Deletion mutagenesis of two ABC transporter genes in *Fusarium graminearum* and characterisation of their roles in azole tolerance and virulence

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List of abbreviations

aa	Amino acid
ABC	ATP-binding cassette
BLAST	Basic local alignment search tool
bp	Base pair
CSPD	Chemiluminescent substrate for detection of phosphat (Disodium 3-(4-methoxyspiro 1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan-4-yl)phenyl phosphate
d	Days
dATP	Desoxyadenosintriphosphat
dCTP	Desoxycytidintriphosphat
dGTP	Desoxyguanosintriphosphat
DIG	Digoxigenin
DJ-PCR	Double joint PCR
DMI	Demethylase inhibitors
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dNPPs	Desoxyribonukleosidtriphosphate
dNTP	Deoxynucleosid triphosphate
DON	Deoxynivalenol
dTTP	Desoxytermedinintriphosphat
dUTP	Desoxyuridintriphosphat
FCR	Fusarium crown rot
FGSC	*F. graminearum* species complex
FHB	Fusarium head blight
FRR	Fusarium root rot
G418	Geneticin
gDNA	Genomic DNA
GER	Gibberella ear rot
GOF	Gain-of-function
h	Hour
Hph	Hygromycin B phospotransferase gene
Hyg	Hygromycin
M	Molar
MDR	Multidrug resistance

MRP	Multidrug resistance–associated protein
NBD	Nucleotide-binding domain
NCBI	National center for biotechnology information
NIV	Nivalenol
Nours	Nourceutricine
nt	nucleotide
Ø	Diameter
ORF	Open reading frame
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDR	pleiotropic drug resistance
PEG	Polyethylene glycol
pH	Lat.: *potential hydrogenii*, hydrogen ion concentration
rpm	Rotation per minute
RT	Room temperature
SBI class	Sterol biosynthesis inhibitors class
SDS	Sodium dodecyl sulfate
SNP	single nucleotide polymorphisms
Sp	Species
STC	Sorbitol-Tris-Calciumchlorid
TAE	Tris/Acetat/EDTA
Taq	*Thermus aquaticus*
TE	Tris/EDTA
Tebu	Tebuconazole
TE-Puffer	Tris-EDTA-buffer
TM	Melting temperature
TMD	Transmembrane domain
trpC	Tryptophan synthase C gene of *Aspergillus nidulans*
UV	Ultraviolet
v/v	Volume/volume (%)
WT	Wild-type
X	Times
YPD	Yeast pepton dextrose medium

ZEA	Zearalenone
Δ	Deletion mutant
µg	Microgram
µl	Microliter
15-ADON	15-acetyl-Deoxynivalenol

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A. Introduction

A.1. The plant pathogen *Fusarium graminearum*: Overview and taxonomy

The ascomycete fungus *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein) Petch), one of the most important and economically relevant plant pathogens, recently ranked fourth on a list of top 10 fungal plant pathogens based on scientific and economic importance (Dean *et al.*, 2012; Geiser *et al.*, 2013). Previously, *F. graminearum* has been divided into sixteen phylogenetically distinct species which were identified within the *F. graminearum* species complex (FGSC) (Boutigny *et al.*, 2011; O'Donnell *et al.*, 2000; O'Donnell *et al.*, 2004; Sarver *et al.*, 2011; Starkey *et al.*, 2007; Yli-Mattila *et al.*, 2009). One of these species (=lineage 7) kept the original name *F. graminearum*, all others received new names. The majority of species within the *F. graminearum* complex have evolutionary origins in the Southern Hemisphere (O'Donnell *et al.*, 2000; O'Donnell *et al.*, 2004) and Asia (Wang *et al.*, 2011; Yli-Mattila *et al.*, 2009). Recent biogeographic data suggest that ecological factors may have had a significant effect on the distribution of these species (Alvarez *et al.*, 2011; Astolfi *et al.*, 2011; Desjardins & Proctor, 2011; Lee *et al.*, 2009; O'Donnell *et al.*, 2004; Sampietro *et al.*, 2011; Zhang *et al.*, 2007). Recently, species of the FGSC have been introduced into non-indigenous areas, as a result of changed agronomy, trade and shipment of cereal products, and global climate change that have influenced the spread of these species, especially in areas that rely extensively on agricultural imports (O'Donnell *et al.*, 2004; Wang *et al.*, 2011). The FGSC comprises strains that may differ significantly in aggressiveness on cereal crops and in mycotoxin production (Goswami & Kistler, 2005). Species in the FGSC infect wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), maize (*Zea mays* L.) and other cereal crops across the world, causing diseases such as Fusarium head blight (FHB) and Gibberella ear rot (GER), and in addition infect several wild herbs and grasses (Boutigny *et al.*, 2011; Goswami & Kistler, 2004; Goswami *et al.*, 2006; O'Donnell *et al.*, 2000; Sampietro *et al.*, 2011; Starkey *et al.*, 2007). Fusarium crown rot (FCR) and Fusarium root rot (FRR) are also important diseases of wheat that can be caused amongst others by *F. graminearum* (Gardiner *et al.*, 2013; McMullen *et al.*, 1997). *F. graminearum* has been discovered in a broad range of environments and it is described to be the main causal FHB agent in the USA, Canada, and Europe (Goswami & Kistler, 2004; McMullen *et al.*, 1997; Trail, 2009; Yli-Mattila *et al.*, 2009).

