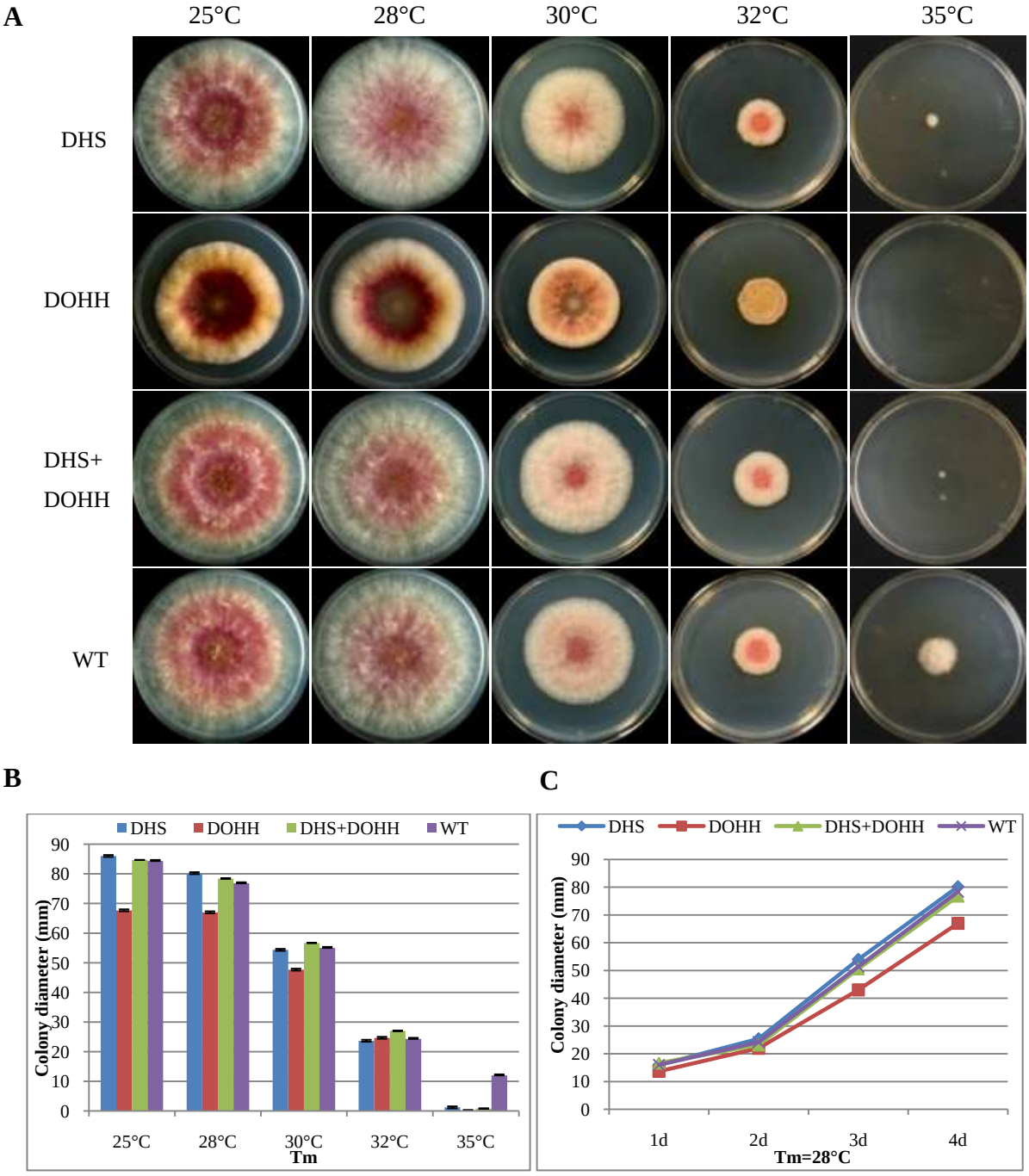

Figure 20. Growth behavior of DHS, DOHH and double overexpressing mutants cultivated on complete medium at different temperatures. A, 92 mm Petri plates were inoculated with a drop of 2000 conidia in 10µl for 5 days at 25°C, 28°C, 30°C, 32°C and 35°C. **B**, Growth rate of the overexpressing mutants on CM at the respective temperatures on day 4. **C**, Growth rate of the overexpressing mutants on CM at 28°C on days 1, 2, 3 and 4. *F.graminearum* wild-type stain 8/1 was served as control. Error bars indicate SE (n=6). Pictures were taken by a digital camera (Canon PowerShot S40).

Characteristics on rich medium-YPG. Plates were also incubated at different temperatures of 25°C, 28°C, 30°C and 32°C. In contrast to CM, all strains on this media showed different behavior. At 25°C, colonies of WT, DHS and double overexpressing strains grew fast and produced relatively large amounts of dense cottony mycelia that vary from white to pale orange in color with yellow rings in the middle (double mutant), subsidence (DHS mutant), or yellow rings with Subsidence (WT). At 28°C, The three strains produced subsidence in the middle, while it was accompanied with clear yellow rings in DHS mutants. All cultures formed yellow to orange pigments in the agar (Figure 21A). Although the three strains were approximately similar in their growth rate at 25°C, a little increase was observed at 28°C, but growth rate of WT was relatively low (Figure 21B). DOHH overexpressing mutant was also different in its morphology and growth rate. DOHH overexpressing colonies grew flat and lacked aerial mycelium, which varies from pale orange tips to orange center at 25°C, and to light orange centre at 28°C. Cultures formed abundant dark orange pigments in the agar with yellow rings of pigmentation (Figure 21). In a similar behavior to CM, the growth rate of DOHH strain was visibly lower than other strains and the colony diameter was one third lower. Also, DOHH strain showed a temperature sensitivity. It grew slower at 25°C than at 28°C (Figure 21).

Similar to CM at higher temperature, all strains showed temperature sensitive and grew slower. The growth rate at 30°C was shrunk to half. DOHH strain had also lower growth rate than other strains. WT, DHS and double overexpressing strains formed less orange pigments in the agar, while the mycelia had whitish aerial tips and orange centers. DOHH strain formed orange flat mycelia with yellow tips, while its growth rate was lower than other strains, too.

At 32°C, growth rated of all strains was shrunk to a fourth and formed small colonies. Similar to CM, growth rate of WT and DHS strains was also the same at 32°C, but was less than DOHH and double overexpressing strains which each showed similar growth rate, too (Figure 21). DHS differed morphologically at this degree from other strains. It formed pale orange aerial mycelia with bright orange ring, while WT and double overexpressing strains formed orange flat mycelia like DOHH strain (Figure 21).

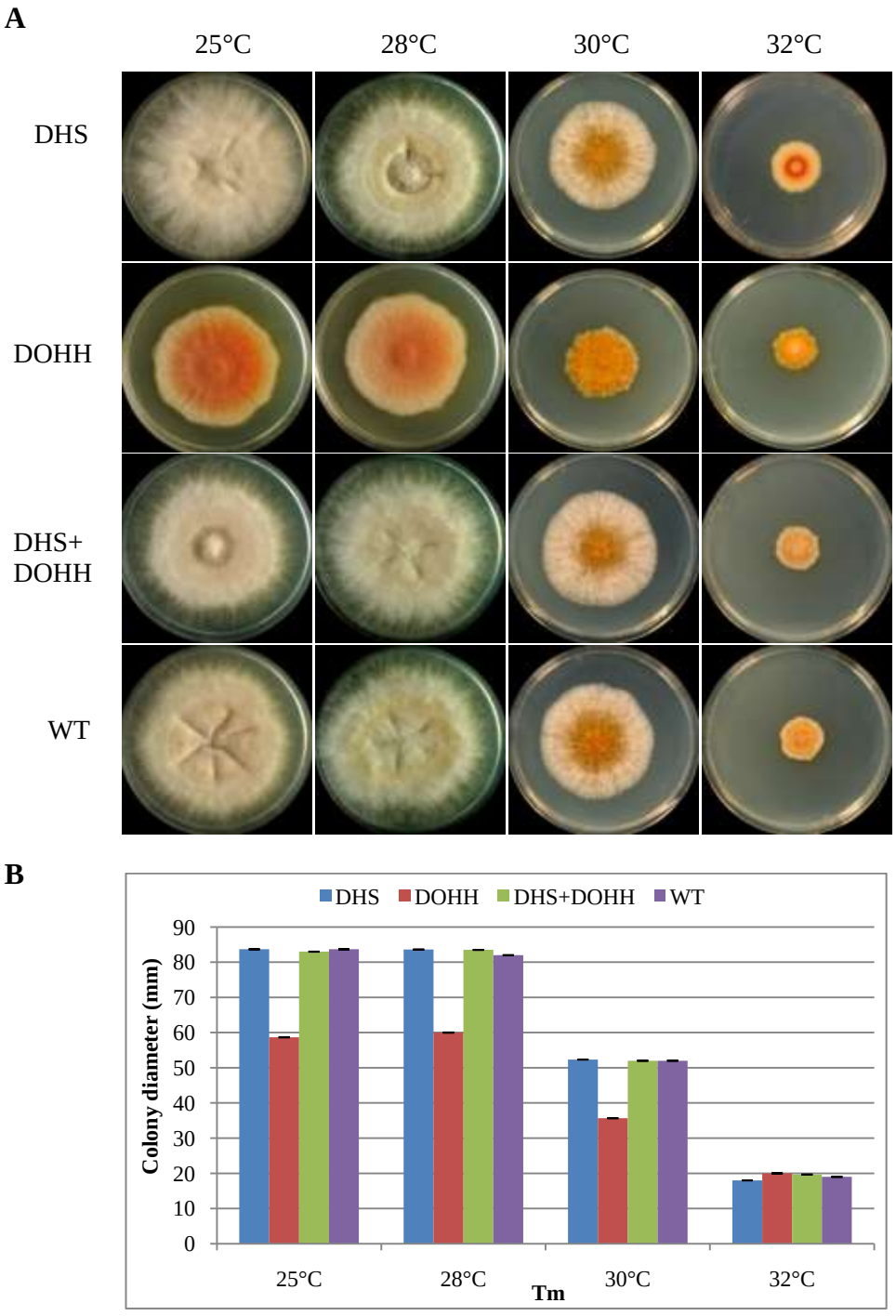

Figure 21. Growth behavior of DHS, DOHH and double overexpressing mutants cultivated on YPG at different temperatures. A, 92 mm Petri plates were inoculated with a drop of 2000 conidia in 10µl for 5 days at 25°C, 28°C, 30°C and 32°C. **B**, Growth rate of the overexpressing mutants on YPG at the respective temperatures on day 4. *F.graminearum* wild-type stain 8/1 was served as control. Error bars indicate SE (n=6). Pictures were taken by a digital camera (Canon PowerShot S40).

Characteristics on poor medium-SNA. For further morphological analysis, 28°C was used as a standard temperature for incubations. Strains were grown on poor medium SNA and SNA supplement with 1%NaCl. All four strains grew slowly on SNA medium compared to CM and YPG medium (Figure 22). On SNA+1%NaCl, the growth rate was doubly increased and overgrown compared to CM and YPG medium. Growth rate of DOHH strain was visible less than other strains on SNA medium, but it increased to have similar patterns on SNA+1%NaCl. As well, DHS strain was superlatively higher expanded with more dense mycelia. On both media, all four strains had whitish mycelia and did not produce pigments, except DOHH strain on SNA. The type resulted in a flat slimy colony that lacked aerial mycelium, but produced abundant red pigmentation (Figure 22).

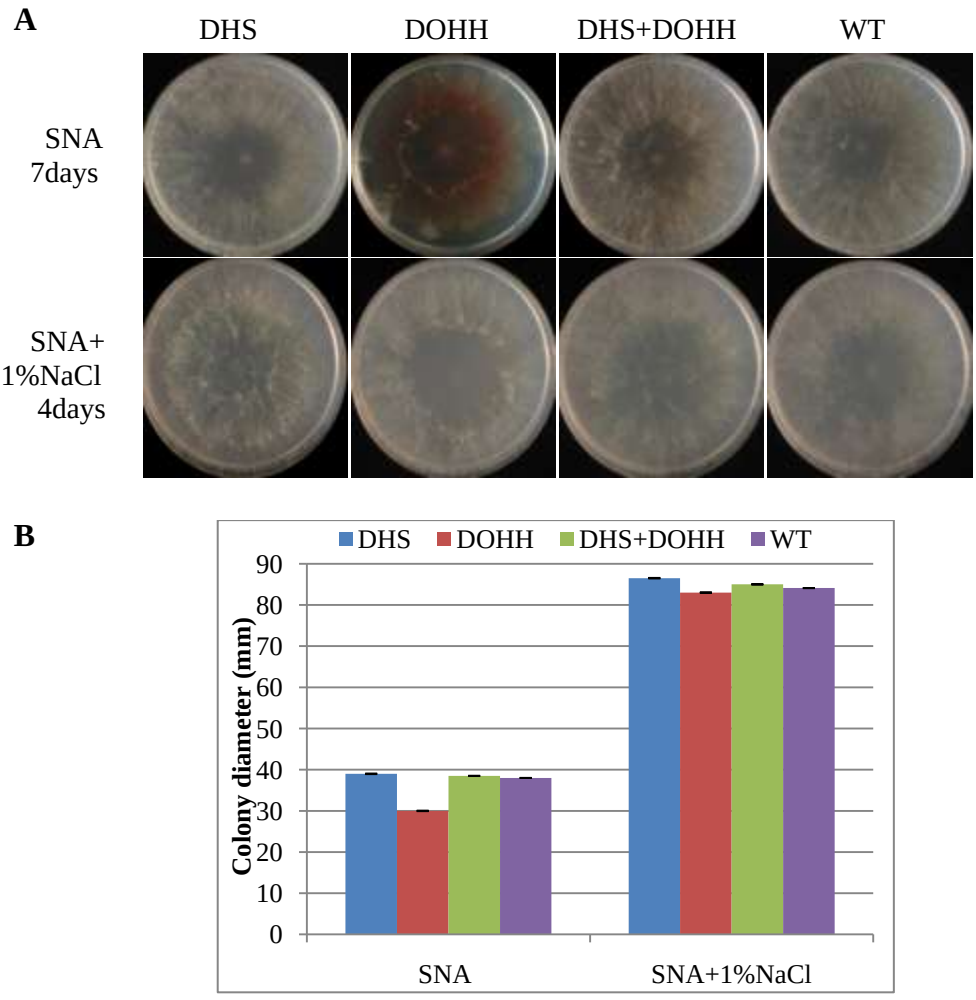

Figure 22. Growth behavior of DHS, DOHH and double overexpressing mutants cultivated on poor medium (SNA) and SNA+1%NaCl. A, 92 mm Petri plates were inoculated with a drop of 2000 conidia in 10µl at 28°C on SNA for 7 days comparing to SNA+1%NaCl medium for 4 days. **B**, Growth rate of the overexpressing mutants on SNA and SNA+1% at 28°C on day 4. *F.graminearum* wild-type stain 8/1 was served as control. Error bars indicate SE (n=6). Pictures were taken by a digital camera (Canon PowerShot S40).

Characteristics to salt stress. To study the strains morphologically under osmotic stress and accumulations of salt, strains were grown on complete medium supplement with 0.5M, 1M, 1.5M and 2M NaCl. DOHH strain was more pigmented than other strains. The strain began to produce red pigments 2 days after inoculation on 0.5M NaCl medium and yellow pigments on 1.5M NaCl medium, while WT, DHS and double overexpressing strains did not begin to produce light red pigments until day 4 (Figure 23). Mycelia growth of all of four strains was severely retarded relative to higher NaCl levels. Similar patterns were observed on 0.5M NaCl medium when all four strains were overgrown than on 0M NaCl medium, but they lacked the aerial mycelia and grew flatly.

At higher concentrations 1M NaCl, the reduction in radial growth of these strains was not as severe as was the reduction in the strains at 1.5M NaCl (Figure 23). The four strains produced 50% less fungal mass and DOHH strain showed susceptibility two times more compared with wild-type strain results at 1M NaCl. In contrast, WT showed susceptibility two times more compared with DOHH strain results at 1.5M NaCl. At 1M and 1.5M, DHS and double overexpressing strains showed to be adapted to growth with high salt concentrations. DOHH strain resulted in a flat slimy colony that lacks aerial mycelium, while WT, DHS and double overexpressing strain resulted in a sterile white mycelium (Figure 23).

At 2M NaCl, Conidia of WT did not germinate at all. Conidia of DOHH strain germinated a little bit then stopped growing. Double overexpressing strain produced a small whitish colony. Surprisely, DHS strain was able to grow on 2M NaCl medium and produced a colony similar to the produced colony at 1.5M NaCl but less mycelial density (Figure 23).

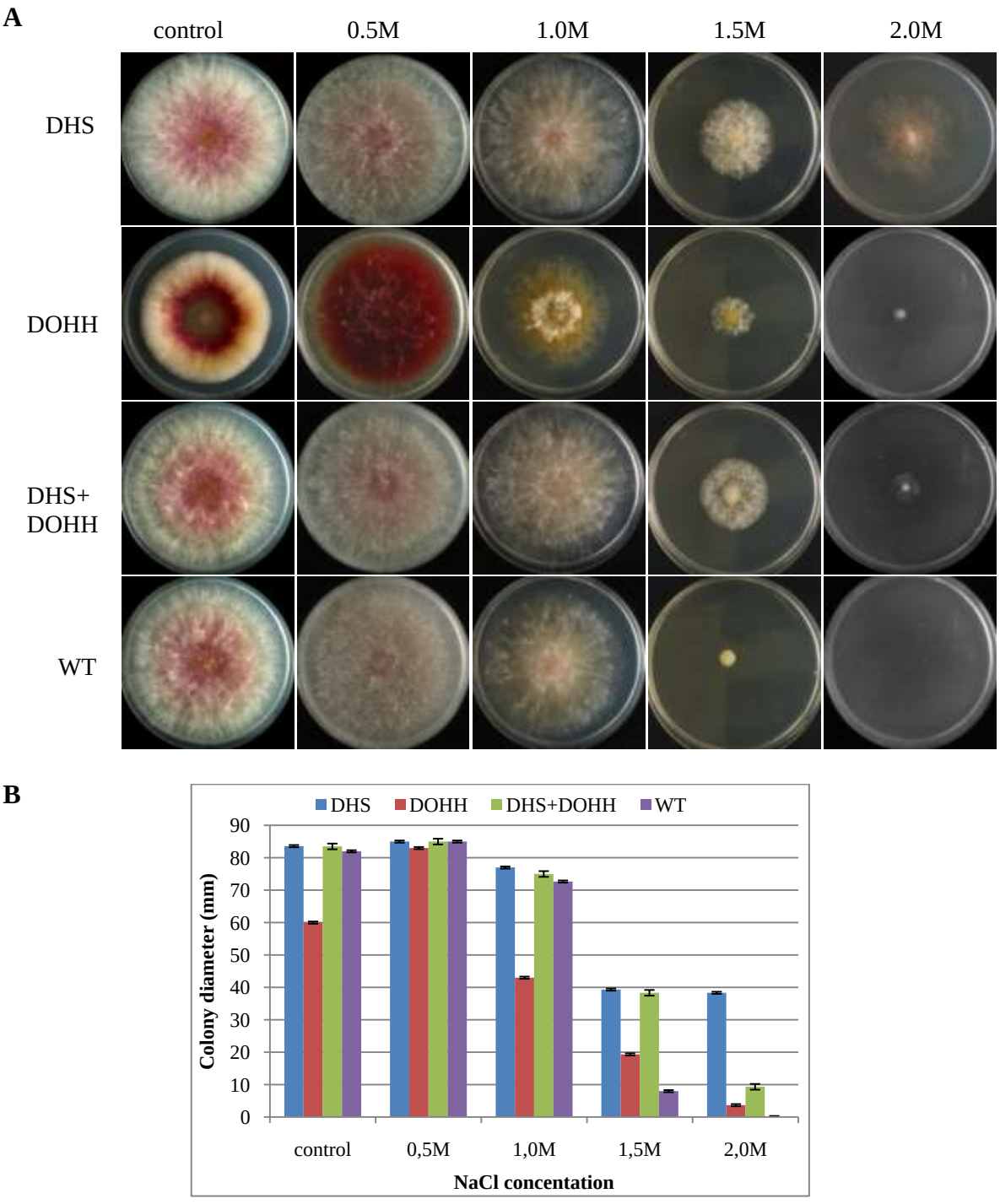

Figure 23. Growth behavior of DHS, DOHH and double overexpressing mutants cultivated on CM under salt stress. A, 92 mm Petri plates were inoculated with a drop of 2000 conidia in 10µl for 5 days at 28°C with different concentration of NaCl: 0M, 0,5M, 1M, 1,5M and 2M. **B**, Growth rate of the overexpressing mutants on CM at the respective salt concentrations on day 4. *F.graminearum* wild-type stain 8/1 was served as control. Error bars indicate SE (n=6). Pictures were taken by a digital camera (Canon PowerShot S40).

Characteristics to the fungicide Azoxystrobin.

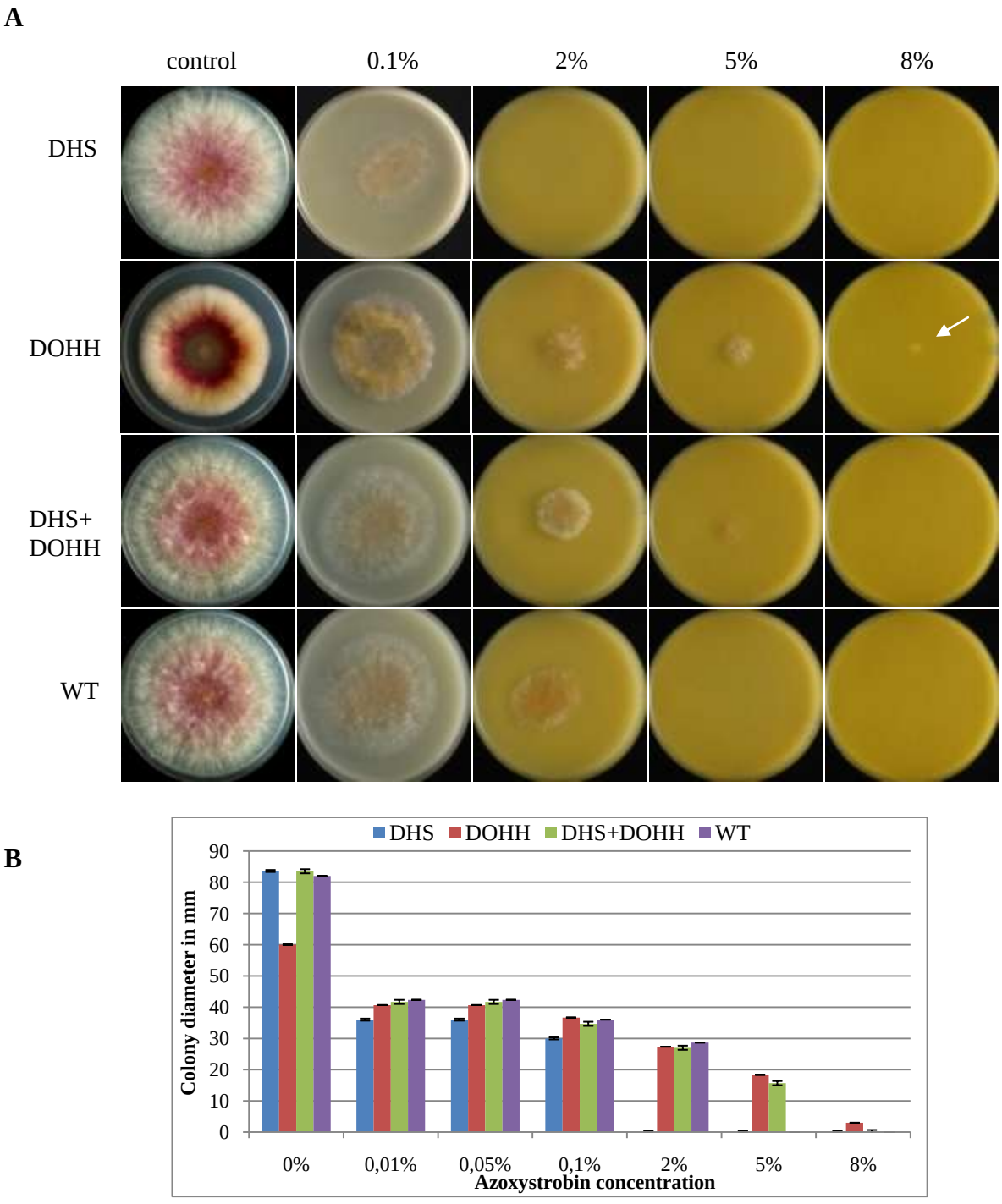

Figure 24. Growth behavior of DHS, DOHH and double overexpressing mutants cultivated on CM with the fungicide Azoxystrobin. A, 92 mm Petri plates were inoculated with a drop of 2000 conidia in 10µl for 5 days at 28°C with different concentration of Azoxystrobin: 0%, 0,1%, 2%, 5% and 8%. **B**, Growth rate of the overexpressing mutants on CM at the respective concentrations: 0%, 0,01%, 0,05%, 2% and 5% on day 4. *F.graminearum* wild-type stain 8/1 was served as control. Error bars indicate SE (n=6). Pictures were taken by a digital camera (Canon PowerShot S40).

To compare the different genotypes morphologically with fungicides, strains were grown on complete medium supplement with 0.01%, 0.05%, 0.1%, 2%, 5% and 8% Azoxystrobin. At low concentrations 0.01%- 0.1%, all four strains did not produce pigments and resulted in a sterile mycelium colored from white, pale orange to orange. Mycelia growth of all four strains was severely retarded relative to Azoxystrobin concentrations. The four strains produced 50% less fungal mass and DHS strain showed susceptibility compared to other strains (Figure 24). At 2% Azoxystrobin, conidia of DHS strain did not germinate. In contrast, DOHH and double overexpressing strains showed a resistance against Azoxystrobin and were able to grow and produce small colonies at 5% Azoxystrobin, while conidia of WT did not germinate at this concentration. At high concentration of Azoxystrobin 8%, double overexpressing strains did not germinate at all. Surprisingly, condidia of DOHH strain were able to germinate a little and stopped growing later at this high concentration (Figure 24).

Taken together, the value of physiological characters placed heavy emphasis on the use of a variety of complex media and the resultant growth patterns and rates. There were some strains that grew considerably slower (or faster) than others, however, and such slow- (fast-) growing strains and the conditions resulting in their slow (fast) growth were noted in the individual strains descriptions. This finding suggested an essential role of DHS and DOHH in fungal development, germination, and regulation of adaptation to environmental conditions such as temperature, starvation, osmotic stress and fungicides.

3.10.3 Development of cell death in fungal filaments

Programmed cell death (PCD), in which cells actively participate in their own death through the activation of defined pathways, can be activated by deleterious environmental stimuli such as starvation, oxidative stress and antifungal agents as well as developmentally during sporulation (Robson, 2006). Compared to standard growth, comparative studies were made to investigate whether exposure to toxic treatments triggers the development of an apoptotic phenotype in the overexpressing strains. The tolerance of overexpressing strains to enviromental stress conditions was determined previously, when DHS overexpressing strain was able to tolerate elevated NaCl concentrations and DOHH overexpressing strain was able to tolerate elevated Azoxystrobin concentrations. Contrary response was observed when DHS strain was completely inhibited with elevated Azoxystrobin concentrations and DOHH strain was suppressed with elevated NaCl concentrations, which are often accompanied by irreversible deleterious effects leading to cell death as an indication for loss of membrane integrity and resulting apoptosis. To obtain functional data on the role of DHS and DOHH

during the stress adaptation, quantitative cell death measurements were performed by Evans blue staining with hyphae of *Fusarium* wild-type and the overexpressing strains grown in liquid complete medium or medium supplemented with 0.5M, 1M NaCl and 0.05%, 0.1% Azoxystrobin for 4 days (Figure 25). Under standard growth conditions the overexpressing strains did not show any differences compared with wild-type, suggesting that under favorable growth conditions, the encoded proteins do not contribute to apoptotic phenotypes. Interestingly, the DOHH overexpressing strain already showed enhanced Evans blue staining under salt growth conditions in contrast to DHS strain, which showed low staining compared to WT. Studies of Azoxystrobin influence showed that DHS strain enhanced Evans blue staining under Azoxystrobin growth conditions in contrast to DHS overexpressing strain, which showed low staining compared to WT. Double overexpressing strain showed approximately the same staining patterns as WT (Figure 25).

Thus, these results are confirming the results obtained from the growth experiments on Azoxytrobin or NaCl-containing medium, which could be indicative of the need of DHS and DOHH expression under stress culture conditions and control of toxin damages.

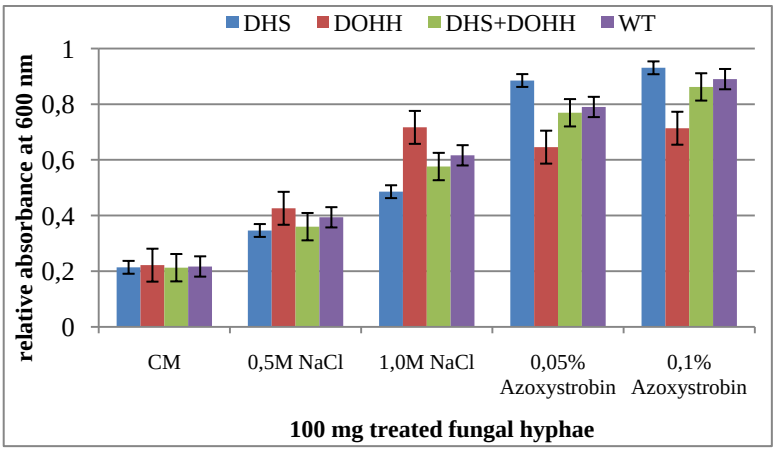

Figure 25. Cell death was measured spectrophotometrically by Evans blue staining in *F. graminearum* wild-type and the overexpressing mutants. Strains were grown in standard growth medium (CM-untreated) or in medium supplemented with 0,5M, 1M NaCl, and 0,05%, 0,1% Azoxystrobin for 4 days. Error bars indicate SE (n= 6).

3.10.4 Comparison of total protein content of the different genotypes

Cell death measurement showed no differences compared with wild-type under standard growth conditions and suggest that proteins were not accumulated to apoptotic phenotype. To correlate the protein composition of the overexpressing strains with growth rate and mycelia density, total protein content was quantified. Total protein extraction was performed with SDS-PAGE and quantified by Bradford staining from hyphae of wild-type and the overexpressing strains grown on standard growth conditions, CM and YPG for 4 days. The protein was quantified from harvested hyphae of $1cm^2$ plug agar at early stationary growth phases. The total protein contents of the three mycelia fungal overexpressing strains and wild-type were approximately the same for harvested cultures under standard conditions with a slight increase in DHS strain (Figure 26). However, this comparison is only tentative, since analysis of yield biomass and protein visualization and under stress conditions are desirable.

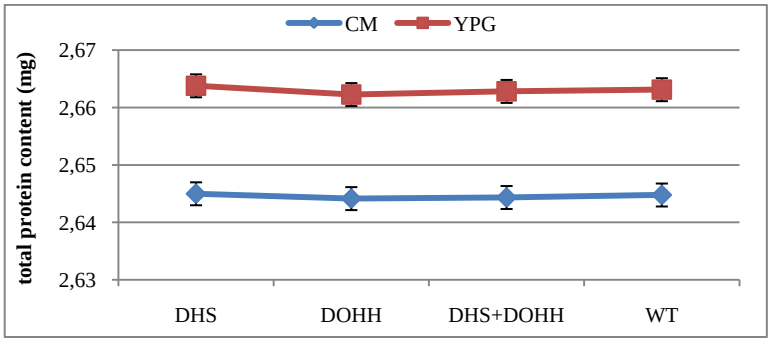

Figure 26. Total protein measurement of DHS, DOHH, and double overexpressing mutants and *Fusarium* wilde-type strain 8/1 cultivated on rich media (CM and YPG). Total protein was extracted from $1cm^2$ plugs of 5 day-old mycelia with SDS-PAGE extraction buffer and quantified with Bradford reagent (Sigma, Germany). Error bars indicate SE (n=6).

3.10.5 Infection patterns on wheat heads

To determine whether DHS and DOHH are involved in pathogenicity, wheat spikes of cultivar Nandu and Amaretto were infected with conidia from the overexpressing strains and the wild-type for three weeks. The chosen cultivar Nandu is susceptible to Fusarium heatd blight, while Amaretto is resistant. In both cultivars, spikes infected with DHS and double overexpressing strain had increased symptoms compared with wild-type infected spikes. Symptoms produced by DHS strain were drastically increased. In Nandu, the strain developed dramatically dense cottony mycelia which covered the whole bleached spike from the top to the stem. In Amaretto, the symptoms were less extreme, but significantly higher than in other

strains. The dense cottony mycelia were formed in the inoculated and the adjacent spikelets. Apart from bleached spikelets, the remaining parts of the spike were not affected and did not differ in appearance compared with uninoculated spikes (Figure 27).

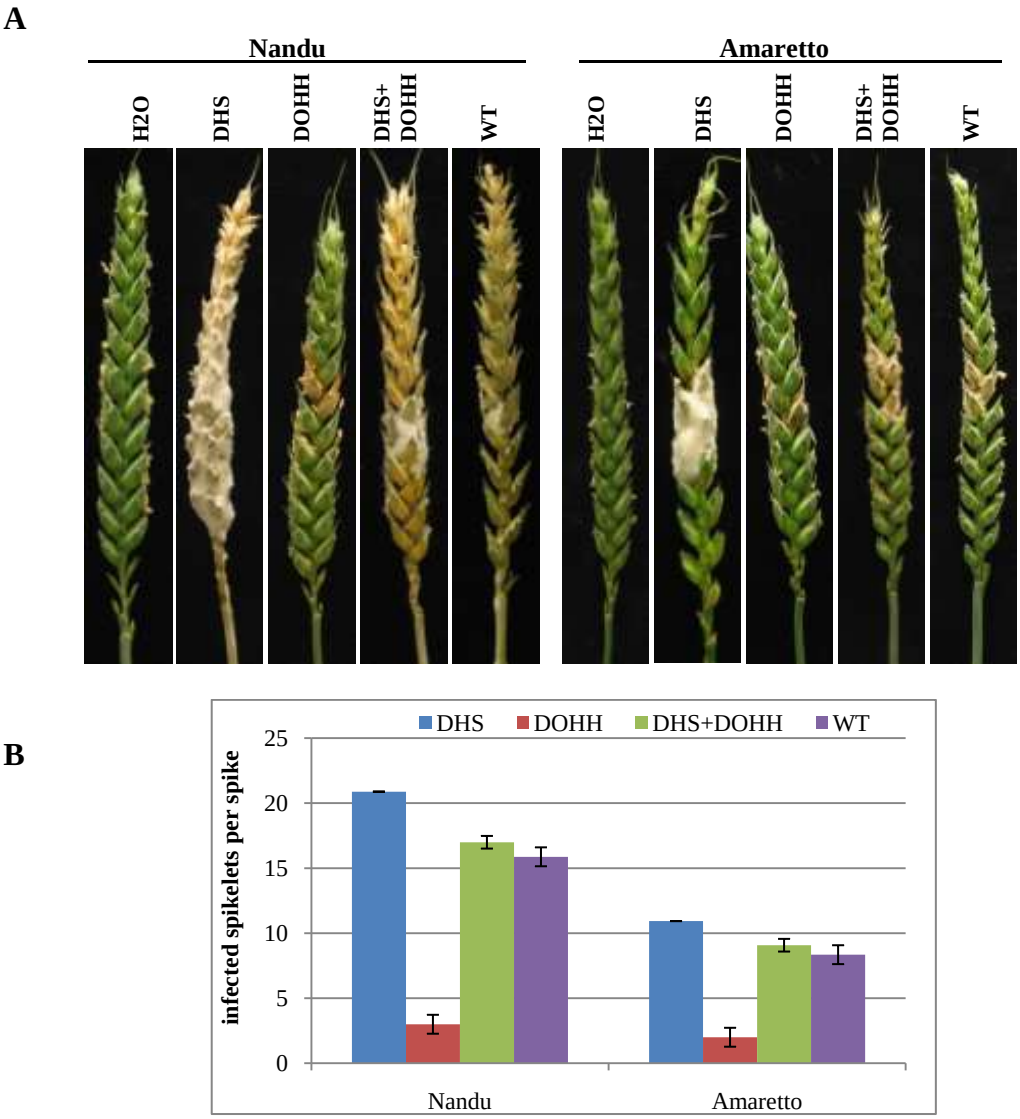

Figure 27. Pathogenicity tests of the overexpressing mutants on wheat spikes. A, Two central spikelets of wheat spikes (Nandu and Amaretto cultivars) were each inoculated with a suspension of 200 conidia of wild-type and the three overexpressing mutants. The infection was monitored for three weeks post inoculation. Then, infected wheat spikelets were quantified (**B**). Pathogenicity tests were repeated 20 times with the four independent strains of each wheat cultivar. Control: pure water-inoculated spike.

Spikes inoculated with wild-type conidia were bleached except for the spikelets at the lower end of the spike in Nandu. In Amaretto, the symptoms were limited to some infected spikelets, where inoculation had occurred. The remaining parts of the spike were not affected

and did not differ in appearance compared with uninoculated spikes (Figure 27). In Nandu, spikes inoculated with conidia of double overexpressing strain were totally bleached with cottony mycelia grown in the inoculated spikelets. In Amaretto, the pattern of symptoms was similar to wild-type with some significant increase (Figure 27). DOHH strain surprisely lost its pathogenicity. The symptoms were limited to the inoculated spikelets (in Nandu) and to the directly inoculated florets (in Amaretto), where inocultion had occurred. The adjacent spikelets of the spike were not affected and did not differ in appearance compared with uninoculated spikes (Figure 27). However, the average percentage of infection of DHS and double overexpressing strains was significantly increased compared with wild-type. In contrast to DOHH strain infection, only directly inoculated spikelets were bleached during infection.

To gain a more detailed picture of the infection pattern of the strains, isolated caryopses were infected. Spikes of wheat cultivar Nandu were harvested at anthesis. After disinfection, separated caryopses were inoculated by pipetting a macroconidia suspension of either wild-type or overexpresing strains on the dorsal side of the caryopses. Compared to other strains, DHS strain showed extensive growth on the wheat kernels, and mycelium covered the caryopses completely at 3 dpi (Figure 28a). Double overexpressing strain developed growing mycelium with significantly greater density than WT and less than DHS strain. In contrast, DOHH strain developed weak mycelium on the caryopses (Figure 28a). Macroconidia of DHS and double overexpressing strains were present on the surface of the caryopses on day 3. Generation of macroconidia was first observed on wild-type on day 4, while no macroconida were formed by DOHH strain.