However, *F. graminearum* infections were also regularly documented in Asia, Australia and South-America (Astolfi *et al.*, 2011; Obanor *et al.*, 2013; Zhang *et al.*, 2007). The FHB has re-emerged over the past two decades as one of the most economically devastating plant diseases around the world causing high losses to the grain industry (Goswami & Kistler, 2004; McMullen *et al.*, 2012; Wang *et al.*, 2011; Windels, 2000). *F. graminearum* impacts humans in two ways. Firstly, the economic importance of FHB results from losses of grain yield and reductions of kernel size and seed quality. Secondly, these fungi can pose serious threats to human and animal health because they produce several types of mycotoxins that may contaminate the grains (Bin-Umer *et al.*, 2014; Goswami & Kistler, 2004; McMullen *et al.*, 1997; Parry *et al.*, 1995; Trail, 2009; Wegulo, 2012).

The most important mycotoxins formed by *F. graminearum* are trichothecenes such as deoxynivalenol (DON), nivalenol (NIV) and their derivatives, and zearalenone (ZEA), which is an estrogenic compound (Sutton 1982; Chelkowski, Visconti et al. 1984; Parry, Jenkinson et al. 1995; Goswami and Kistler 2005; Becher, Hettwer et al. 2010). Without control of FHB, these well documented mycotoxins may accumulate in grains at levels that may be considerably higher than the legally allowed thresholds. DON and ZEA levels are regulated in food supplies of many countries (McMullen, Bergstrom et al. 2012; Pieters *et al.*, 2002; Desjardins & Proctor, 2007).

Crops that are contaminated with mycotoxins may be downgraded from food to feed grade, and in order to reduce contamination, additional cleaning processes may be required, which increase the processing costs. In case of highly contaminated crops it may be processed to a non-feed use. In some cases destroying highly contaminated crops in the field may be more economical than harvesting it as unmarketable products. Improper drying and storage can lead to increased mycotoxin contamination further down the production chain (Charmley *et al.*, 1995; Hussein & Brasel, 2001; Kendra & Dyer, 2007; Marroquin-Cardona *et al.*, 2014).

Trichothecenes are characterized by their chemical structure as sesquiterpenes that have epoxy rings at C-12 and C-13 positions. The toxic effect of trichothecenes is mainly due to their ability to bind to the 60S ribosomal subunit of eukaryotes, resulting in inhibition of protein synthesis followed by the induction of apoptosis (Cundliffe *et al.*, 1974; Li & Pestka, 2008; Rocha *et al.*, 2005).

Trichothecenes are also potent phytotoxins for certain plants since they act as a virulence factor aiding the spread of the fungus during disease development and produce symptoms including wilting, chlorosis and tissue necrosis (Arunachalam & Doohan, 2013; Dyer *et al.*, 2005; Pasquet *et al.*, 2014; Proctor *et al.*, 1995). In wheat, DON was shown to promote the spreading of *F. graminearum* from the initially infected to the neighbouring spikelets. In barley, a *TRI5* mutant, which is unable to make DON, spread as efficiently to the neighbouring spikelets as the wild type strain did indicating that DON is a virulence factor for only some hosts (Jansen *et al.*, 2005).

The mycotoxin ZEA is a polyketide produced by several *Fusarium* species that has structural similarity to estrogen allowing its binding at estrogen receptors in human and animals. Studies revealed that ingestion of contaminated feed causes alterations in the reproductive tract of laboratory and domestic animals. Various estrogenic effects such as decreased fertility, increased embryo lethal resorptions, reduced litter size, changed weight of adrenal, thyroid and pituitary glands and altered serum levels of progesterone and estradiol have been observed(Diekman & Green, 1992; Schwartz *et al.*, 2013; Trail, 2009; Zhao *et al.*, 2014 a). Therefore, strict threshold levels were introduced by legislation in several countries.