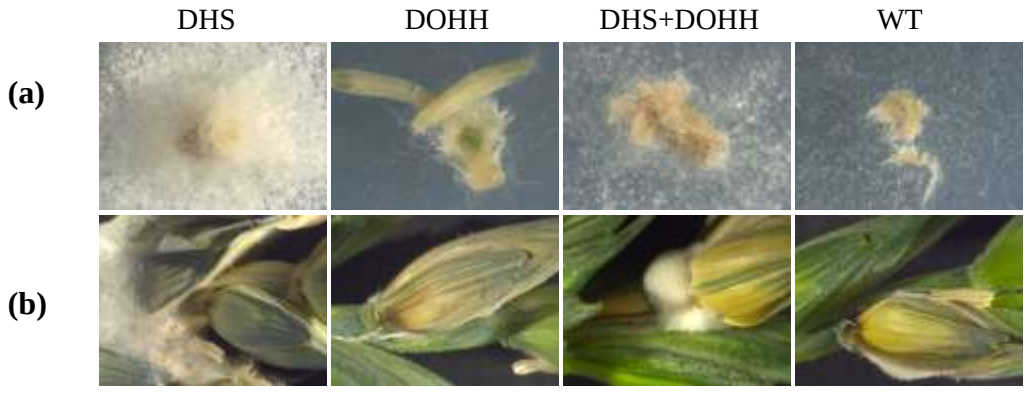

Figure 28. infection patterns of DHS, DOHH and double overexpressing mutants. (a), Caryopses of wheat cultivar Nandu were inoculated with a suspension of 200 conidia of wild-type and the three overexpressing mutants for 3 days. **(b)**, Close view of 7 dpi-spikelets. Two central spikelets of wheat spikes (cultivar Nandu) were each inoculated with a suspension of 200 conidia of wild-type and the three overexpressing mutants.

In addition, spikes were studied 7dpi. Close view of inoculated wheat spikelets and adjacent spike stem regions proved the previous results. Figure 28b shows the overgrown dense cottony mycelium and extent of bleached and necrotic tissue as results of DHS strain infection. Both the inoculated spikelet of the wild-type and double overexpressing strain started to bleach, necrotize and produce mycelium at the glume and rachis node, which was significantly greater by double expressing strain. The symptoms of DOHH strain infection was rarely observed except some bleached and necrotic tissue of the glume (Figure 28b). Hence, although lose of pathogenicity was limited to DOHH overexpressing strain, DHS overexpressing strain developed the most severe blight from the inoculated spikletes. The overexpression of these genes showed to be affected in penetration and colonization of host tissue.

3.10.6 Production of the mycotoxin Deoxynivalenol (DON) in infected spikes

The production of secondary metabolites, including mycotoxins, was also used as an important character in *Fusarium*. A strain's potential ability to form a mycotoxin was often a reason for seeking to identify it. In general, isolates of *F. graminearum* may produce three important mycotoxins, zearalenone, nivalenol and deoxynivalenol-DON. Mycotoxin production of the overexpressing strains and wild-type was measured *in vitro* through DON quantification of 7 dpi-wheat spikelets (Figure 29).

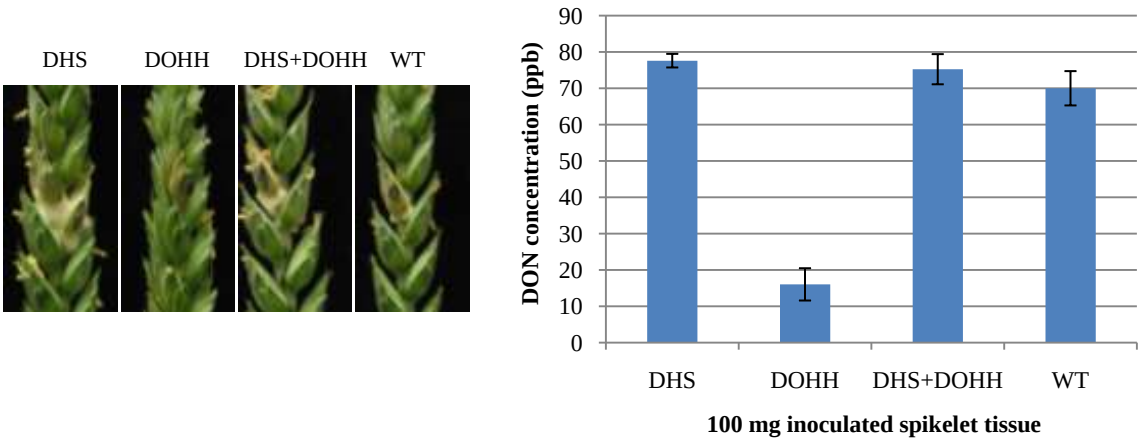

Figure 29. DON measurement of 7 dpi-wheat spikelets with DHS, DOHH, and double overexpressing mutants and *F.graminearum* wild-type strain 8/1. Two central spikelets of wheat spikes (cultivar Nandu) were each inoculated with a suspension of 200 conidia of wild-type and the three overexpressing mutants for 7 days. Then, inoculated spikelets were harvested and quantified *in vitro* for mycotoxin production by RIDASCREEN DON Kit (R-Biopharm AG, Germany). This experiment was repeated four times with three repetitions each.

The results showed that DHS and double overexpressing strains produced more DON compared to wild-type, as was the production of DON the greatest in DHS strain. In contrast, DOHH strain produced very low DON. The production of DON decreased to a fifth compared to wild-type (Figure 29). These results correlated strongly to the infection patterns of the strains.

3.10.7 Generation of reactive oxygen species (ROS) in infected spikes and germinating conidia

Both secondary metabolites and reactive oxygen species (ROS) have important roles in the dialogue between fungal pathogens and their plant hosts. The great elevated chlorophyll bleaching in the infected wheat spikes with DHS and double overexpressing strains compared with wild-type observed during infection course suggested an enhanced generation of reactive oxygen species (ROS) in the mutants. ROS are often damaging, yet are now known to provide developmental cues in fungi and, thus, serve a valuable function of fungal penetration through plant cell walls (Gessler *et al*, 2007). Quantitative H2O2 measurements were performed with wheat spikes infected with *Fusarium* wild-type and the overexpressing strains after 3 days and 7 days of inoculation. The H2O2 levels were elevated with both DHS and double overexpressing mutants compared with wild-type and the H2O2 levels were higher with DHS mutant. In addition, the H2O2 levels with DOHH mutant were the lowest as expected (Figure 30a). The results obtained lead to the conclusion that the balance between ROS as a signal and as a stress provides another point where virulence could be regulated.

Furthermore, ROS formation is essential for fungal development, germination regulation, and intercellular communications in fungi (Gessler *et al*, 2007; Semighini and Harris, 2008). The formation of ROS at every stage of fungal development is indicative of an increase in level of intracellular oxygen radicals. Quantitative H2O2 measurements and detection of superoxide $O2^-$ by staining with nitro blue tetrazolium (NBT) were performed with *Fusarium* wild-type and the overexpressing mutants after exposure to 0.5M and 1M NaCl. The H2O2 levels were elevated in both DHS and double overexpressing mutants compared with wild-type plants after 4 days NaCl treatment with significant higher increase in DHS mutant. Surprisely, the H2O2 level was the lowest in DOHH mutant (Figure 30b). These measurements were further confirmed by the NBT staining of the overexpressing mutants as well as *F. graminearum* wild-type 4 days after exposure to 0.5 NaCl (Figure 30c).

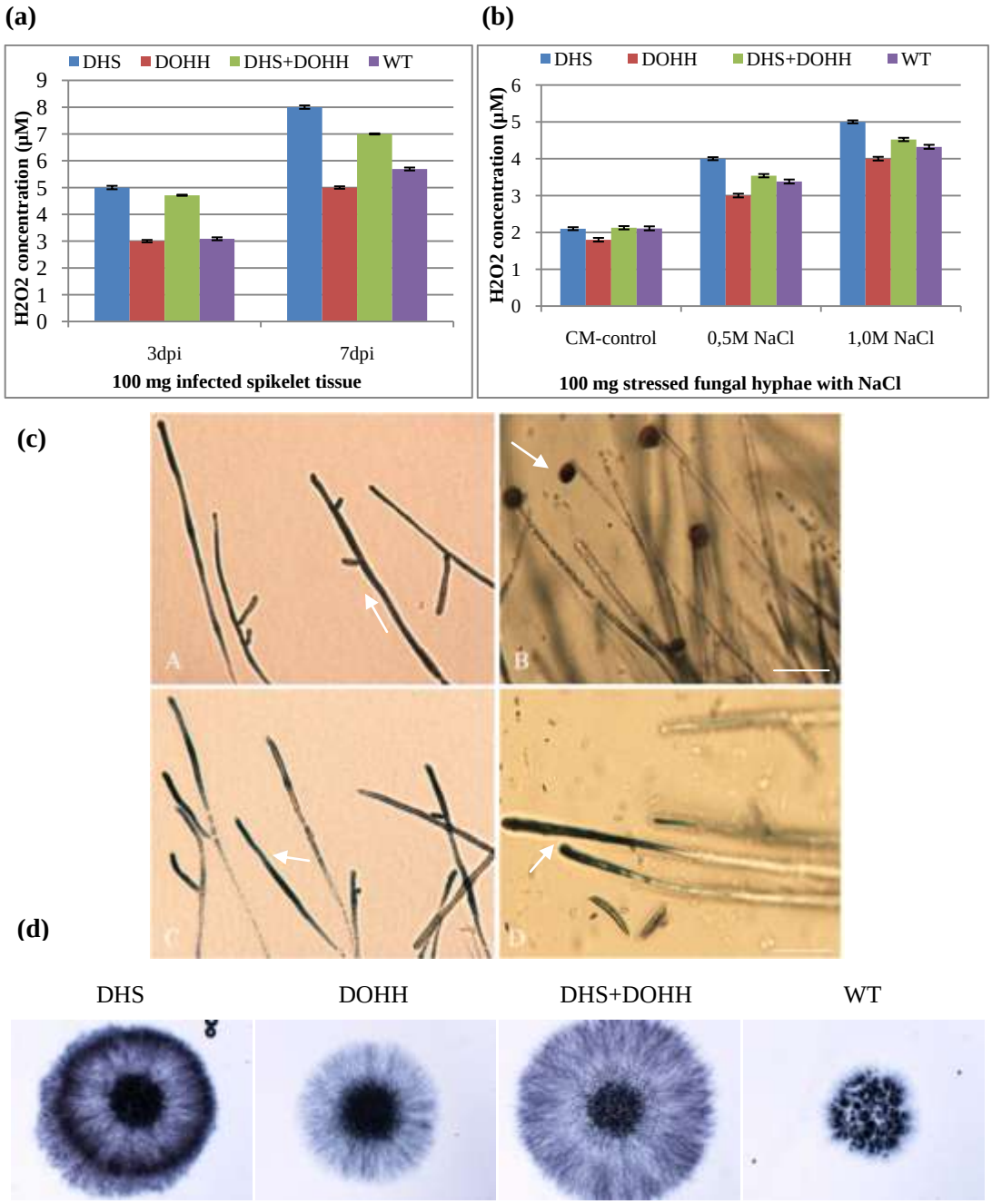

Figure 30. H2O2 measurement, ROS localization and response to oxidative stress. (a), H2O2 measurment of 3 dpi- and 7dpi-wheat spikelets (cultivar Nandu) with a suspension of 200 conidia of the overexpressing mutants and wild-type. **(b)**, H2O2 measurement of the mycelia of overexpressing mutants and wild-type grown in standard liquid medium (CM-untreated) and liquid medium supplemented with 0.5M and 1M NaCl. Error bars indicte SE (n=6). **(c)**, ROS localization was visualized with nitro blue tetrazolium (NBT) in *F. graminearum* wild-type and the overexpressing mutants: A; DHS, B; DOHH, C; DHS+DOHH and D; WT. The strains were grown in the presence of 0.5M NaCl for 4 days. A-D Scale bar = 200 µm. **(d)**, ROS levels were visualized in response to 50 mM H2O2 for 5 days with nitro blue tetrazolium (NBT) in wild-type and the mutants. This experiment was repeated three times with similar results.

The characterization of ROS localization displayed different patterns: (i) Localization at hyphal tips in wild-type, (ii) formation and distribution at apical germinating hyphae in double mutants, (iii) intensive accumulation in germinating hyphae in DHS mutants and (iv) bubbly forms and excreting ROS through tips of germinating hyphae in DOHH mutants.

A dominating role of ROS in self-regulation of fungal development was surprising. The results obtained lead to the conclusion that germinating hyphae excrete ROS in DOHH mutants, which suppress spore and mycelium development under conditions unfavorable for germination. At the same time, self-regulation of ROS development can serve as a positive factor for the causative disease agent in which excreted-ROS DOHH mutants proved to loose its pathogenicity (DOHH).

Finally, plates were used to determine whether mutants display altered responses to oxidative stress in germinating conidia. To analyze the effect of ROS in the overexpressing mutants, NBT staining was determined to oxidative stress after exposure conidia to 50 mM H2O2 (Figure 30d). Wild-type strain suffered under oxidative stress while conidia germinated a little bit, and then stopped growing. In contrast, although the reduction of a growth was significant compared to control media, the overexpressing strains succeeded to grow and develop small colonies. Colony diameter was in the order DOHH<DHS < double mutants, while ROS accumulation was in the order DOHH<double<DHS strains. NBT staining affected mycelium color in which ROS distributes. DOHH strain gained light colored mycelium and double strains gained darker mycelium. DHS strain had different patterns. It gained dark colored mycelium with darker ring color at the tips.

However, the tolerance degree of oxidative stress measured in the overexpressing strains was above the wild-type. These findings are in agreement with the observed elevated levels of H2O2 in the overexpressing mutants, and imply a role of DHS and DOHH in regulation of ROS formation, scavenging or excrement of ROS.

3.11 Constructing strategy to silence DHS in wheat

3.11.1 Vectors for generating transgenic wheat and inducing RNAi of DHS

Three vectors were generated for conditional, Cre-lox-regulated, RNA interference. One vector allows for conditional activation through regulating the *Cre recombinase* by heat shock inducible promoter, whereas the other permits conditional inactivation of short hairpin RNA (shRNA) expression by excising the selectable marker *bar* gene between lox P sites and allows the expression of sense-linker-antisense of the target gene DHS. The third vector predicts the transgenic plants with the selectable marker *neo* gene after activating the Cre-lox system and excising the selectable marker *bar* gene (Figure 31).

The binary vectors designed by Dr. Dirk Becker, University of Hamburg, which serve as vectors of choice for generating transgenic wheat RNAi lines, are pHSP-Cre, pD1-ubi-bar-lox, and pCay-neo. pCay-neo is a constructed plasmid utilizing the constitutive cauliflower mosaic virus (CaMV) 35S promoter to drive expression of protein coding sequence of the *neomycin phosphotransferase* resistance gene (*neo*) from transposon Tn5, which confers antibiotic resistance to geneticin. pHSP-Cre is a constructed plasmid utilizing the Soybean heat shock promoter to regulate the *Cre recombinase* (bacterial phage), while pD1-ubi-bar-lox, adjust to left border LB, is a *phosphinothricin acetyl transferase* gene and the cauliflower mosaic virus (CaMV) 35S terminator driven by the constitutive Ubi promoter and first intron of the maize ubiquitin (Ubi-1) gene (Christensen *et al*, 1992). *Phosphinothricin acetyl transferase* confers herbicide resistance for bialaphos/Basta and phosphinothricin and is commonly known as the *bar* (Basta resistance) gene. *Bar* gene and T35S region are flanked between two similar directions of lox P site, the recognition sequence of *Cre recombinase*. The Ubi promoter and intron1 drive expression of the target gene inverted repeat when Cre-lox system is activated and excise the region of *Bar* gene and T35S .The Arapidopsis intron for *Beta-glucuronidase* (Gus) serves as a spacer between the inverted repeats (Figure 31). The Ubi promoter is expressed in most plant cells, and this strong promoter drives the expression of the various inverted repeat (IR) transgenes engineered into the pD1-ubi-bar-lox vector. The Ubi sequence most likely is irrelevant for RNAi, which depends on RNA production rather than protein translation, but makes the vector adaptable to other purposes such as overexpression of protein.

To construct an RNAi-inducing transgene in pD1-ubi-bar-lox, cDNA fragment (376 bp) of targeted DHS gene was cloned in two steps into multiple cloning sites that flank a 775 bp region of the spacer Gus. The two cDNA fragments were cloned in a way they are in opposite orientations relative to one another, with the Gus intron acting as a spacer between them

(Figure 31). Expression of this IR transgene results in a transcript that terminates within open reading frame (ORF) of wheat DHS gene and folds back on itself by virtue of the inverted repeats, thus generating a dsRNA. The dsRNA is then a substrate for the Dicer and RISC complexes that process the dsRNA into short interfering RNAs (siRNAs) that target homologous mRNAs for degradation.

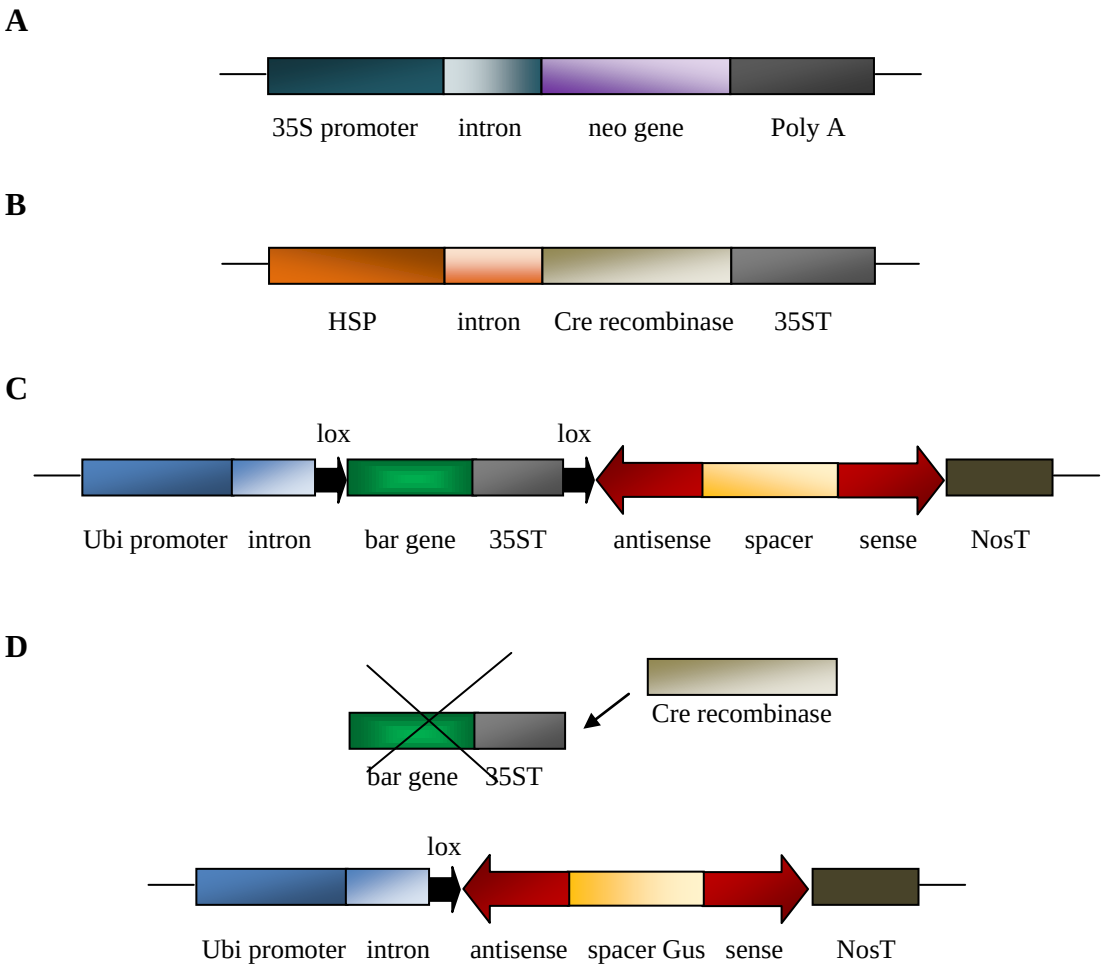

Figure 31. Organization of the wheat ransformation vectors. **A**, pCay-neo is constructed of a polyadenylation (poly A) trap vector in which the expression of a poly A less neo gene, a selectable marker gene conferring geneticin resistance, is driven by the constitutive CaMV 35S promoter. **B**, pHSP-Cre regulates the expression of *Cre recombinase* under the control of the inducible Soybean heat shock promoter (HSP) and the CaMV 35S terminator. **C**, pD1-ubi-bar-loxDHS is a self-complementary hp RNA construct derived from DHS transgene used in the bombardment experiments. In addition, the expression cassette flanked between two lox P sites includes a selectable marker gene conferring Basta resistance (the *bar* gene) under the control of the Ubi promoter and the CaMV 35S terminator. **D**, DHS-specific sequences (red arrows indicating the orientation, antisense-spacer Gus-sense) were controlled by the constitutive maize Ubi promoter (blue box) and the nopaline synthase terminator (brown box) related to activation of *Cre recombinase* and excision the sequence between loxP sites.

3.11.2 Analysis of inverted repeat (IR) sequence context

Since RNAi is a homology-mediated process, an IR sequence designed to target one gene might also silence a homolog. Transcription of an inverted repeat sequence is thought to result in a dsRNA that is recognized and processed by dicer-like proteins. Therefore, a given IR sequence will be processed to generate a population of siRNAs, with the number of siRNAs directly related to the length of the IR fragment. These siRNAs interact with argonaute-like proteins (Vaucheret, 2006), leading to degradation of target gene mRNA. For example, a variety of features have been shown to be important predictors of an siRNA's silencing ability in human embryonic kidney cell culture (Reynolds *et al*, 2004). These include GC content, melting temperature and presence of adenine at position 3 or 19, guanine at position 13, and uridine at positions 10 and 19 for each predicted siRNA (positions numbered consecutively with 1 indicating the 5` most end of the siRNA relative to the presumed direction of transcription). To determine if the specific sequences of the component siRNAs would correlate with silencing ability of wheat IR sequences, the siRNA Target Finder, a computer program by Ambion, was used to generate a list of all possible 21 bp siRNAs for each IR sequence and each siRNA was evaluated for the characteristics listed above. To identify such potential secondary targets, sequences were searched for matches of at least 21 bp (size of many typical siRNAs) between primary IR segments and closely related gene family members (Vaucheret, 2006). Thus, a 376 pb sequence of wheat DHS gene would be judged to be a potential target for silencing by an IR transgene, if it shared a 21 bp stretch of identity with the IR sequence.

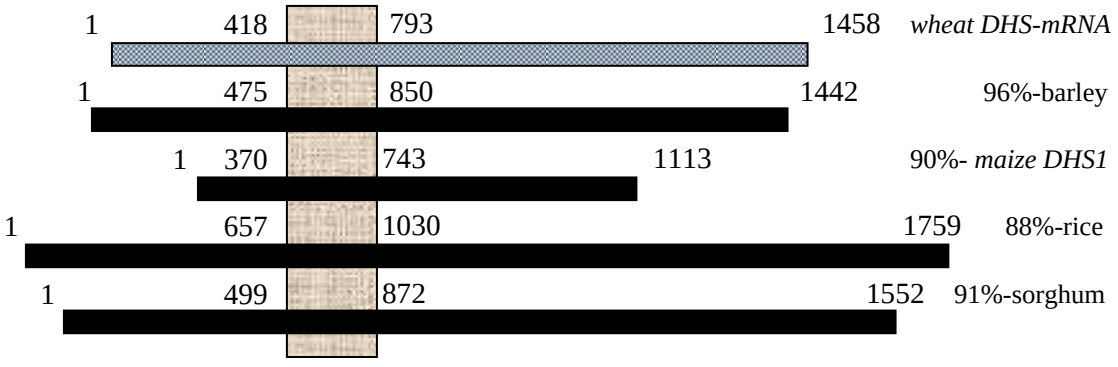

Figure 32. Location of the wheat DHS fragment used to construct the RNAi vector in the corresponding wheat DHS sequence and in the corresponding full-length DHS cDNAs identified in barley; AK248438, maize; GU735677, rice; NM_001057753.1, and sorghum; XM_002444888.1. Base pairs, percentage of identity, and overlapping areas (shaded box) are indicated (not on scale).

In designing the RNAi construct to silence DHS, the inverted repeat (IR) sequence was selected from a region which has the highest identity to other plant DHS genes, so that this gene and its alleles could be silenced by this transgene (Radicella *et al*, 1992; Selinger and Chandler, 2001). While the selected region was checked against the siRNA Target Finde program as mentioned above, a BLASTN 2.2.23 program (Zhang *et al*, 2000) was run to predict the percent identity against the Genbank.nucleotide database. Figure 32 shows the locations of the DHS region used in the experiment described above and the relationship of wheat DHS mRNA sequence utilized in the IR construct relative to other plant DHS sequences.

3.11.3 Clone cDNA fragment corresponding to targeted wheat DHS

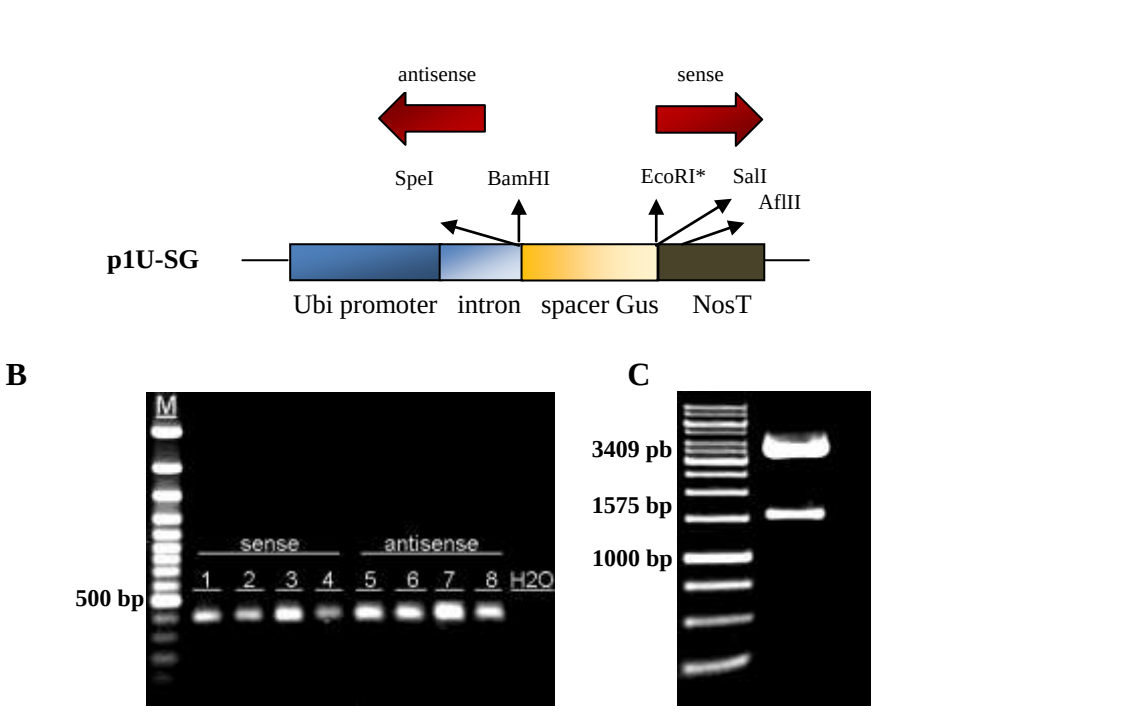

Figure 33. Cloning of the wheat DHS fragment selected for RNAi construct. **A**, p1U-SG is a plasmid indicated the two multiple cloning sites flank the spacer Gus, providing sites for cloning target gene sequences in an inverted orientation (open arrows). Only sites used for the two-step cloning procedure for this construct are indicated in this map. Specific sequences (red arrows indicating the orientation) were cloned and controlled by the constitutive maize Ubi promoter and the nopaline synthase terminator. **B**, The 376 bp fragment was amplified by pairs of restriction endonuclease sites included in the primer sequences that constitute the ends of the PCR fragment by gradient PCR. M; 100bp plus DNA ladder from Fermentas. **C**, Digestion test of the cloned DHS sense and anti sense to p1U-SG. SpeI and AflII enzymes were used to digest a fragment of 1575 bp of sense, spacer Gus and anti sense sequences. M; DNA ladder mix from Fermentas.

A 376 bp portion of wheat DHS was selected concerning the analysis of IR results. Using RT-PCR, two fragments were amplified from senescence-induced cDNA with a proof reading enzyme facilitated by pairs of restriction endonuclease sites included in the primer sequences that constitute the ends of the PCR fragment. Theses fragments were cloned, sequenced, and ligated into p1U-SG vector provided by Dr. Becker-University of Hamburg. Plasmid p1U-SG contains the spacer Gus and was constructed for inverted repeat transgenes. The resulting plasmid then served as a template for further cloning (Figure 33). This plasmid was delivered to DNA Cloning Service (Hamburg) to generate the sense and antisense of DHS with the spacer Gus into pD1-ubi-bar-lox vector.

The three binary vectors described previously for producing transgenic wheat RNAi lines were carried out by using transformation of *E. coli* following selection of ampicillin resistant colonies. Plasmid DNA maxi prep was prepared from scale cultures of LB medium (~100 ml) and identification of recombinant plasmids was checked concerning the plasmid maps.

3.11.4 Biolistic transformation of wheat

Biolistic transformation is a unique process in which DNA or RNA is introduced into cells on micron-sized particles. These microparticles are accelerated to supersonic speeds utilizing forces generated by a gunpowder discharge or cold gas explosion. This technique was first developed for transformation of plant cells. It has since been used successfully with bacteria, fungi, and mammalian cells, both *in vitro* and *in vivo* (Johnston and DeVit, 1996).

Microprojectile biolistic delivery of DNA is used to produce transgenic wheat lines expressing the inverted repeat construct. Bombardments were carried after (Day *et al*, 2005; Kikkert *et al*, 2005) with some modification by Dr. Becker, University of Hamburg, (unpublished). Briefly, small gold microcarrier particles (0.4-1.2 µm) were used and coated with a lower concentration of plasmid DNA (0.4µg plasmid DNA/1 mg microcarriers) so as to achieve lower copy number transgene insertions. The plasmid DNA had a ratio 1:1:1 of the three binary vectos: pHSP-Cre, pD1-ubi-bar-lox, and pCay-neo.

Modifications also included the use of 1350-psi rupture discs, a rupture disc-to-macrocarrier distance of 6.5 mm, a macrocarrier to- stopping screen distance of 8.0 mm, and a stopping screen-to-target tissue distance of 10 cm. The target tissues for bombardment-mediated wheat transformation were immature embryos derived from self-pollinated, greenhouse-grown Florida cultivar (winter cultivar). Embryos were precultured axis-side up on callus induction medium (without amino acid) for 1-2 days in the dark at 26°C. 6 hours perior

to bombardment, embryos were cultured axis-side down on osmotic medium containing macro salts, microsalts, FeNa-EDTA, and maltose. Following bombardment, plates were placed overnight in the dark at 26°C.

3.11.5 Selecting transgenic Calli and plant regeneration

Over 5000 embryos were bombarded and the bombarded tissues were cultured again axis-side up on callus induction medium for 14 days in the dark at 26°C. Later, they were individually transferred to selection medium containing 1.5 mg/ml Basta (20% phosphinothricin acetyltransferase-PPT), and following five to six weekly subculture and subdivision steps on selection medium containing 1.5 mg/ml Basta (20% PPT) at 26°C in the light (Figure 34).

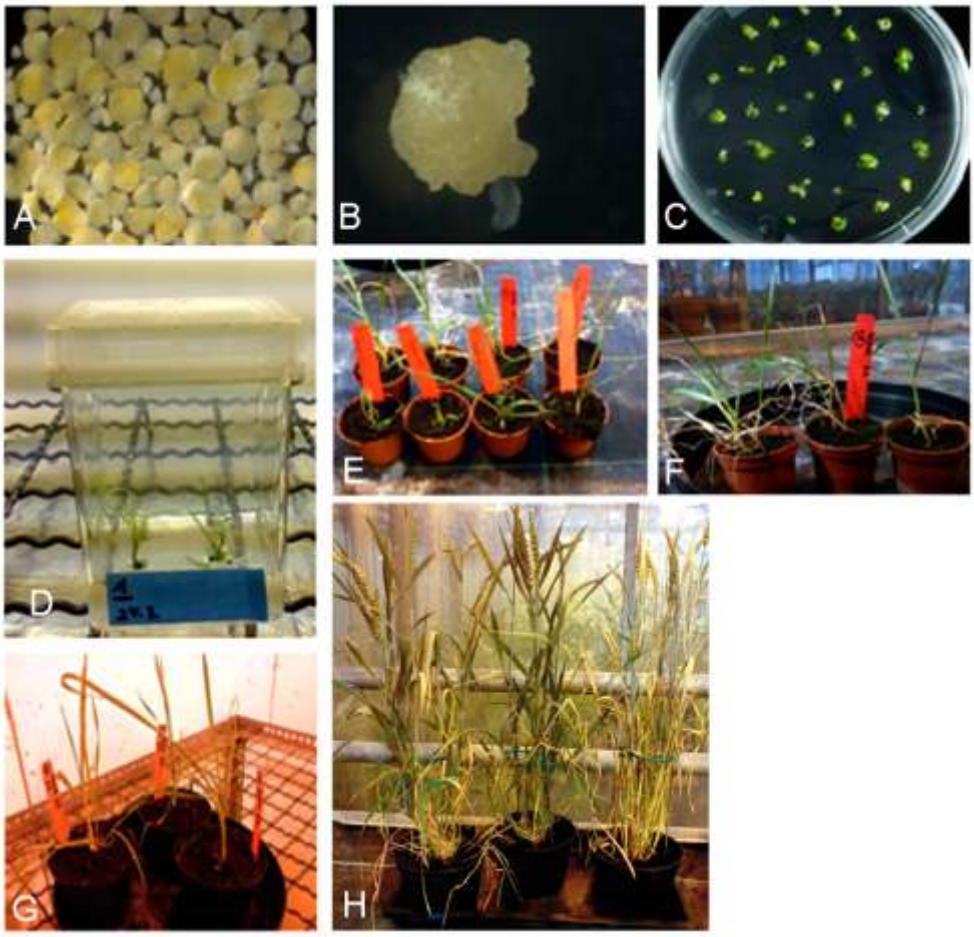

Figure 34. Response of Florida to the tissue culture process. A, Embryos after shooting with gold particles. **B**, Embryogenic callus induction. **C**, Plant regeneration on selective medium. **D**, Plant regeneration on regeneration medium. **E**, Plant regeneration in greenhouse. **F**, Three transformants resistant to herbicide/Basta. **G**, Transformants were placed in the cold champer for 6 weeks. **H**, Plant production of T_1 seeds.

Before transferring embryos to selective medium, the effect on scutellum embryogenesis is summarized. The majority of bombardment embryos produced a high yield of embryogenic callus, while minority not reacting to the induction. Generally, first globular stage embryogenic callus was observed 4–5 days after the transfer, and the globular stage was usually formed directly from the scutellum. This was followed by a high frequency of repetitive embryogenesis. Early globular stages were followed by the full differentiation of the callus.

After selecting on selection medium, independent transgenic callus lines were identified, placed in magenta boxes on generation medium containing no herbicides, and grown for 3 weeks in the light. The putative transgenic seedlings were moved to the greenhouse. After 1-2 weeks, plants were examined for tolerance to herbicide /Basta. 250 mg/L Basta and 0.1% tween were sprayed once every week for three times. Three putative transgenic plants were just produced by particle bombardment from over 5000 bombarded embryos after herbicide test in the greenhouse. The surviving plants were moved to a cold champer for 6 weeks to induce the reproductive stage and moved later to the greenhouse to continue growing (Figure 34).

The three putative transgenic plants were produced from the early shooting experiments by particle bombardment. After induction of the reproductive stage, these plants were analyzed by PCR at an older stage to avoid sensitivity to wounding and leave cut. Genomic DNA from these plants was extracted, while *Cre recombinase*, *bar* gene, and *neo* gene were detected by PCR primers corresponding to the transformed binary vectors. Figure 35 shows the present of *Cre recombinase* and *bar* gene in the transformants, while *neo* gene is absent. This means that PD1-ubi-bar-loxDHS and pHSP-Cre vectors were transformed with the self-complementary DHS, *bar* gene, and the inducible *Cre* gene, while pCay vector, which regulates the second selectable marker was, not transformed.

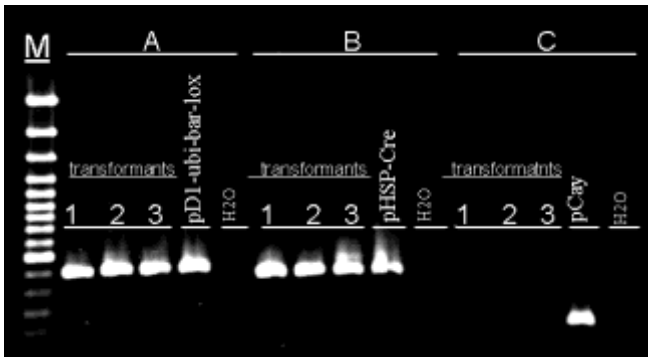

Figure 35. PCR screening of wheat transformants. A, B, C, Amplification of *bar* gene (427 bp), *Cre* gene (437 bp), and *neo* gene (131 bp), respectively. Vectors were served as control. M; 1KB DNA ladder (Fermentas, Germany).

The *bar* gene is the selectable marker, which allows the presence of the transgene to be tracked by testing for bialophos/Basta resistance. Herein, the term "resistant" is used to describe plants that are resistant to Basta, and the term "susceptible" is used to describe plants that are not herbicide resistant. The transgenic individuals continued to cosegregate with the herbicide resistance Basta in all subsequent generations, whereby DHS mRNA is produced after activation of Cre-lox system, but the mRNA is degraded by transgene-induced RNA silencing before a protein can be translated.