A.2. Epidemiology of FHB

F. graminearum over-winters on or within plant tissue residues including stems and roots of small grain cereals as well as stalks and ears of maize (*Zea mays* L.) (Osborne & Stein, 2007; Parry *et al.*, 1995; Sutton, 1982). During overwintering the fungus typically forms perithecia that represent the sexual stage *Gibberella zeae*. These fruiting bodies contain club-shaped asci each containing eight haploid ascospores resulting from meiosis (Guenther & Trail, 2005). Matured ascospores are actively ejected from the perithecium to serve as inoculum for infecting the next host (Boenisch & Schafer, 2011; Trail, 2009). Fungi in the *F. graminearum* species complex also produce abundant mycelium as well as conidia, and some of the species produce also chlamydospores on and within the crop debris. During crop growth, the conidia and ascospores produced on debris are dispersed in the air, then infect and colonize wheat spikes, and stems, leaf sheaths and ears of maize. At harvest, plant debris contaminated with the fungus is left on the field and the fungus then continues with a new life cycle (Anderson, 2005; Gilbert *et al.*, 2008; Parry *et al.*, 1995; Sutton, 1982).

Ascospores are formed in ephemeral perithecia during periods of milder temperatures and moist conditions. They are forcibly discharged from mature perithecia and dispersed by wind, rain or insects to a new host plants (Goswami & Kistler, 2004; Parry *et al.*, 1995; Trail *et al.*, 2002). The forcibly discharged ascospores are adequate for long-distance dispersal, whereas the asexually formed macro- and microconidia are entirely depend even more on the kinetic energy of wind and rain-splash (de Luna *et al.*, 2002; Fernando *et al.*, 1997; Keller *et al.*, 2010; Paul *et al.*, 2004). Perithecia production and maturation and ascospore release are affected by environmental factors (Gilbert & Fernando, 2004; Guenther & Trail, 2005; Manstretta & Rossi, 2015; Trail *et al.*, 2002). Studies revealed that temperatures between 15°C and 25°C benefit perithecial development (Gilbert *et al.*, 2008; Manstretta & Rossi, 2015). In addition, their development is influenced by moisture levels and high humidity is required for the discharge of ascospores from perithecia (Parry *et al.*, 1995). *F. graminearum* is more broadly adapted to environmental variability than many other *Fusarium* species. However, warmer and wetter conditions increase infection rates of this fungus. Infection by *F. graminearum* occurs across a broader range of temperatures in comparison to related species, and infection may occur more rapidly (Osborne & Stein, 2007; Rossi *et al.*, 2007). Epidemics on wheat may initiate when warm and moist weather conditions coincide with the onset of anthesis (Cowger *et al.*, 2009). Barley heads become most susceptible to FHB once the heads are out of the leaf sheath and exposed to *F. graminearum* spores (mainly ascospores) (McMullen *et al.*, 2012). During crop flowering, primary infection occurs when ascospores or macroconidia are released from soilborne debris and deposited on or inside flowering spikelets at conducive temperature and humidity (Brown *et al.*, 2010; Pritsch *et al.*, 2000; Sutton, 1982). The fungus initially does not penetrate directly through the epidermis. Instead, hyphae develop on the exterior surfaces of florets and glumes, allowing the fungus to grow toward stomata and other susceptible sites within the inflorescence such as developing caryopses as well as the adaxial surfaces of lemma and palea that are only accessible in open florets and are susceptible to infection (Goswami & Kistler, 2004). In most cases *F. graminearum* colonization of wheat spikes initiates on dead anthers, which may be either extruded or captured between floral bracts. In the presence of moisture, the spores germinate, hyphae enter via the cracked anthers and grow down the anther filament into the host plant, followed by hyphal penetration of the ovary and eventual infection of the floral bracts including glume, palea, and lemma (Brown *et al.*, 2010; Jansen *et al.*, 2005; Pritsch *et al.*, 2000). After penetrating and colonizing the floret tissue the fungus spreads systemically through the parenchyma and vascular tissue of wheat and reaches remaining florets of the

spike (Ilgen *et al.*, 2009; Jansen *et al.*, 2005). At the point of infection symptoms begin to appear as water soaking spots with partially necrotic and chlorotic tissue of the spike and eventually spread up- and downwards in the rachis (Brown *et al.*, 2010; Goswami & Kistler, 2004; Trail, 2009). Bleaching is a common symptom of this disease and can be seen on wheat heads before senescence (Guenther & Trail, 2005; McMullen *et al.*, 2012; Parry *et al.*, 1995) (see Fig.A.1). Studies revealed that, when fungal infection takes place, cell wall degradation occurs that is often responsible for the typical FHB symptoms such as the discontinued kernel development and the discoloured and shrivelled seeds. During fungal proliferation in the spike, cellulose, xylan, and pectin are reduced in the cell walls of colonized ovaries, lemma, and rachis (Chappell Matthew, 2001; Kang & Buchenauer, 2000; Kikot *et al.*, 2009; Zhang *et al.*, 2012; Zhao *et al.*, 2014 b).