Southern blot analysis of integration and copy numbers of T-DNA, generation of progeny (T_1 and T_2), and activation of Cre-lox system is continued by other colleagues, since this project is to date still running and the time frame of each generation is 9 months of transgenic Florida plants.

3.12 Constructing strategy to overexpress DHS in maize

3.12.1 Vector for generating transgenic maize inducing overexpression of DHS

The method of choice for genetically engineering maize is *Agrobacterium tumefaciens*-mediated genetic transformation. *A. tumefaciens* is a common soil bacterium that can transmit a segment of DNA, known as the T-DNA (transferred DNA), from a large resident plasmid, called the Ti plasmid, to a recipient plant cell through a process that closely resembles bacterial conjugation (McGinnis *et al*, 2005). The binary vector, which served as a vector of choice for generating transgenic maize overexpression lines, is p7i-HSP-Cre-Ubi-Npt-Bar generated for conditional, Cre-lox-regulated, gene overexpression. This vector allows for conditional activation through regulating the *Cre recombinase* by heat shock inducible promoter. However, it permits conditional inactivating of targeted maize DHS1 expression by excising the selectable marker *nptII* gene between lox P sites, and allows the expression of DHS open reading frame (Figure 36).

The vector is a derivative of the pPZP binary vector (Hajdukiewicz *et al*, 1994) that contains the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (aadA) for bacterial selection. The expression cassette in p7i-HSP-Cre-Ubi-Npt-Bar, adjusted to right border RB, is constructed of a *phosphotransferase II* gene (*nptII*), kanamycin resistance, and the cauliflower mosaic virus (CaMV) 35S terminator driven by the constitutive Ubi promoter and first intron of the maize ubiquitin (Ubi-1) gene (Christensen et al, 1992). The region of

nptII gene and T35S is flanked between two similar directions of lox P site, the recognition sequence of Cre recombinase. The Ubi promoter and intron1 drive expression of the target gene (DHS) and its nopaline synthase terminator when Cre-lox system is activated and excise the region of *nptII* gene and T35S. The inducible Soybean heat shock promoter (HSP) regulates the expression of *Cre recombinase* and the CaMV 35S terminator, while left border LB is adjusted to a *phosphinothricin acetyl transferase* gene and the cauliflower mosaic virus (CaMV) 35S terminator driven by the constitutive (CaMV) 35S promoter. *Phosphinothricin acetyl transferase* confers herbicide resistance for bialaphos/Basta and phosphinothricindesigned. p7i-HSP-Cre-Ubi-Npt-Bar-DHS was designed by Dr. Becker, (University of Hamburg) and synthesized by DNA Cloning Service (Hamburg).

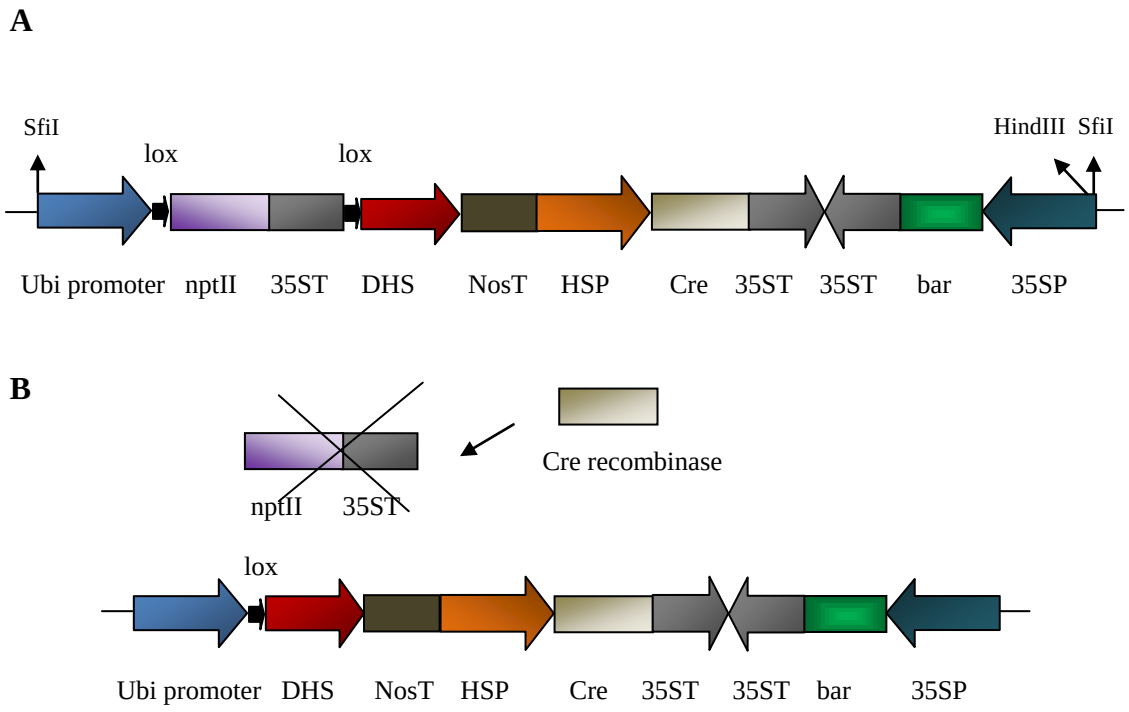

Figure 36. Organization of the maize transformation vector. **A**, p7i-HSP-Cre-Ubi-Npt-Bar-DHS is constructed for conditional activation of maize DHS1 overexpression through regulating the Cre recombinase by heat shock inducible promoter, whereas it permits conditional inactivating of targeted DHS expression by excising the selectable marker *nptII* gene between lox P sites and allows the expression of DHS open reading frame (**B**). *Bar* gene is a selectable marker conferring herbicide/Basta under the control of the CaMV 35S promoter and its terminator. Open arrows refer to expression orientation. Black arrows refer to restriction sits for southern blot analysis.

3.12.2 Mobilize plasmid vector into *A. tumefaciens*

Once the overexpression vector has been verified, purified plasmid DNA isolated from *E. coli* was used to transform *A. tumefaciens,* strain LB4404 by freeze-thow method (Jyothishwaran et al, 2007). Spectinomycin- Tetracyclin resistant colonies were isolated and plasmid DNA was prepared from small-scale cultures (2–5 ml) grown to stationary phase in YEB medium (Godoy-Hernández et al, 2006). Purified plasmid DNA was then subjected to restriction mapping to verify that the plasmid is correct and had not suffered any obvious rearrangements due to growth in *A. tumefaciens*. Large-scale cultures (~500 ml) were then grown for plant transformation.

3.12.3 Maize transformation

The target tissues for *A. tumefaciens*-mediated maize transformation were immature embryos derived from cross-pollinated, greenhouse-grown HiIIA X HiIIB (F1) hybrids. Maize plants were transformed by a modified protocol of Amati *et al*, unpublished. In brief, twenty to one hundred precultured, immature embryos (1.5-2 mm, isolated 11–13 days postpollination) were targeted by means of transformation. Embryos were then inverted and submerged for 5 min in a solution of *A. tumefaciens* that had been grown to mid-log phase, pelleted by centrifugation, and resuspended in a solution that includes detergent (0.02% Silwet L-77). The detergent allowed the solution to thoroughly coat the cells and infiltrate into the intercellular spaces. Immature zygotic embryos infected with *A. tumefaciens* strain LB4404 harboring the standard binary vector p7i-HSP-Cre-Ubi-Npt-Bar-DHS were cocultivated after (Frame *et al*, 2002; Frame *et al*, 2006).

It should be noted that two types of embryogenic callus, type I and type II, may proliferate from the scutellum of immature embryos, depending on the genotypes of maize. The type I callus is a relatively hard and compact embryogenic callus, whereas the type II callus is relatively soft and friable. A type I callus is usually obtained from inbreds A188, A634, H99 and W117 on media described in general protocols. For genotypes such as Hi-II, from which the type II callus is frequently produced, modification of the media is recommended (Ishida *et al*, 2007). Protocols recommended for Hi-II and some other genotypes have been reported by other protocols. These protocols are similar to those presented in this work and differ essentially only in the composition of the media. While the macro components in all of the media in this work are based on the N6 medium, those in the protocols of other groups are based mostly on the LS medium. In addition, Fram *et al*, 2004 used media that contained

cysteine for cocultivation. Thus, media based on N6 and media with cysteine should also be considered, especially when other genotypes are to be transformed. Bialaphos-resistant callus events were regenerated on medium containing 10 mg/L bialaphos. Derived transgenic events regenerated to plants were grown in the greenhouse (Figure 37). Because the T-DNA includes the *bar* gene, transgenic plants that express this selectable marker gene survive herbicide spraying whereas, nontransgenic plants were killed. Seedlings were typically sprayed three times, first when they were approximately one week old and again when they were 2 and three weeks old.

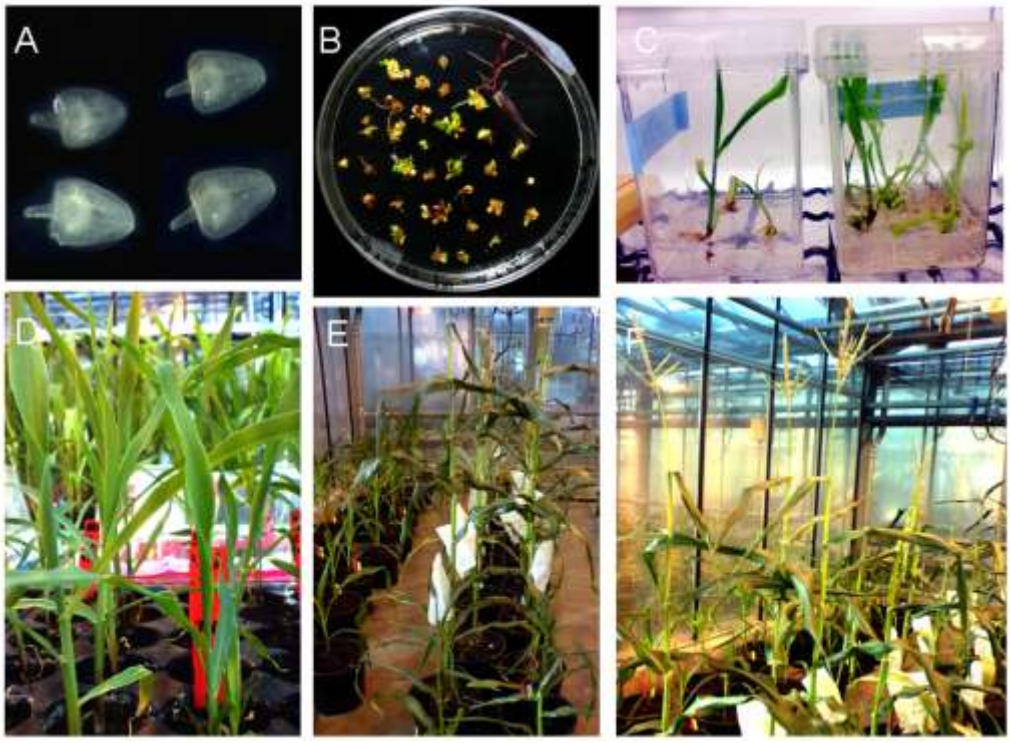

Figure 37. Generation of transgenic maize plants. A, Embryos precultured for *A. tumefaciens*-mediated transformation. **B**, Plant regeneration on selective medium. **C**, Plant regeneration on regeneration medium. **D**, Transformants resistant to herbicide/Basta. **E**, Plant generation and production of T_1 seeds. **F**, Transgenic plant cosegregation; back crossing, self pollination, or crossing with A188 inbred.

3.12.4 Molecular screening of transgenic maize plants

Shoot tissue harvested from PPT-resistant plants was screened to verify the presence of the *nptII* gene in the plant genome. From over 400 transformed embryos, ten independently transformed plants that successfully produced plants resistant to the herbicide treatment were analyzed for *nptII* gene copy number by Southern blot analysis. Results indicated that among the plants analyzed from all experiments, seven plants contained the *nptII* gene (Figure 38).

Southern-blot analysis was carried out to assess the integration of the *nptII* transgenes in the T_0 generations of numerous independent explants. Total genomic DNA was extracted from leaf tissue, digested separately with the restriction enzyme HindIII (unique site on the plasmid of 15214 bp), and with the restriction enzyme SifI which cuts the plasmid twice and produce a fragment of 4295 bp containing the *nptII* gene cassette. DNA blots were prepared and hybridized with the *nptII* probe. As illustrated in (Figure 38), Hind III and SifI restriction digestion of genomic DNA derived from transgenic material not only liberated a fragment containing the *nptII* gene cassette, but also yielded various band sizes (>4295 bp) that hybridize to the *nptII* probe. The number of bands hybridizing with the *nptII* probe reflects, in turn, the different sites of transgene integration and represents an estimate of transgene copy number in the maize genome.

Samples of genomic DNA from 10 independent events were hybridized with the *nptII* probe. Seven of these events showed the expected 4295 bp dropout band (plants 1, 3, 4, 5, 6, 7 and 9), suggesting the intactness of the *nptII* gene cassette in the maize genome. In the case that more than one plant was selected from the same callus, Southern analysis was performed immediately. In the case that the band segregation patterns in the Southern blot were similar, the plants were counted as one event. In the case that the hybridizing bands bigger or smaller than 4295 bp, the *nptII* gene cassette was considered rearranged. Plants 3, 5 and 9 were considered a single event, while plants 1, 4, 6 and 7 were considered independent events. Plants 8a, 8b and 10 were considered to be escaped.

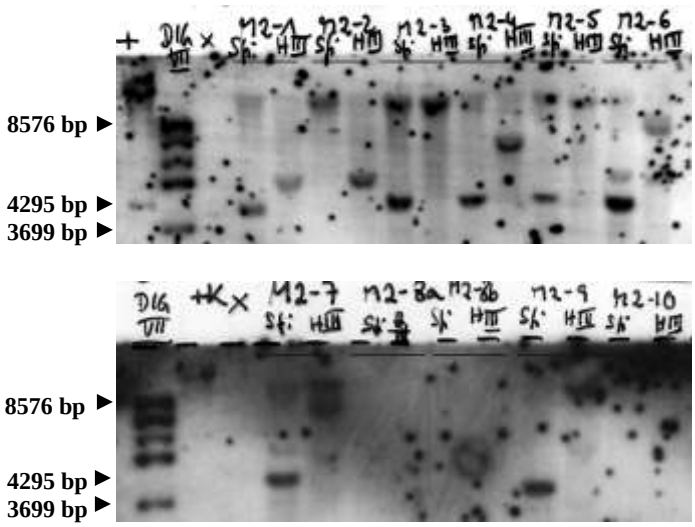

Figure 38. **Southern blot analysis of regenerated T_0 maize plants.** The DNA of 10 plants was separately digested with SfiI (cuts the plasmid twice), and with HindIII (unique site), then probed with the digoxygenin-labeled *nptII* gene (30ng/ml). Dig-VII DNA ladder was used as marker (Roche Applied Science). (+); Vector as control. Plants 1, 3, 4, 5, 6, 7and 9 were considered positive; plants 3, 5 and 9 were considered a single event.

3.12.5 Production of T_1 seeds and assessment of transgene segregation

The inclusion of a linked selectable marker, the *bar* gene, in all transgenes means that crossing hemizygous T_0 plants with an intact transgene to non-transgenic should produce T_1 plants that segregate 50% non-transgenic, susceptible plants and 50% hemizygous transgenic, resistant plants.

Approximately seven transgenic maize plants (T_0 generation) were regenerated for each independent callus line. T_1 seed was produced by crossing regenerated transgenic T_0 plants as the male or female parent with maize inbred line HiIIA, HiIIB, or A188, producing on average 3 ears with seed for each independent event. Plants were back-crossed, outcrossed rather than self-pollinated to keep the transgene hemizygous and, thus, to potentially increase the stability of transgene expression, resulting in T_2 seed that is hemizygous for each transgene insertion (Table 1). Progeny screening of T_1 generation seedlings was carried out on a subset of transgenic events to assess whether the *bar* transgenes were inherited in a normal Mendelian fashion. Seed for these tests was derived from female transgenic plants that had been pollinated (outcrossed) with nontransgenic pollen. As such, the expected segregation ratio for inheritance of the transgene as a single locus was 1:1.

Table 1. Crossing and segregation of transgenic maize plants in the T_1 progeny.

Transgenic ID	Crossing Events
M2-1	M2-1 X HiIIA
M2-2	M2-2 X M2-2 M2-2 X A188
M2-3	M2-3 X M2-3 M2-3 X A188
M2-4	M2-4 X M2-4 M2-4 X HiIIA M2-4 X HiIIB M2-4 X A188
M2-6	M2-6 X HiIIB
M2-7	M2-7 X HiIIA
M2-9	M2-9 X HiIIA

T_1 plants for each transgenic line were planted in the greenhouse and tested for herbicide resistance to verify that the transgene is active. The selected progeny was evaluated again for resistance to PPT. Resistant and sensitive seedlings were clearly distinguishable after spraying with 250mg/L Basta. All lines (randomly taken) were studied for inheritance of the transgenes. The Basta-resistant progeny of the plants that gave segregation ratios of 1:1 or 1:0 were analyzed: (M2-1 X HiIIA), (M2-3 X A188), and (M2-4 X M2-4). To date the project is running. Activation of Cre-lox system of selected plants by heat shock to induce the overexpressed DHS gene and analyzing the progeny is continued by other colleagues.

4.0 Discussion

4.1 Sequence conservation of eIF5A, DHS and DOHH

eIF5A and the hypusine biosynthetic enzymes DHS and DOHH occur in all eukaryotes and are highly conserved as illustrated in Figures 6-8. The high sequence conservation of each of the three proteins may have been dictated by the structural requirement of eIF5A for its interaction with cellular effector molecules as well as with the modification enzymes.

As shown in Figure 6, eIF5A is highly conserved in eukaryotes (with amino sequence identity of 61% between the human eIF5A-1 and *F.graminearum* eIF5A. It is comprised of two domains, the N-terminal (aa 1–113) and C-terminal (aa 127–163) domains for *Fusarium* protein as example. The sequence identity is especially high in the region surrounding the hypusine modification site in the basic N-terminal domain. This finding suggests an importance of this site for the interaction of eIF5A with the modification enzymes or with its binding partners (proteins or RNA) (Boeke *et al*, 1987; Wolff *et al*, 2007). The C-terminal domain, which is less highly conserved than the N-terminal domain, contains a stretch of highly conserved amino acids (120-134 upper numbering, Figure 6) and was proposed as a potential effector domain involved in protein-protein interaction (Liu *et al*, 1997). Although the N-terminal domain alone is sufficient as a substrate for DHS and for DOHH as well, both the N- and C-terminal core domains are required for its activity (Wolff *et al*, 2007; Park *et al*, 2010).

Like eIF5A, DHS is highly conserved (Figure 7).The amino acid identity between the *F. graminearum* and the human enzyme is 56%, while the identity among the human, the wheat, and the maize enzyme is 60%. The active site amino acid residues involved in the binding of its amine substrate spermidine, and the cofactor NAD have been identified from the crystal structure (Umland *et al*, 2004) and their roles were confirmed by site-directed mutagenesis (Lee *et al*, 2001a).The active site for binding of spermidine is strictly conserved (Figure 7). Alanine substitution of any single amino acid of these residues abolishes spermidine binding and DHS activity (Wolff *et al*, 2007). The amino acid residues predicted to be involved in the binding of NAD are also highly conserved. This finding suggests maintenance of a fundamental cellular function of eIF5A and DHS through evolution. The unique feature of the hypusine modification is the strict specificity of the enzymes toward its substrate protein, eIF5A. Moreover, DHS exhibits a narrow specificity toward spermidine.