Fig.A.1. Fusarium head blight symptoms. A, Wheat head infection with bleached spikelets. B, Six-row barley head infection. C, infected durum wheat kernels (McMullen, 2012)

During prolonged wet periods, salmon-orange to pink masses of conidiophores grouped into a *sporodochium* are formed from the vegetative mycelium and can often be observed at the base of infected spikelets of wheat and barley, which may lead to secondary infections within the same vegetation period, resulting in severe FHB epidemics (Boenisch, 2013). Stems of host plant extensively colonized after head infections increase the potential for inoculum production and survival of *G. zeae* in the field, as these tissues remain intact through the winter and serve as the basis for inoculum production in the spring (Gilbert *et al.*, 2008; Guenther & Trail, 2005) (Fig.A.2). *F. graminearum* may infect cereals at other developmental stages in the following season if infected seed is planted, resulting in seedling blight, foot, crown or root rot diseases (Becher *et al.*, 2013).

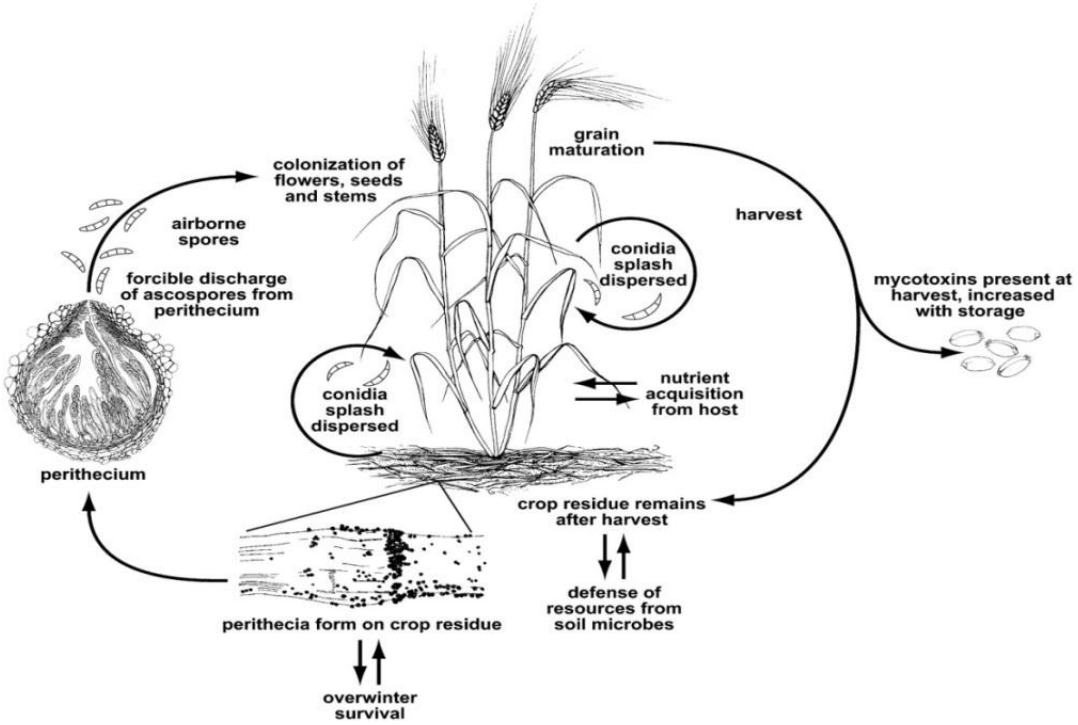

Fig.A.2. The life cycle of *F. graminearum* (sexual phase, *G. zeae*), causal agent of *Fusarium* head blight on wheat (Trail, 2009)

A.3. Management strategies of FHB

Control of FHB can be achieved through various crop management practices that reduce factors favouring epidemics. The most important practices, which may reduce the severity of FHB and DON contamination are agronomic practices such as appropriate crop rotation in conjunction with ploughing, weed control, the utilisation of resistant cultivars, and fungicide application. However, the use of a single management strategy often fails to control the disease and to keep mycotoxins at acceptable levels. Therefore, integrated approaches have been used worldwide in which several control measures were combined (Bai & Shaner, 2004; Blandino *et al.*, 2012; Landa *et al.*, 2004; McMullen *et al.*, 2012; Yuen & Schoneweis, 2007). If environmental conditions are highly favourable for infection, agronomic practices cannot completely control FHB. In this case, and as no fully FHB-resistant wheat cultivars exist, application of azole fungicides becomes even more important for control. Although QTLs providing resistance against *F. graminearum* are known (Becher et al., 2013), at present, no fully FHB-resistant high-yield wheat cultivars exist. Therefore, control of FHB relies on the use of commercial cultivars with at best partial resistance. The use of such cultivars, which have been generated in several breeding programs, still must be coupled with other

management strategies to prevent FHB epidemics, which may arise if disease pressure is high (McMullen *et al.*, 2012; Mesterhazy *et al.*, 2011; Mesterhàzy *et al.*, 2005; Snijders, 2004).

Chemical control by application of fungicides is still considered an effective way to protect cereal crops, and it is included in integrated programs as a priority based on the risk predicted by FHB forecasting models (Blandino *et al.*, 2012; McMullen *et al.*, 2012; Mesterhazy *et al.*, 2011). Factors such as the susceptibility of the planted cultivar, the cultural practices applied, and local weather forecasts are important in determining the need for a fungicide spray to control FHB.

A.4. Azole fungicides

The effectiveness of chemical management of FHB is differing widely and depending on the fungicide classes, the biological attributes of cultivars, the environment and methodological problems related to the way of fungicide application in the field. Due to the complex interaction between fungicide treatment, mycotoxin production and weather conditions, application of fungicides to control FHB provided varying results (Blandino *et al.*, 2006; Blandino *et al.*, 2012; Simpson *et al.*, 2001). Azoles (imidazoles and triazoles) are the largest group of sterol 14a-demethylation inhibitors (DMI) fungicides and the most widely used class of antifungal agents for the control of pathogenic fungi of humans and plants (Becher & Wirsel, 2012b; Fan *et al.*, 2013; Sheehan *et al.*, 1999). Triazoles such as tebuconazole, prothioconazole and metconazole, have revealed the most consistent results in reducing scab symptoms and DON content. This was demonstrated in experimental *in vitro* conditions and also in practice (Blandino *et al.*, 2006; Edwards *et al.*, 2001; Matthies *et al.*, 2000; McMullen *et al.*, 2012; Paul *et al.*, 2007; Paul *et al.*, 2010). The timing of fungicide application revealed to be very important for successful disease reduction. However, the timing of fungicide application may differentially affect FHB severity and mycotoxin levels. Fungicide application at anthesis was found crucial for reducing FHB, while treatments at the late milk stage may be optimal for controlling mycotoxin levels in wheat (Parry *et al.*, 1995; Paul *et al.*, 2008; Yoshida *et al.*, 2012). The best efficacy of azole treatment to control for FHB development and mycotoxin production was achieved when fungicide applications were combined with the planting of partially resistant wheat cultivars, agricultural practices and field management strategies, which decreased the severity of the disease and eliminated

sources of primary inoculum (Becher & Wirsel, 2012b; Blandino *et al.*, 2012; Lehoczki-Krsjak *et al.*, 2010; Mesterházy *et al.*, 2003; Paul *et al.*, 2008).

A.4.1. Mode of action

The first azole fungicides, also referred to as DMI (= demethylase inhibitor) fungicides, were patented in the 1970s (Buchel, 1986). Today DMIs are a group of one of the most important antifungals in medicine and agriculture (Becher *et al.*, 2011; Gisi, 2014; Kuck *et al.*, 2012; Tsuda *et al.*, 2004). The chemical structures of agricultural DMIs can be found in the FRAC (Fungicide Resistance Action Committee) Code List 'Modes of action of fungicides' (www.frac.info). Azoles inhibit cytochrome P450-dependent C14α demethylase (the *ERG11* gene product), which is referred to also as CYP51 (synonym ERG11). It belongs to the Cytochrome P450s (CYPs), constituting a superfamily of heme-containing monooxygenases, which comprise many different P450-dependent enzymes involved amongst others in the synthesis of endogenous substrates and in metabolism of xenobiotics (Chen *et al.*, 2014; Kelly *et al.*, 2001; Kelly *et al.*, 2003; Lamb *et al.*, 1998; Park *et al.*, 2008; Yoshida, 1988; Yoshida *et al.*, 2000). Fungal CYP51 catalyses a key step in the biosynthesis of ergosterol, a sterol in fungal membranes that regulates membrane fluidity and integrity (Becher *et al.*, 2011a; Becher & Wirsel, 2012b; Fan *et al.*, 2013; Lamb *et al.*, 1998; Nakayama *et al.*, 2001).