DOHH also showed to be highly conserved (Figure 8). DOHH monomer is a dyad of symmetrical N-terminal (HEAT-repeats 1–4) and C-terminal (HEAT-repeats 5–8) domains connected by a variable loop. Four strictly conserved His-Glu motifs in the N-terminal and the C-terminal domains are required for binding of iron, and the substrate protein, eIF5A intermediate containing deoxyhypusine, and for deoxyhypusine hydroxylation (Kang *et al*, 2007). Moreover, the high conserved sequences of eIF5A, DHS and DOHH proteins in the eukaryotic kingdom may reflect evolutionary of studying this class of genes in all species and genuses.

4.2 eIF5A, DHS and DOHH are essential genes in *F. graminearum*

The essentiality of DHS and DOHH genes for *Fusarium* growth was examined by gene disruption and their rescue with the normal genes. Deletion of *Fusarium* DHS or DOHH produced lethal phenotype. The genotype was not viable and an arrest in growth most likely resulted from depletion of DHS or DOHH and, consequently, a reduction in eIF5A below the minimum level required to support cell growth. The finding that this hypusination/ hydroxylation are vital in *F. graminearum* suggests a specialized eIF5A function and/ or a critical hypusination/ hydroxylation-dependent regulation mechanism involving eIF5A.

Also, interestingly, these genes displayed different expressions on growth media. On rich medium eIF5A, DHS and DOHH are expressed in germinating conidia and rapidly dividing young mycelia. After 6 days in older mycelium, just DHS transcription is clearly reduced. In contrast, on poor medium the DHS and DOHH transcripts are upregulated throughout from germinating conidia to older mycelium, while eIF5A transcript is constitutively induced on both media. The high expression of fungal eIF5A, DHS and DOHH during nutrition stress assumes an important function of these genes in germination, cell viability and fungal proliferation.

Although the precise cellular function of eIF5A is unclear, the necessity for hypusine-containing eIF5A for proliferation is well established. Yeast with rapidly degraded eIF5A is blocked in G1 and yeast knockout mutants produce non-viable spores (Schnier *et al*, 1991; Sasaki *et al*, 1996; Park *et al*, 1998). Mammalian cells treated with inhibitors of deoxyhypusine synthetase or deoxyhypusine hydroxylase fail to proliferate (Chen *et al*, 1996; Park *et al*, 2010). Although DOHH is not an essential gene in *S. cerevisiae* (because the DOHH null strain is viable (Thompson *et al*, 2003; Park, 2006)), inactivation of the DOHH gene is recessively lethal in multicellular eukaryotes, e.g. *Caenorhabditis elegans* (Sugimoto, 2004) and

Drosophila melanogaster (Spradling *et al*, 1999; Patel *et al*, 2009), suggesting a requirement for the fully modified eIF5A in higher eukaryotes. Gene disruption and mutation studies in yeast and higher eukaryotes have provided valuable information on the essential nature of eIF5A and the deoxyhypusine/hypusine modification in cell growth and in protein synthesis (Kim *et al*, 2006b; Park *et al*, 2010). It is also conceivable that eIF5A in higher eukaryotes is multi-functional and performs a hypusination/ hydroxylation-dependent function in addition to a hypusination/ hydroxylation-independent basic function common to yeast and human (Wolff *et al*, 2007).

4.3 Virulence role of *Fusarium* eIF5A, DHS and DOHH

In the present study, the pathogenic fungus *Fusarium graminearum* and its interactions with host plants was investigated. *F. graminearum* is a necrotrophic, filamentous ascomycete, which causes Fusarium head blight (FHB) and cob rot of maize, and is one of the most destructive crop pathogens worldwide. *F. graminearum* causes quantitative losses by reduction of yield and quality losses by contaminating the remaining harvest with mycotoxins. Therefore, fusarioses of cereals pose a major threat to food supply worldwide. *Fusarium* eIF5A, DHS and DOHH proved to be strongly induced during its pathogenic interaction with its host plant, wheat, especially during the early stages of infection. Moreover, the expression of eIF5A, DHS and DOHH during the early stages of infection showed to be stronger than the expression of known secreted virulence factors trichothecenes (Maier *et al*, 2006) and lipases (Voigt *et al*, 2005). Disruption of trichothecene genes, regulates production of the mycotoxins DON and NIV, showed in different *F. graminearum* wild-type strains a reduced virulence on wheat and mutants lacking DON colonized only the inoculated wheat spikelets (Ilgen *et al*, 2009). Disruption mutants of Fgl1, a secreted lipase regulated by MAP kinase, showed similar symptoms on wheat spikelets as the trichothecene mutants (Ilgen *et al*, 2008). The lipase mutants produced a higher amount of DON compared to the wild-type during initial infection of wheat spikelets, despite its inability to further colonize the spike (Ilgen *et al*, 2008). The two virulence factors, lipases and trichothecenes, showed to be present for an effective colonization of wheat (Ilgen *et al*, 2008).

Many studies involved eIF5A in mRNA degradation, translation as well as linking eIF5A with both poly (A)-binding protein and protein kinase C, suggesting important connections between these three proteins and their roles in RNA metabolism, including translation, mRNA decay, and ribosome biogenesis (Valentini *et al*, 2002). Furthermore, depletion of eIF5A causes a reduction in the total protein synthesis and an increase in the average time necessary for

ribosomes to transit along mRNAs (Gregio *et al*, 2009). The yeast homolog of the metazoan PKC gene encodes a regulator of one of the four mitogen- activated protein-kinase cascades characterized so far in *S. cerevisiae* (Valentini *et al*, 2002). PKC-signaling pathways can be activated by hypotonic and heat shock and they are involved with cell cycle control and cell wall biosynthesis, and are upstream regulators of the cell wall integrity signaling pathway WSC (Valentini *et al*, 2002). Despite ribosomal RNA and ribosomal protein mRNA transcription being inhibited during the stress response caused by a secretion block and this downregulation requires PKC and the WSC genes (Valentini et al, 2002), several results point to the role of eIF5A in regulating ribosome synthesis and, therefore, to the secretory pathway (Valentini *et al*, 2002; Zanelli and Valentini, 2007; Frigieri *et al*, 2008).

Thus, taken together, the high expression of fungal eIF5A, DHS and DOHH during plant infection assumes an important function of hypusine pathway in pathogencity, process of infection and may have a role in regulating other secreted virulence factors.

4.4 Inhibition of hypusine pathway is a possible application for suppression of fungal phytopathogens

The transcriptional activation of DHS of wheat and fungus during their pathogenic interaction was demonstrated and application of the guanylhydrazone CNI-1493, DHS inhibitor, during infection of wheat and maize flowers resulted in strong inhibition of the pathogen without interference with kernel development (Woriedh *et al*, in press). CNI-1493 inhibits fungal DHS and, thereby, prevents fungal germination and, consequently, fungal virulence without disturbing plant growth. The activity of eIF5A protein can indirectly be monitored by inhibition of its activating enzyme DHS. The enzyme DHS performs the first of two spermidine-dependent reactions. These reactions modify a lysine of eIF5A to hypusine, a highly conserved reaction in all eukaryotic cells (Park, 2006; Wolff *et al*, 2007).

During infection of wheat spikes by *F. graminearum*, DHS transcription was induced in wheat as well as in the fungal cells. Interestingly, DHS is not transcribed in wheat spikes during normal growth without stress. In fungal cells, however, the transcript of fungal DHS increased remarkably in infected spikes. This suggests that in the host as well as in the pathogen, the protein eIF5A transports, upon activities, mRNAs specifically needed through the pathogenic interaction. The notion that eIF5A participates in the posttranscriptional processing (i.e. stabilization, transport and translation) of a subpopulation of specific mRNAs is supported by the finding that this protein exhibits RNA-binding properties (Xu and Chen,

2001) and acts as a cellular cofactor of the HIV-1 Rev regulatory protein, which mediates the nucleocytoplasmic transport of specific retroviral transcripts (Bevec *et al*, 1996). In consequence, interference with the hypusine-modification of eIF5A effectively inhibits the replication of HIV-1 or feline immunodeficiency virus (FIV), another Rev-encoding lentivirus (Andrus *et al*, 1998; Hart *et al*, 2002; Hauber *et al*, 2005; Schaefer *et al*, 2006). Thus, eIF5A proved to selectively transport a subset of cellular mRNAs out of the nucleus to the ribosomes, thereby initiating their translation (Zanelli and Valentini, 2007; Lee *et al*, 2009). Furthermore, immunocytochemical analyses showed that the hypusinated eIF5A is primarily localized in cytoplasm, while the non-hypusinated eIF5A precursor distributes in both cytoplasm and nucleus (Lee *et al*, 2009).

In accordance with the inhibition of fungal germination, CNI-1493 displayed a powerful antifungal activity by controlling *Fusarium* infections of wheat and maize. When added to the fungal inoculum at a concentration of 10 µM, *Fusarium* infection was prevented completely and no visible symptoms were detectable. Surprisingly, the concomitant development of the plant kernel remained unaffected in spite of the application of the DHS inhibitor. The CNI-1493 treated wheat and maize plants did not show any negative effects, they displayed normal growth and normal kernel development. Similarly, when wheat kernels were incubated in CNI-1493, even a concentration of 50 µM effected neither kernel germination nor further seedling development (Woriedh *et al*, in press).

The mechanism underlying the different susceptibility of wheat and *Fusarium* to CNI-1493 can be speculated that the plant cuticle might prevent the uptake of the inhibitor. All aerial parts of plants are covered by a cuticle, whose major component is cutin, an insoluble lipid-derived polyester. These plant cuticles function as permeability barriers for water and water-soluble materials, playing important roles in plant growth, environmental adaptation and plant protection against environmental stresses (Kolattukudy, 1996). In agreement with this notion, it has been previously demonstrated that different cell lines take up DHS inhibitors differently and that their inhibitory effect varies in dependence of the uptake efficiency (Lee *et al*, 1995; Lee *et al*, 2003). This might reflect different inhibition levels of DHS enzyme activity in plants, where complete suppression or deletion of the gene is lethal (Duguay *et al*, 2007). Therefore, the phenotype of a mutant plant with down regulated DHS expression depends on the remaining cellular amount of DHS (Thompson *et al.* 2004; Wang *et al.* 2001; 2003; 2005). In contrast, the selective suppression of DHS in leaves enhanced growth without negative pleiotropic effects (Duguay *et al*, 2007).

A different explanation might be the observed induction of DHS transcription during infection of wheat. DHS transcripts of the invading fungus were already detectable one day after inoculation while, in contrast, the corresponding wheat gene transcripts were detectable only after 3 days. Keeping in mind that DHS transcripts are not detectable in the spike during stress-free development, it might be reasonable that the early inhibition of fungal development by CNI-1493 prevents the induction of the wheat DHS gene. Therefore, a DHS inhibitor is without effect in wheat, because its DHS target is lacking.

Thus, the DHS inhibitor CNI-1493 selectively inhibits fungal development and effectively prevents *Fusarium* infection of wheat and maize, without disturbing plant development. Like eIF5A, DHS genes are highly conserved (Wolff *et al*, 2007); therefore, targeting DHS in the fungal kingdom by inhibitors, such as CNI-1493, appears to be a novel and valuable approach for protection of plant growth against fungal infection and a lead substance for the development of new anti-fungal agents.

4.5 Overexpression of *Fusarium* DHS and DOHH reveals contrary singnaling pathways and a dual balanced signaling for double overexpression

The ideal test of a virulence factor is to compare biological responses in fungi with and without the factor. Such comparisons have traditionally required the isolation of mutant strains. Gene knockout mutations of *Fusarium* DHS and DOHH resulted in lethal mutants as discussed above; therefore, gain of function of these two genes was studied and characterized.

4.5.1 Aggressiveness of DHS mutansts, nonpathogenicity of DOHH mutants and correlation to DON and ROS

Gene overexpression of DHS resulted in mutants with massively increased production of DON and increased aggressiveness toward wheat. In contrast, DOHH overexpressing muntans lost their pathogenicity and the ability of infection toward wheat with massively decreased production of DON. The balance was achieved in double overexpressing mutants that showed a similar virulence infection toward wheat like WT with a slight increased aggressiveness and a slight increased production of DON. The results not only identify a novel mechanism of regulation of DHS, DOHH, DON production and virulence in *F. graminearum*, but also point out the potential of this pathogen to evolve with an ability to produce massive amounts of toxins and virulence.

Other factors contributing to aggressiveness were correlated. Correlation was significant between DON production and severity of strain colonization. Mycotoxin DON was found to elicit ROS production and programmed cell death in infected wheat tissue. These results were observed when the aggressive DHS mutants produced more DON and, in turn, more ROS and programmed cell death in infected wheat spikes than the DOHH mutants produced. Also, the production of ROS and programmed cell death in infected wheat spikes with double overexpresssing mutants was slightly higher than in infected tissue with WT. This agrees with the correlation between DON production and aggressiveness of double mutants and WT.

Previous work has shown that production of DON plays an important part in fungal colonization of host tissues in both FHB and maize red cob caused by the fungal pathogens *F. graminearum* (Jansen *et al.*, 2005; Maier *et al.*, 2006). Interestingly, the work described here indicates that DON can also induce a range of classical plant defence responses in wheat, including the production of ROS that may be at least partially responsible for the induction of host defence gene transcripts, their protein products and programmed cell death. This suggests that DON may assist necrotrophic growth of the pathogen by promoting host cell death, while also stimulating an antimicrobial defence response in the host. These contrasting effects of DON may influence the rate and extent of disease development during FHB of wheat. Host cell death and H2O2 production all appeared to require the application of relatively high concentrations of DON. The experiments suggest that the level of production of DON affects wheat cellular responses in a way that could either promote necrotrophic fungal growth by initiating programmed cell death or reduce fungal growth by triggering defence gene expression and accumulation of antimicrobial proteins (Desmond *et al*, 2008).

The effect of DON on cell function has been most extensively studied in animal cells in attempts to understand its toxicity (Pestka *et al*, 2004). These studies have resulted in a model where trichothecenes inhibit protein synthesis by binding to the 60S ribosomal subunit, activating a cell signaling programme that results in apoptosis. The role of DON and other trichothecene mycotoxins during pathogenesis has been analysed using mutant fungal strains that do not produce toxin (Hohn and Desjardins, 1992). Studies on a strain of *F. greaminearum* that has a mutation in the Tri5 gene encoding a DON biosynthetic enzyme have shown that *F. greaminearum* strains unable to produce DON are less aggressive during FHB in both wheat and barley (Boddu *et al*, 2007). More specifically in wheat, the mycotoxin appears to be necessary for fungal passage from infected florets into the rachis from where it can further colonize the head (Jansen *et al*, 2005). These DON-non-producing *F. greaminearum* strains are unable to prevent thickening of host cell walls after penetration and so their movement from

the point of infection is hindered (Jansen *et al*, 2005; Maier *et al*, 2006). There is also evidence that FHB-resistant wheat genotypes accumulate far less DON than susceptible ones (Goswami and Kistler, 2005; Miedaner *et al*, 2006). For FHB, it therefore appears that occurrence of disease may not be dependent on the toxin, but DON production does affect disease levels, and so improving resistance to DON may improve resistance to the disease. Several studies have shown that fungal derived toxins can elicit responses in plants that have aspects in common with well-known pathogen-induced responses (Desmond *et al*, 2008). In wheat, differential display analysis has been used to demonstrate that DON affects transcript levels of a few specific host genes in roots, including peroxidase genes (Desmond et al, 2008). However, the physiological and molecular responses of wheat cells to DON exposure have not been described. The effects of DON on wheat defence responses observed the production of H2O2, a well-known signaling molecule, followed by cell death, a phenomenon frequently observed in mammalian cell lines exposed to DON (Pestka *et al*, 2004). In addition, DON induced a range of well-known defence genes and, interestingly, a DON-producing fungal strain induced higher levels of defence transcripts than a DON-non-producing mutant during disease development. Both cell death and defence gene induction were reduced by co-treatment of DON and an antioxidant that would scavenge free radicals such as H2O2. These results suggest that DON produced during *Fusarium*-related diseases may play a role in the activation of wheat defence responses and cell death, at least partially via the signaling molecule H2O2 (Desmond *et al*, 2008).

It has been documented that, in some *Fusarium* species and with the same conditions, toxin production by *Fusarium* strains may vary, drastically. Some strains are able to produce large amounts of trichothecenes, while others produce small or undetectable amounts of trichothecenes (Bakan *et al*, 2002). Aggressiveness is the quantity of disease induced by a pathogenic isolate on a susceptible host (Van der Plank, 1968). However, little is known about the genetic basis of aggressiveness and DON production by *F. graminearum*. Knowledge of genetics of aggressiveness and DON production of *F. graminearum* is important in developing resistant cultivars and in estimating durability of resistance. Resistance to *F. graminearum* head blight in wheat is quantitatively inherited. All known cultivars are infected but the degree of infection varies greatly (Bai and Shaner, 2004). The finding that trichothecenes enhance the aggressiveness of *F. graminearum* suggests that it could be possible to reduce head blight of wheat and mycotoxin problems caused by this fungus by breeding trichothecene-resistant crops. Disease-causing capacity of the trichothecene-deficient mutant of *F. graminearum*, however, indicates that there are other factors that contribute to aggressiveness, such as cell-wall degrading enzymes (Jenczmionka *et al*, 2003; Voigt *et al*,

2005), other metabolites and hypusine pathway as a new discovered factor in this study. Studies on host resistance and genetic modification of the pathogen are required to understand the role of DON, DHS and DOHH in plant pathogenesis. Resistant cultivars will remain the most practical and effective control against head blight of wheat. Correlation between head blight rating, DON production, and overexpression of DHS and DOHH was high. For the latter, however, correlation with fungal colonization and ROS production was also high. In pathosystems, quantitative variation of aggressiveness and resistance was tightly correlated with *Fusarium* DHS and DOHH content. These genes are either fungal virulence factors or are involved in various fungal developmental processes or secondary metabolism, such as polyketide synthases and signaling components.

Hypusinated eIF5A is required for the translation of certain mRNAs in yeast cells, the products of which presumably regulate progression through the cell cycle (Gentz *et al*, 2009). Yeast eIF5A interacts with the structural components of the 80S ribosome, possibly playing a role in translation elongation (Gentz *et al*, 2009). The protein may also be involved in ribosome synthesis, RNA metabolism and mRNA turnover (Gentz *et al*, 2009). Experiments using SELEX RNA have shown that eIF5A binds specific RNAs containing the two consensus sequence motifs, UAACCA AAAUGUCACAC, and that RNA binding is hypusine dependent (Gentz *et al*, 2009). Initially, eIF5A was characterized as a translation initiation factor, based on its activity in stimulating methionyl-puromycin synthesis, a model assay of the first peptide bond formation (Dias *et al*, 2008). Because depletion of eIF5A in yeast causes an increase in the number of G1-arrested cells, and inhibitors of DOHH cause cell cycle arrest in mammalian cells at the G1⁄ S boundary, it was hypothesized that eIF5A may be important for the translation of mRNAs encoding specific proteins required for cell cycle progression, probably those involved in S-phase onset (Dias *et al*, 2008). It has been demonstrated recently that eIF5A physically interacts with structural components of the 80S ribosome as well as with the translation elongation factors. eIF5A was shown to co-fractionate with monosomes in a translation-dependent manner, and eIF5A mutant strains show altered polysome profiles and are sensitive to translation inhibitors. Thus, these results point to a function for eIF5A in translation, although it is still not known whether eIF5A affects the translation of all mRNAs or a subset of specific mRNAs (Dias *et al*, 2008). Moreover, eIF5A was shown to associate physically with membranes and this association is ribosome-dependent, while results confirrm the link between translation and vesicular trafficking and reinforce the implication of eIF5A in protein synthesis (Frigieri *et al*, 2008). This suggested a model in which these proteins work together in the cell to allow proper protein synthesis and secretion necessary for bud formation during G1/S transition (Frigieri *et al*, 2008).

Nevertheless, in contrast to the eIF5A (intermediate), no reversal was observed with hypusinated eIF5A (Park, 2006). Park *et al.* suggested that the first step of hypusine synthesis by DHS is reversible, and the second step, DOHH-mediated hydroxylation, locks eIF5A into an active hypusine form, thereby making the overall reaction an irreversible protein modification (Park, 2006). Lee *et al.* found that the precursor is localized in both cytoplasm and nucleus, while the hypusinated eIF5A is primarily localized in cytoplasm (Lee *et al*, 2009). These results support the finding that overexpresion of DHS may activate the reversible reaction in two directions, which can facilitate interconversion of spermidine, eIF5A (intermediate) and in turn, interferes in activation of eIF5A as nucleocytoplasmatic shuttle protein of a subset of mRNAs. Also, overexpression of DOHH may activate and highly precede the accumulation of hypusinated eIF5A, which may reflect the contrary signaling of the overexpressed DHS and DOHH. Finally, Kang *et al.* found that protein kinase CK2 phosphorylates and interacts with DHS and suggested that DHS and its phosphorylation modification may have other independent cellular functions because of versatile roles of DHS (Kang and Chung, 2003).

4.5.2 DHS and DOHH mutations involved in conidial differentation, growth rate and anastomosis which showed a requirement for virulence

The isolation of overexpressed DHS and DOHH conidia enabled the corresponding gene to be characterized and the transcript to be identified in germinating conidia. DHS/ DOHH-dependent activity was detected in ungerminated conidia. These data suggest that DHS and DOHH are involved in conidial development. To substantiate this, the responses of developing conidia to various concentrations, various temperatures and various surfaces, including exposure to the host leaf, nutrient agar (fully inductive formation), and glass (non-inductive) revealed that DHS/ DOHH either promoted or inhibited conidial differentiation.

SNA is a weak nutrient agar used for the identification and maintenance of strains of *Fusarium*, and promotes sporulation and good conidiogeneous cell development (Leslie and Summerell, 2006). Culture degeneration, which is common on many synthetic media, usually does not occur on SNA, but it did in DOHH overexpressing mutants. All *Fusarium* strains grew well vegetatively on rich media, but growth rates varied upon changing the temperature. No growth was observed at low and high temperatures. Overexpression of DOHH aggressively reduced the growth rate on all media, while mycelial density and protein content of all strains were approximately the same. Overexpression of DOHH increased production of the red pigment aurofusarin and reduced mycelial growth. Moreover, overexpression of GIP2, a transcription factor that regulates the aurofusarin biosynthetic gene, was also found to

increase aurofusarin production and reduced mycelial growth (Kim *et al*, 2006a). Thus, Aurofusarin production was negatively correlated with vegetative growth by *F. graminearum* (Kim *et al*, 2006a). This correlates tightly the overexpression of DOHH, in turn, hypusine pathway, with aurofusarin production, vegetative growth, and regulation of GIP2 and other polyketides of secondary metabolites.

On SNA, overexpression of DHS promotes conidial septation and elongation. The events regulating septum formation have been critically examined in yeast cells, particularly temperature sensitive mutants of *S. cerevisiae* and *S. pombe* (Cole, 1986). Septation is the final and essential event of the budding process which culminates in secession of daughter cell from maternal cell (Cole, 1986). Therefore, the timing of septation would be synchronized with events which partition cytoplasmic components between these two cells. Indeed, mutants arrested in deoxyribonucleic acid synthesis and nuclear division fail to construct a septum. An additional mutant has been identified in which bud formation occurs but cytokinesis is blocked. The dividing cells were unable to form a normal septum (Cole, 1986). Frigieri *et al.* suggested a model in which eIF5A works in the cell to allow proper protein synthesis and secretion necessary for bud formation during G1/S transition (Frigieri *et al*, 2008). Recent findings re-established a function eIF5A in translation and suggested a role for this factor in translation elongation instead of translation initiation (Zanelli *et al*, 2006; Saini *et al*, 2009). Other results showed that a temperature-sensitive mutant of eIF5A is suppressed by high-copy genes involved in actin cytoskeleton polarization and eIF5A function is essential for polarized growth, a process necessary for the G1/S transition in yeast (Zanelli *et al*, 2006; Saini *et al*, 2009). However, the mechanism triggered by eIF5A to accomplish this cell cycle function remains unclear and although this essential factor has been associated with different cellular events, its role could not be elucidated so far.

Conidial anastomosis tubes (CATs) can be recognized in 73 species of filamentous fungi covering 21 genera, and develop in culture and in host-pathogen systems. They have been shown to be morphologically and physiologically distinct from germ tubes in *Colletotrichum* and *Neurospora*, and under separate genetic control in *Neurospora* (Gabriela Roca *et al*, 2005). CATs are short, thin, usually unbranched and arise from conidia or germ tubes. Their formation is conidium-density dependent, and CATs grow towards each other. MAP kinase mutants of *Neurospora* are blocked in CAT induction (Gabriela Roca *et al*, 2005). Nuclei pass through fused CATs and are potential agents of gene exchange between individuals of the same and different species. CAT fusion may also serve to improve the chances of colony establishment (Gabriela Roca et al, 2005). A fungal mycelium is typically composed of radially extending

hyphal filaments interconnected by bridges created through anastomoses. These bridges facilitate the dissemination of nutrients, water, and signaling molecules throughout the colony(Craven *et al*, 2008). Overexpression of *Fusarium* DOHH resulted in both the loss of self-anastomosis and pathogenicity on wheat, while overexpression of *Fusarium* DHS resulted in a high self-anastomosis and aggressivity on wheat as well as capability of non-self-fusion. Anastomosis events in DHS mutants are higher than in double mutants and wild-type. These results tightly correlate anastomosis and pathogenicity. Although Di Pietro *et al*. suggested that anastomosis is not essential for plant infection by *F. oxysporum* (Prados Rosales and Di Pietro, 2007), anastomosis showed to be required for virulence of the fungal necrotroph *Alternaria brassicicola* (Craven *et al*, 2008). This finding, combined with the discovery that a nonpathogenic *A. brassicicola* mutant defective for a mitogen-activated protein kinase gene (amk1) also lacked the capacity for self-anastomosis, suggests that self-anastomosis is associated with pathogenicity in *A. brassicicola* (Craven *et al*, 2008). In *Fusarium graminearum*, nuclear migration between conidia of the same strain and between conidia belonging to different strains was observed in this study. This suggests that genetic exchange may occur through intermingling of nuclei during anastomosis formation and opens the way to studies of vegetative compatibility in natural populations of *F. graminearum* under separate genetic control of DHS/ DOHH that promotes/ blocks CAT induction. Taken collectively, these data suggest that DHS and DOHH play a role in conidial differentiation, anastomosis regulation and colonization in this obligate, plant-pathogenic fungus.

4.5.3 Salt tolerance of DHS mutants, fungicide resistance of DOHH mutants and regulation of ROS in fungal development

F. graminearum mutants reacted in various ways when incubated in the presence of sub-lethal concentrations of salt, H2O2 and fungicides such as Azoxystrobin. The total amount of programmed cell death in the strains increased with increasing the concentration of saline or fungicide in the growth medium and was tightly correlated to the sensivity of the strains to salt and Azoxystrobin concentration. Reactive oxygen species (ROS) showed to play a regulation role in fungal development under these conditions and under oxidative stress.

A low concentration of salt increased growth rate of all strains, but with an increasing salinity, a decrease in the growth rate of mutants was observed. The percentage of germinated conidia decreased with increasing salinity. Concentration of 0.5M NaCl produced structures in all strains and increased fusarin production in DOHH mutants, which decreased with increasing salt stress. Concentration of 1.5M NaCl decreased growth rates of DOHH and double mutants

and suppressed growth of WT. DHS mutants were more tolerant to the toxin than other strains and grew at 2M NaCl. The present study suggests that there are some differences in tolerance with regard to both germination and mycelial extension in response to osmotic imposed water stress. The total cell water potential is the sum of the solute potential and the turgor pressure of the cell wall. When cells are exposed to water stress, low molecular mass compounds often are synthesized or accumulated intracellularly to equilibrate the cytoplasm with that of the surrounding environment (Ramirez and Chulze, 2004). This is the first detailed study and measurement of the levels of *F. graminearum* mycelia as effected by osmotic water stress. The results showed that mycelia decreased with decreasing medium water potential, regardless of overexpression of DHS or DOHH. The decrease in total cellular water potential is necessary for the extraction of water from the substrate and its translocation to the growing mycelial front. This can be done effectively only by maintaining a water potential gradient from the substrate into the hyphal cells, which also facilitates the functioning of enzyme systems (Ramirez and Chulze, 2004).

There is a wealth of information on the physiological adaptation of yeast and some filamentous fungi to solute stress, which shows that the patterns of accumulation of sugar alcohols and sugars by *F. graminearum* are modified significantly by osmotic and matric water stress, perhaps via passive diffusion and some endogenous synthesis (Blomberg and Adler, 1992; Ramirez and Chulze, 2004). It was demonstrated that *F. graminearum* accumulates a combination of different sugar alcohols like many fungi in response to osmotic stress (Ramirez and Chulze, 2004), and an organism might produce a cocktail of osmolytes rather than a single compound (Davis *et al*, 2000). For example, mixtures of sugar alcohols increased the water pressure in a more efficient way and also may reduce the toxicity associated with high concentrations of a single osmolyte and obviate feedback mechanisms that down regulate metabolic pathways in the presence of high concentrations of product (Ramirez and Chulze, 2004). These studies enable a better understanding of the survival and growth strategy employed by *F. graminearum* overexpressing DHS in regulating metabolic pathways for survival, growth and establishment in natural ecosystems.

Furthermore, resistance to Azoxystrobin was significantly observed in DOHH and double mutants, while DHS mutants showed sensitivity compared to WT. DOHH mutants were strongly resistant and germinated at high concentration. In *Alternaria* isolates from pistachio in California, Azoxystrobin resistance correlated with a single mutation in the cytochrome *b* (cyt *b*) gene causing a change of glycine to alanine at amino acid position 143 (Luoa *et al*, 2007). Kaneko *et al*.showed that *F. graminearum* and *Microdochium nivale* have distinct sensitivities

to strobilurin fungicides, which inhibit activity of complex III in the mitochondrial electron transport chain. When mycelia were cultured in medium with the strobilurin fungicide azoxystrobin, *F. graminearum* increased its oxygen-consumption, but *M. nivale*, which is more sensitive than *Fusarium* species to strobilurins, did not (Kaneko and Ishii, 2009). There was no increase in oxygen consumption in *F. graminearum* by the treatment with Azoxystrobin and alternative oxidase (AOX) inhibitor n-propyl gallate. Azoxystrobin was found to enhance the generation of intracellular H2O2 in both fungi (Kaneko and Ishii, 2009). Activities of antioxidant enzymes in *M. nivale* were consistently higher than in *F. graminearum* and transcription of AOX in *F. graminearum* and *M. nivale* was induced by Azoxystrobin, suggesting that AOX activities in both fungi are regulated at the transcriptional level (Kaneko and Ishii, 2009). AOX transcription in *F. graminearum* was rapidly induced by Azoxystrobin. On the contrary, induction in *M. nivale* was low and slow (Kaneko and Ishii, 2009). These results suggested that differential ability of *F. graminearum* to activate AOX transcription is involved in the difference of their sensitivity to Azoxystrobin. Studies of the fitness of azoxystrobin-resistant isolates of *Ustilago maydis* showed that these mutations appeared to be pleiotropic, having significant adverse effects on growth in liquid culture and pathogenicity on young corn plants (Ziogas *et al*, 2002) with the same adverse effects of DOHH mutants. Moreover, DOHH enzyme recently showed to have a nonheme diiron active center that activates O2 (Vu *et al*, 2009), which may explain the resistance of DOHH overexpressing mutants to Azoxystrobin. Recombinant human deoxyhypusine hydroxylase (DOHH) has been reported to have oxygen- and iron-dependent activity. Also, DOHH has an antiferromagnetically coupled diiron center with histidines and carboxylates as likely ligands that carries out the hydroxylation of the deoxyhypusine residue present in the elF5A substrate, too (Vu *et al*, 2009).

The generation of reactive oxygen species (ROS) is considered one of the primary events under a variety of stress conditions, but data obtained from this study reported that DHS mutants accumulated more ROS, while DOHH mutants produced less ROS. NBT staining showed different localization patterns of ROS among the mutants as well as mutants showed tolerance to oxidative stress compared to WT. Surprisingly, double mutants had the highest tolerance. Suppression of the spore germination by high H2O2 can be easily explained by the severe cell damage, but this is unlikely for its very low concentrations. It seems likely that H2O2 suppressed the germination at a regulatory level in the second case. Lack of the effect at 50mM H2O2 possibly means that such concentration was insufficient for immediate damage but it was too high for participation in regulatory mechanisms. Decrease in surface area is one

of the mechanisms of fungal mycelial adaptation to an increase in ROS in the environment upon oxidative stress.

ROS are formed by fungi in the course of metabolic activity. ROS production increases in fungi due to various stress agents such as starvation, light, mechanical damage, and interactions with some other living organisms (Gessler *et al*, 2007). DHS and DOHH appear to be involved in ROS accumulation and localization, while regulation of ROS level appears to be very important during development of the fungal organism. In fungal hyphae, apical dominance refers to the suppression of secondary polarity axes in the general vicinity of a growing hyphal tip. The mechanisms underlying apical dominance remain largely undefined (Semighini and Harris, 2008). Localized accumulation of ROS in the apical region of *Aspergillus nidulans* hyphae revealed a correlation between localized production of ROS and enforcement of apical dominance and altered patterns of ROS localization correlate with the polarity and branching phenotypes (Semighini and Harris, 2008). Studies in other highly polarized cell types strongly suggest that ROS play a key role in the spatial regulation of polar growth. In Arabidopsis, the formation of root hairs from epidermal cells is preceded by the localized accumulation of ROS at the polarization site (Foreman *et al*, 2003). ROS may promote calcium influx by activating plasma-membrane calcium channels. The subsequent accumulation of calcium at hyphae would presumably affect the dynamics of the actin cytoskeleton as well as the vesicle trafficking machinery (Semighini and Harris, 2008). In addition, ROS may also modulate MAP kinase-signaling pathways that impinge upon morphogenesis (Takemoto *et al*, 2007). Hydrogen peroxide has been implicated in the localized softening of the cell wall during the extension of highly polarized plant cells (Hovav *et al*, 2008) and could conceivably play a similar role in the hyphae. Experimentally detected relationships of developmental processes with the action of factors increasing intracellular ROS concentration indicated that ROS act as signaling molecules regulating physiological responses and developmental processes in fungi (Gessler *et al*, 2007). The toxicity of radicals and their role in pathological processes and aging are well known. However, recently ever more data are accumulated on specific intracellular enzymes producing ROS to regulate proliferation, differentiation, extracellular signal transduction, ion transport, immune response, and asexual and sexual reproduction (Droge, 2002), which may positively dominstrate the correlation between hypusine pathway and regulation of ROS. Thus, generation of $O2^{-}$ is related to specific stages of development in fungi. Intracellular enzyme systems produce ROS required for differentiation in fungi. Increase in intracellular ROS concentration prior to the next differentiation stage leading to formation of a new cell type has been shown in different classes of molds and fungi (Gessler *et al*, 2007).

It was reported recently that H2O2 was produced at the interface between mycelium cells and the plant in tomato leaves with the penetrated fungus *Botrytis cinerea* (Schouten *et al*, 2002). Clear evidence of fungal contribution to ROS production common with the plant has been obtained with fungi showing such activity outside the plant. For example, spores of *M. grisea*, the activator of rice blast disease, germinating in water generated H2O2, $O2^-$, and OH extracellularly (Gessler *et al*, 2007). Recognition of self and alien species upon hyphal contact appears to by a crucial point in development of mycelial fungi. Silar analyzed H2O2 generation by contacting mycelia in a large group of fungi. This activity increased upon interaction of *Coprinopsis cinerea* and *P. anserina*. Enhancement of activity was not observed upon their interaction with other fungi. Increase in activity was also not observed upon the contact of the colony of one of these types with initially nonliving material-killed hyphae or yeast. It is of interest that the same reaction was provoked by some bacteria. It has been concluded that H2O2 serves as one of the signals for identification of alien organisms by hyphae (Silar, 2005; Gessler *et al*, 2007).

Recent results concluded that ROS might serve as regulators of development of fungal population depending on its density. It is known that fungal development is inhibited not only in excessively dense spore suspensions, but also in overly diluted ones (Blakeman, 1980). Self-suppression of spore germination in dense spore suspension could be explained by deficiency of vital resources and self-intoxication by vital activity products. It turned out unexpectedly that upon too high or extremely low spore concentrations, self-uppression of fungal development was decreased or completely eliminated upon addition of an antioxidant (Blakeman, 1980). The results led to the conclusion that germinating spores excrete ROS, which suppress spore development depending on its density. (Gessler *et al*, 2007). At the same time, self-regulation of spore development will serve as a positive factor for the causative disease agent, if it suppresses useless spore germination under conditions unfavorable. Intracellular ROS increase is accompanied by the cessation of growth, and it provokes morphological changes leading to cell adaptation to changes in life conditions as well as the decrease in intracellular oxidants. Numerous experimental data support the relationship between triggering of differentiation processes and an increase in intracellular ROS concentrations (Aguirre *et al*, 2005). H2O2 is considered as one of the most important metabolites in all respiring cells that promotes transition to filamentous growth in *U. maydis* and development of its pathogenicity (Leuthner *et al*, 2005). It is generally accepted that antioxidants are an important ROS counterbalance and defend the organism from exceeding stresses. Sugar alcohols, e.g. mannitol and arabitol, which are abundant in fungal cells, are also apparently found to play an antioxidant role (Gessler *et al*, 2007).

It has been shown that more than half of the *S. Cerevisiae* genome is related to yeast adaptation and to such stress factors as starvation, temperature shifts, oxidative stress, and variations in pH value and medium osmolarity. As a response to different oxidants, specific changes in gene expression occur (Pocsi *et al*, 2005). In response, various oxidants not only ROS origin but their concentration as well influences the response of the organism, which points to specificity of ROS signal transduction (Pocsi *et al*, 2005). ROS signal transduction to the transcription factors in fungi might follow different pathways common to all living systems: two-component phosphorylation system (histidine/aspartate phosphorylation), G-proteins, mitogen-activated protein kinase (MAPK) cascade (Gessler *et al*, 2007). Finally, adaptation to environmental cues and organism survival in extreme conditions appear to be provided by cell efficiency in bringing into action ROS signal transduction rapidly and specifically.