Thus, inhibition of demethylation at the 14-α-C-lanosterol or 24-methylene dihydrolanosterol in fungi by DMI fungicides leads to the accumulation of sterol precursors, including 14α-methyl-3,6-diol. The latter impairs membrane function by increasing its fluidity resulting in generation of a plasma membrane with modified structure and function (Bean *et al.*, 2009; Becher & Wirsel, 2012; Ghannoum & Rice, 1999; Kelly *et al.*, 1995; Lupetti *et al.*, 2002; Martel *et al.*, 2010; Watson *et al.*, 1989) (Fig.A.3). CYP proteins contain a prosthetic heme group that consists of a porphyrin ring centring an iron atom. Azoles bind to the heme iron and thus inhibit oxygen activation that is necessary for substrate demethylation by the lanosterol 14α demethylase enzyme in yeasts, also called eburicol 14α demethylase in filamentous fungi (Balding *et al.*, 2008; Hartman *et al.*, 2013; Lupetti *et al.*, 2002).

A.4.2. Fungal azole resistance

Since the introduction of target site-specific plant protection agents, resistance of various phytopathogenic fungi including *Fusarium* species against fungicides of different chemical classes has emerged in field populations (Hollomon, 1993; Hollomon & Brent, 2009; Klix *et al.*, 2006; Yin *et al.*, 2009). Different factors such as their application frequency, the mode of action of their active ingredient(s), the rate of mutations within a pathogen population, the reproduction mode and rate of the pathogen, and the fitness cost of a mechanism conferring resistance affect the development of resistance towards fungicides (Andersen *et al.*, 2014; de Waard *et al.*, 2006; Klix *et al.*, 2006). The widespread and long-term use of DMIs has selected for fungal populations with polygenic resistances or so called 'quantitative resistance'. This resistance is usually characterised by the accumulation of several mutations, mostly SNPs (single nucleotide polymorphisms), leading to an increased tolerance over an extended period of time. Such resistance develops gradually less susceptible populations (Becher & Wirsel, 2012b; Deising *et al.*, 2008; Hollomon & Brent, 2009; Klix *et al.*, 2006; Musiol & Kowalczyk, 2012). Field and greenhouse studies have shown that different mechanisms led to reduced fungicide effectiveness and to DMI-resistance in different fungal species (Becher & Wirsel, 2012b; Hamamoto *et al.*, 2000; Kretschmer *et al.*, 2009).

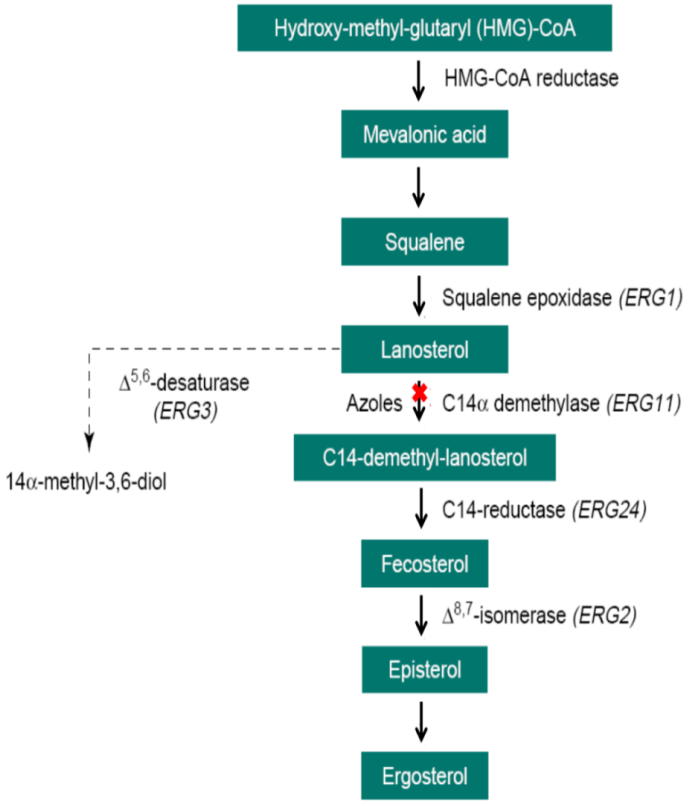

Fig.A.3. Mechanism of action of azole drugs and their target site *ERG11*in the ergosterol biosynthetic pathway. Important enzymes are reported on the right with encoding genes in parentheses. Modified from (Lupetti, 2002)