4.6 Plant DHS is involved in tolerance to environmental stress

In this study, wheat and maize DHS were identified after inducing senescence, salt stress and drought stress. Wheat DHS was also induced and upregulated during pathogenic interactions, i.e. fungal infection, suggesting a critical role for DHS expression in plant growth, stress and defense.

Upregulation of eIF5A and DHS expression during environmental stress was reported in previous studies (Wang *et al*, 2001; Wang *et al*, 2003; Thompson *et al*, 2004). Recombinant plant DHS, like its mammalian counterpart, has been shown capable of catalyzing the formation of deoxyhypusine in precursor plant eIF5A (Ober and Hartmann, 1999; Wang *et al*, 2001). In Arabidopsis, there is only one DHS gene, but there are multiple members of the eIF5A gene family. It has, therefore, been proposed that the single DHS enzyme activates all of the eIF5A isoforms in Arabidopsis, and that DHS function is tightly correlated with the cellular requirement for activated eIF5A (Thompson *et al*, 2004). In light of this, DHS expression is likely under tight developmental regulation, presumably by a multi-element promoter that enables its upregulation, when there is a need to activate any or all of the eIF5A isoforms (Duguay *et al*, 2007).

Levels of DHS protein are upregulated during senescence, whether it occurs naturally or is induced prematurely by environmental stress. In this study, there is a pronounced high level of DHS protein in the treated plants of wheat and maize (leaves, stems, and roots) during salt and drought stress, while no induction of DHS is reported in the control plants of wheat and maize.

In a similar result, levels of DHS and eIF5A protein have been found to increase in tomato leaves, in which senescence was induced prematurely by chilling stress (Wang *et al*, 2001). Moreover, constitutive suppression of DHS has been shown to delay the onset of senescence in leaves of Arabidopsis and canola and in tomato fruit (Wang *et al*, 2003; Wang *et al*, 2005a; Wang *et al*, 2005b). However, in each case there were additional pleiotropic effects depending on the degree of suppression. These included enhanced growth and increased tolerance to environmental stress. These observations were interpreted as indicating that DHS plays a role in mediating natural senescence as well as premature senescence induced by environmental stress. Wheat and maize are sensitive to environmental stress. This was demonstrated in the present study by treating the plants with PEG and NaCl, a treatment that results in osmotic stress analogous to that imposed by conditions of drought and salinity. When plants were treated with 5% PEG or 2% NaCl on alternate days, anthocyanin formation, which is a symptom of stress (Chalker-Scott, 1999), was clearly evident in the leaves within 15 days. This was also evident from the fact that anthocyanin was not visible in the leaves of control plants. This type of programmed cell death in leaves is sometimes referred to as necrosis rather than senescence, because chlorosis, which defines the progressive yellowing of leaves, reflects chlorophyll degradation during natural leaf senescence (Figure 9).

External factors that induce premature initiation of senescence, which is also referred to as necrosis or apoptosis, include environmental stresses such as temperature, drought, salinity, poor light or nutrient supply, as well as pathogen attack. Plant tissues exposed to environmental stress also produce proline and ethylene, commonly known as stress ethylene (Narayana *et al*, 1991; Buchanan-Wollaston, 1997; Cao *et al*, 2007). Ethylene is known to cause senescence in some plants. Senescence is not a passive process, but rather an actively regulated process that involves coordinated expression of specific genes. The osmolite amino acid proline is a unique function of a pathway regulated by ABA and accumulated in response to osmotic, salt, and drought stresses, as a part of the machinery responsible for the adjustment of plants to these stresses (Claussen, 2005).

During senescence, the levels of total RNA decrease and the expression of many genes is switched off. However, there is increasing evidence that the senescence process depends on de novo transcription of nuclear genes. For example, senescence is blocked by inhibitors of mRNA and protein synthesis and enucleation (Hensel *et al*, 1993; Procházková and Wilhelmová, 2007). Similarly, plants exhibiting increased resistance to environmental stress, e. g., decreased susceptibility to low temperature (chilling), drought, infection, etc., and/or

increased resistance to pathogens, are selected as superior products (Wang *et al*, 2003; Duguay *et al*, 2007).

It is also consistent with reports that there is an accumulation of eIF5A within the nuclei of mammalian cells undergoing apoptosis (Tome *et al*, 1997; Tome and Gerner, 1997; Beninati *et al*, 1998; Caraglia *et al*, 2003; Taylor *et al*, 2007). eIF5A is also reported to selectively transport a subset of cellular mRNAs out of the nucleus to the ribosomes, thereby initiating their translation (Zanelli and Valentini, 2007; Lee *et al*, 2009). DHS is known to catalyze the first of two reactions that hypusinate eIF5A resulting an activation of eIF5A (Cooper *et al*, 1983; Park *et al*, 1993a; Park *et al*, 2010). Therefore, the objective of this investigation was to evaluate the effects of salt and drought on growth, and at the same time try to correlate these effects with changes in DHS expression. It is clear from the present study that DHS and, in turn eIF5A, is highly expressed at the onset of senescence, whether or not there is mobilization of nutrients out of the senescing tissue, and also in the event of necrosis by pathogen attack. It seems likely, therefore, that plant DHS and eIF5A facilitate the translation of mRNAs required for programmed cell death regardless of how it is initiated, but they may not be involved in the regulation of the mobilization of nutrients that accompanies some types of programmed cell death (Thompson *et al*, 2004; Hopkins *et al*, 2008). In addition, the pleiotropic effects of DHS suppression in *Arabidopsis thaliana* indicate that DHS protein plays a central role in plant development, senescence, and tolerance to environmental stress.

4.7 Factors controlling transformation efficiency in wheat and maize

Numerous transgenic events were produced from Florida and HiIIA X HiIIB immature zygotic embryos transformed using Gun bombardment and *A. tumefaciens* standard binary vector system. Southern blot analyses confirmed integration of the transgenes in the wheat and maize genome. Transgenic plants from these experiments were fertile and normal inheritance and stable expression of the *bar* transgenes in T_1 and T_2 progeny was observed.

In previous studies, all analyzed events estimated transgene copy number ranged from one to five copies per genome. A similar range in copy number estimate was reported in transgenic events derived from *A. tumefaciens* super binary vector transformation of maize (Zhao and Ranch, 2006). In contrast, copy number estimates for biolistic gun-derived transgenic events of maize can range as high as 20.

The aim was also to produce high quality, stable DHS-RNAi and DHS-overexpressing lines that are available for further research. Because multicopy transgenes are often associated

with transcriptional silencing, transgenic lines that have single-copy T-DNA insertions were only identified and distributed. This decision was based on the premise that expression from such single-copy transgenes is likely to be more stable and reliable than from multicopy lines, especially after several generations. There is a report that single-copy RNAi lines silence target genes more efficiently than multicopy lines (Kerschen *et al*, 2004). An RNAi-based approach offered two main advantages. First, RNAi-induced mutations are dominant. Many of the assays needed to monitor the effect of reduced gene expression on epigenetic phenomena require a multiple-generation crossing scheme; dominant mutants reduce the number of crossing generations needed. The second advantage of RNAi is the potential to reduce expression of multiple, closely related genes with a single transgene locus (McGinnis et al, 2007). In a study that targeted a large number of genes, one of the parameters found to be important for silencing efficiency was transgene copy number (Kerschen et al, 2004; McGinnis et al, 2007). In Arabidopsis, lines bearing a single copy of an inverted repeat transgene silenced the endogenous target more efficiently than lines with multi-copy insertions (Kerschen et al, 2004). This parameter, together with several others, required optimization for large-scale study in crop plants. Transgene-induced RNAi was used to trigger specific silencing of genes in wheat and maize. In some cases, this technique was highly effective, resulting in a properly transmitted transgene that led to thorough reduction of target gene mRNA. In other cases, effectiveness was compromised by reduced transmission, transgene silencing, or failure of the IR construct to silence its gene targets. These inconsistencies in transgene transmission and silencing ability might result from the innate variability of RNAi as a biological system, from stochastic silencing or spontaneous rearrangement of the bombarded transgenes, or from inadvertently biased selection during tissue culture (McGinnis et al, 2007). The use of biolistic transformation, which frequently results in complex transgene loci, might also explain some of the transgene behaviors, including changes in transgene activity throughout development and biased transmission. In order to induce successful RNAi using an inverted repeat transgene, at least three distinct steps must occur: production of the dsRNA from the transgenic locus, processing of the dsRNA to form siRNAs, and degradation of the target RNA guided by the siRNAs. Although RNAi is a widely used technology, relatively little is known about the optimal application of this technique in plants. There are many potential factors that can contribute to a given sequence's ability to cause RNAi induced silencing of an endogenous target. The "21-bp rule" is commonly used to predict whether a given IR will target a given gene, meaning that 21 base pairs of 100% identity should be enough to trigger silencing. Additional analysis will be required to determine what characteristics can be used to model and predict the silencing effectiveness of a given inverted repeat sequence in plants (McGinnis et

al, 2007). In addition, experiments with dsRNA suggested that the 21-mers are produced in wheat at about one-quarter the rate of the 24 to 25-nt small RNAs with the observation that de novo dsRNA synthesis in wheat germ extracts is linked to production of siRNAs that are almost exclusively 25-nt long (Hamilton et al, 2002; Tang et al, 2003). The starting point for generating a given IR vector is a bioinformatics effort. By using known cDNA sequences or predicted gene sequences corresponding to the target gene of interest, primers are designed to amplify a portion of the cDNA by using reverse transcription-PCR (RT-PCR). If the target gene is a member of a multigene family, multiple alignments of family members will be needed to help guide the design of PCR primers. The region of the gene to be amplified and its similarity to other genes dictates whether the resulting IR construct is likely to target a single mRNA or transcripts of multiple related genes. It is demonstrated that RNAi-mediated gene silencing is effective in hexaploid wheat and can efficiently induce reduction of mRNA levels of homoeologous genes. RNAi can resolve the issue of genetic redundancy in hexaploid wheat in an efficient way, as it was also suggested by studies in the two allotetraploid species *Arabidopsis suenica* and cotton *Gossypium hirsutum* (Travella *et al*, 2006). In this study, a wheat DHS sequence of around 376 bp was used to construct the RNAi vector. A specific fragment is derived from a specific region, but it is known that homoeologous genes in wheat share up to 99% identity at the nucleotide sequence level in the coding regions (Kimbara *et al*, 2004). Therefore, there is a high chance that homoeologous genes will retain regions of identity, resulting in silencing of all the genes. The suppression of the homoeologous genes was probably also facilitated by the use of a sequence within the conserved regions identified among homologous wheat ESTs. Concerns have arisen that siRNA could cause other effects than those related to the knockdown of the target gene due to cross hybridization or binding in a sequence-dependent manner to various cellular proteins (off-target effects) (Jackson *et al*, 2003; Scacheri *et al*, 2004; Travella and Keller, 2009). Standard software can now be used for improving detection of sequence identity to accurately and systematically evaluate and minimize RNAi off-target effects between siRNA sequences and target genes (Qiu *et al*, 2005). The effectiveness of silencing is gene dependent and could reflect accessibility of target mRNA or the relative abundance of the target mRNA and the hpRNA in cells in which the gene is active (Travella *et al*, 2006). It was suggested that RNAi efficiency and the endogenous transcription level of the target gene are not necessarily related (Kerschen *et al*, 2004). In the early development of newly transformed T_0 seedlings, RNAi is not fully established, and; therefore, the mutant phenotypes differ from later generations. Thus, it might be advisable to not only study the T_0 generation in wheat RNAi projects but also in later generations. Previous results indicated that the most efficient silenced phenotypes are stably recovered in

homozygous lines, suggesting that the effect of RNAi in hexaploid wheat is gene-dosage dependent. This is possibly due to the progressive repression of the target gene with increasing allelic concentration of the transgene.

The Ubi-1 promoter has been shown to be highly active in monocots. These constructs may be useful for germinating high level gene expression of selectable markers to facilitate efficient transformation of monocots, to drive expression of reference reporter gene in studies of gene expression, and to provide expression of biotechnologically important protein products in transgenic plants.

Most overexpression studies employ a strong, constitutive promoter, such as the cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al*, 1985; Himmelbach *et al*, 2007), followed by phenotypic analysis of the transgenic plant. In many cases, ectopic expression experiments gave important insight into gene function (Jack *et al*, 1994). However, as possible consequence of ubiquitous overexpression and misdirection of gene products, undesirable pleiotropic effects on the plant may be caused. In addition, strong accumulation of unnecessary proteins leads to wasteful energy consumption, which could, in turn, generate phenotypes that are not directly correlated with the recombinant protein itself (Himmelbach *et al*, 2007). To avoid such unwanted pleiotropic effects that occlude phenotypic analysis, transgene expression can be controlled temporally and spatially by the use of cell- and tissue-specific (Vickers *et al*, 2006) or chemically inducible (Holtorf *et al*, 1995; Maizel and Weigel, 2004) promoters. Most promoters available to date are derived from dicotyledonous plants. Unfortunately, such promoters are typically dysfunctional in cereal species. Thus, expression of transgenes in cereals has been largely driven by ubiquitous promoters, such as those from the maize (*Zea mays*) ubiquitin 1 (Ubi1); (Christensen *et al*, 1992) or the rice (*Oryza sativa*) actin 1 gene (Act1); (Vickers *et al*, 2006). However, a few specific promoters derived from cereal species have been characterized and used to drive transgene expression (Himmelbach *et al*, 2007). To confine transgene expression to the cereal seeds, several grain-specific promoters, such as the oat (*Avena sativa*) AsGlo1 (Vickers *et al*, 2006), the barley hordein (Hor2-4, Hor3-1); (Cho *et al*, 2002), and the rice glutelin B1 (GluB1) (Himmelbach *et al*, 2007) promoters, have been employed recently. Even though drought inducible promoters were described for barley and rice (Xiao and Xue, 2001), stress-induced expression systems that are functionally verified in cereals or other monocotyledonous species are not yet available. As a consequence, there is growing demand for transformation technology that permits controllable expression of transgenes in cereals, which this work aimed to verify using a Cre-lox approach for conditional expression of the target gene by regulating of an inducible promoter.

In the course of doing high-throughput transformation of DHS into wheat and maize, promoter strength appears to be correlated with silencing and overexpression efficiency. For instance, the Meyerowitz laboratory (Chuang and Meyerowitz, 2000) showed that the strong CaMV 35S promoter is more efficient at inducing RNAi than is the weaker nopaline synthase promoter. A potential problem with an RNAi-based gene knockdown strategy is that a given target gene may be an essential gene whose knockdown is lethal, such as DHS. In this case, it may be impossible to generate and maintain a stably silenced line expressing the inverted repeat from a strong promoter. To circumvent such problems, an inducible gene expression system for inducing RNAi is desirable (McGinnis *et al*, 2005). Complete silencing of genes encoding a key element in basic cell functions or at particular developmental stages may result in lethality, whereas the reduced gene expression may give viable plants with phenotypes indicative of the role of the target gene. Systems to deliver inducible RNAi offer the advantage of silencing gene expression at specific developmental stages or in specific tissues, because they provide flexibility for the timing and the degree of gene inactivation and have the potential for reversal of silencing by withdrawal of the inducer (Guo et al, 2003; Wielopolska et al, 2005).Therefore, to characterize DHS in wheat and maize, the regulation of DHS-RNAi and DHS-overexpression was controlled by Cre-lox system with the inducible Soybean heat shock promoter.

The efficiency of wheat biolistic transformation and *A. tumefaciens*-mediated maize transformation depends strongly on the transformation parameters, the condition of the donor plant, and the plant genotype chosen for the transformation process (Witrzens *et al*, 1998; Pellegrineschi *et al*, 2002). A highly responsive wheat and maize genotype is advantageous, because it can enhance the efficiency of biolistic transformation and *Agrobacterium*-mediated transformation. To identify highly responsive genotypes, it is necessary to optimize and standardize tissue culture conditions and transformation efficiency, as well as identify the specific physiological conditions of the starting material for transformation (Pellegrineschi *et al*, 2002). For screening the material, the standardization of the physiological status of the donor plants was one of the most critical factors for comparing the transformation abilities among the selected plants. After testing under various conditions, a uniform non-stressed growth environment was selected for the optimal growth of the donor plants.

The choice of the zygotic embryo development stage was another important factor. Various development stages were screened for their response to the transformation process. The dimension of the embryo (1.5-2 mm on the longest side) was taken as standard in all accessions regardless of the number of days after pollination, because at this developmental stage the

scutella are more responsive to the tissue. The ability of the particle gun to consistently transform wheat has been previously reported (Vasil *et al*, 1992; Lonsdale *et al*, 1998; Witrzens *et al*, 1998).

Current transformation techniques in wheat and maize use tissue culture systems that involve disorganized cell growth, such as passage through a callus phase before plant regeneration. This callus phase usually induces somaclonal variation, i.e., mutations caused by the tissue culture process. Somaclonal variation found in wheat and maize regenerants include gross chromosomal changes such as aneuploidy, breakage, and rearrangements (Karp and Maddock, 1984; Dahleen *et al*, 2001), and changes in gene expression caused by point mutations, altered methylation patterns, or other modifications (Karp and Lazzeri, 1992). The majority of changes caused by tissue culture is undesirable for breeding improved cultivars, and includes reductions in test weight, 1000-kernel weight, percent plump seed, and yield (Bregitzer and Poulson, 1995). Examined somaclonal variation in the progeny of transgenic wheat showed that the transformation process (particle bombardment) increases variation above the level induced by the tissue culture process alone (Maheshwari *et al*, 1995). The extent of variability is partially a function of genotype (Carver and Johnson, 1989) and time in culture (Hartmann *et al*, 1989). A recent report (Dahleen *et al*, 2001) indicates that improvements in tissue culture, transformation techniques and targets may reduce the amount of somaclonal variation generated, decreasing the time needed for transgenic cultivar development. However, it has been noted that cereal transformation is still difficult because of the number of parameters involved in the technique (Vasil *et al*, 1992; Lonsdale *et al*, 1998; Pellegrineschi *et al*, 2002).

4.8 Prospects for transgene-mediated disease and abiotic stress resistance in wheat and maize

Both *Triticum aestivum* and *Zea mays* are capable of mounting defense responses, best characterized by interactions with diseases and abiotic stress. In these cases, resistance is governed by gene-for-gene interactions, and is accompanied by the hypersensitive response and the induction of mRNAs encoding pathogenesis-related (PR) proteins in case of *Fusarium* infection or abiotic stress-induced proteins for salinity and drought. This defense system increases in steady-state levels of some PR protein transcripts detected in pathogen-challenged wheat and maize and abiotically-stressed wheat and maize (Dahleen *et al*, 2001). It is possible that the abiotic and biotic environments have evolved mechanisms for evasion of or tolerance to the wheat and maize defense systems. To achieve some degree of protection against the FHB

pathogen and abiotic stress, native wheat and maize PR proteins may need to be engineered to accumulate earlier, more abundantly, faster, or in tissues other than currently occurs in challenged plants. Alternatively, heterologous genes for proteins with better anti-activity need to be introduced. Generation of transgenic plants from a new species has depended mainly on two factors: first, the development of transformation methods for cultivated plants and second, the knowledge of genes and their function. As mentioned previously, there are now transformation protocols available for the most important crops.

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