A.4.2.1. Alteration in the target-encoding gene

Resistance connecting to the molecular target of azole action, CYP51, has been described for several pathogenic fungi. DMI resistance in fungi may result from mutations in the coding regions of the *CYP51* genes that change some details of the structure of the encoded proteins. This will make CYP51 less sensitive to the drug without considerably affecting its enzymatic activity (Becher & Wirsel, 2012; Cools *et al.*, 2013; Hollomon, 1993; Kretschmer *et al.*, 2009; Lamb *et al.*, 1998; Leroux *et al.*, 2007; Sanglard *et al.*, 1998). In medicine, the molecular mechanisms of DMI resistance have been comprehensively studied in human fungal pathogens, which showed for example that in *Candida albicans* point mutations in *CYP51* are often responsible for DMI resistance (Morio *et al.*, 2010; Sanglard *et al.*, 1998; Warrilow *et al.*, 2012; White, 1997). Also in some plant pathogens, *CYP51* mutations have been correlated with the emergence of azole resistance. Such mutations leading to changes in the target protein(s), which remain functional but less sensitive to the drugs, seem not uncommon in plant pathogenic fungi. Resistance against DMIs that is based on *CYP51* mutations was reported from *Venturia inaequalis* (Kunz *et al.*, 1997), *Ustilago maydis* (Joseph-Horne *et al.*, 1995), *Penicillium digitatum* (Sun *et al.*, 2011), *Blumeria graminis* (Wyand & Brown, 2005), *Pyrenopeziza brassicae* (Carter *et al.*, 2014) and *Mycosphaerella graminicola* (Cools *et al.*, 2011; Cools *et al.*, 2013; Leroux *et al.*, 2007; Stergiopoulos *et al.*, 2003 a).

Some filamentous fungi, particularly in the Ascomycota, subphylum Pezizomycotina, possess two or more paralogous *CYP51* genes (Becher *et al.*, 2011). Studies showed that species with multiple *CYP51* are intrinsically less sensitive to some azoles, and mutations causing azole resistance are usually restricted to one paralogue, most often *CYP51A* (Becher & Wirsel, 2012). The *F. graminearum* genome reveals the existence of three paralogous *CYP51* genes (designated *CYP51A*, *-B*, and *-C*) in this fungus. Recently, it has been demonstrated that deletions of two of these genes (*FgCYP51A* and to a lesser extent *FgCYP51C*) resulted in increased sensitivity to DMIs (Becher & Wirsel, 2012; Liu *et al.*, 2011).

A.4.2.2. Over-expression of the target-encoding gene

Enhanced expression levels of *CYP51* may also contribute to the development of DMI resistance as shown for several phytopathogenic fungi, such as *Mycosphaerella graminicola* and *Botrytis cinerea* (Cools *et al.*, 2013; Kretschmer *et al.*, 2009; Ma & Michailides, 2005).

A significant increase in *CYP51* expression can be caused by substitutions, insertions, and duplications in its promoter regions (Becher & Wirsel, 2012; Cowen & Steinbach, 2008; Hamamoto *et al.*, 2000; Selmecki *et al.*, 2006) or by gain-of-function (GOF) mutations in transcription factors regulating *CYP51* expression (Coste *et al.*, 2007; Dunkel *et al.*, 2008; Sanglard *et al.*, 2009). Consequences of overexpressing *CYP51* seem to be rather unspecific since tolerance against different types of azoles is similarly increased. In contrast, target site mutations reveal a more specific effect since tolerance gains are higher for some azoles than for others. However, normally mutants overexpressing CYP51 show resistance levels that are lower than those having target site alterations (Cools *et al.*, 2013).

A.4.2.3. Alterations in specific steps of the ergosterol biosynthesis pathway

Mutations of specific genes by which some steps of the ergosterol pathway can be blocked in the presence of the fungicide have been reported. Fungal strains carrying such mutations do not synthesize toxic sterol intermediates in the presence of azoles (Cannon *et al.*, 2009; Sanglard *et al.*, 2003). *ERG3* mutants are resistant to azole fungicides, as they are able to modify the biosynthesis of toxic metabolites and accumulate less toxic intermediate 14-methyl-fecosterol under azole treatment. This has been shown for some strains of *S. cerevisiae* in which azole resistance is linked to inactivation of Erg3p conferred by mutation in the sterol C-5 desaturase-encoding gene *ERG3* (Kelly *et al.*, 1995; Sanglard *et al.*, 2009; Taylor *et al.*, 1983). Also, in *Candida* spp., azole-resistant isolates with inactivated *ERG3* alleles have been characterized (Cannon *et al.*, 2007; Chau *et al.*, 2005; Pinjon *et al.*, 2003).

A.4.2.4. Enhanced fungicide efflux

Other mechanisms to increase azole tolerance in pathogenic fungi are mutations in and overexpression of plasma membrane-bound efflux transporters, which often correlates with high-level of azole resistance. These efflux proteins pump the drug out of the cell and reduce its intracellular concentration to levels at which the target is barely inhibited. Increased cellular membrane export of azoles has been considered to explain slowly decreasing sensitivity in laboratory and field strains (Becher *et al.*, 2011; de Waard *et al.*, 2006; Del Sorbo *et al.*, 2000; Goffeau, 2008). The ATP-binding cassette (ABC) transporters and the major facilitator superfamily (MFS) are the two most extensively studied families of transporters involved in efflux. Together, these two superfamilies account for approximately

half of all genes encoding transporters in fungal genomes (Coleman & Mylonakis, 2009; Del Sorbo *et al.*, 2000; Prasad & Goffeau, 2012). Members of the ABC transporter superfamily are the most common of the primary transporters that couple hydrolysis of ATP to translocation of substrates across cellular membranes, typically against a concentration gradient (Coleman & Mylonakis, 2009; Jones *et al.*, 2009; Ren *et al.*, 2007).

A.5. Fungal ABC transporter superfamily

Members of the ATP-binding cassette (ABC) transporter superfamily exist in bacteria, fungi, plants, animals and constitute one of the largest protein superfamilies. Through their transport function, these integral membrane proteins are involved in diverse cellular processes, such as osmotic homeostasis, nutrient uptake, resistance to cytotoxic drugs and antibiotics, cell division, pathogenesis, sporulation and the efflux of xenobiotic compounds and intracellular metabolites (Bouige *et al.*, 2002; Davidson *et al.*, 2008; Higgins, 1992; Holland & Blight, 1999; Jones *et al.*, 2009). Prokaryotes harbour both ABC transporters that import nutrients (including amino acids, sugars and metal ions) and that export xenobiotics (drugs, toxins) and polysaccharides, lipids and proteins, while the majority of eukaryotic ABC family members function as exporters (Cannon *et al.*, 2007; Jones *et al.*, 2009). Some ABC proteins transport specific substrates, while others have evolved broad specificity for compounds, including different types of toxic compounds, which is usually referred to as multidrug resistance (MDR). In MDR strains resistance is not restricted to the compounds it was selected by but also covers other types of structurally and functionally unrelated drugs. This phenomenon is also called cross-resistance (Cannon *et al.*, 2009). Functional studies of fungal ABC proteins were initially performed with model species such as the budding yeast *Saccharomyces cerevisiae*, the fission yeast *Schizosaccharomyces pombe* and the human-pathogenic dimorphic yeast *Candida albicans*. These studies showed that the majority of these transporters are involved in the ATP-dependent transport of a broad range of substrates across the biological membranes. Some of these transporters also function as ion channels or receptors or are involved in the secretion of the mating a-factor peptide, mRNA secretion, biogenesis of ribosomes and cell protection against different classes of toxic compounds (Ernst *et al.*, 2005; Gulshan & Moye-Rowley, 2007; Kovalchuk & Driessen, 2010; Prasad *et al.*, 2002; Prasad & Goffeau, 2012; Wolfger *et al.*, 2004). In plant pathogenic fungi, it has been well documented that ABC drug transporters may provide protection against antibiotics, plant defence compounds and fungicides. Furthermore, they may export fungal molecules that

function in virulence on host plants (de Waard *et al.*, 2006; Kovalchuk & Driessen, 2010; Kretschmer *et al.*, 2009).

There exist full-length and half-size ABC transporters. The former comprise two transmembrane domains (TMDs) and two cytosolic ATP-binding cassettes, which are also known as nucleotide-binding domains (NBDs). These four domains can be made up by one, two or four polypeptide chains, encoded by the same or different genes. Half-size ABC transporters have only one of each domain (Coleman & Mylonakis, 2009; Higgins, 1992; Jones *et al.*, 2009). Beside the prototypical arrangement of the domains, which is characterised as (NBD-TMD_6), several deviations exist, which provided the opportunity to divide the ABC transporter family in several subfamilies. According to a classification scheme, which was initially proposed for human proteins (Klein *et al.*, 1999; Quentin & Fichant, 2000; Vasiliou *et al.*, 2009) there exist seven subfamilies (ABC-A, ABC-B, ABC-C, ABC-D, ABC-E, ABC-F and ABC-G) of which three are involved in the efflux of toxic compounds, i.e. ABC-B, ABC-C, and ABC-G. Based on an alternative classification scheme used in the yeast literature, these three families ,were classified as the multidrug resistance (MDR), multidrug resistance–associated protein (MRP), and the pleiotropic drug resistance (PDR) families, respectively (Coleman & Mylonakis, 2009; Rogers *et al.*, 2001; Sipos & Kuchler, 2006).

A.5.1. Contribution of ABC transporters to azole resistance in plant pathogens

The MDR phenotype in fungi is usually correlated with increased drug efflux (Cannon *et al.*, 2009; Gulshan & Moye-Rowley, 2007; Kretschmer *et al.*, 2009). In filamentous fungi, the literature indicates the presence of a constitutive efflux system that contributes to tolerance to various fungicides and natural antifungal compounds in fungicide resistant strains of *Botrytis cinerea*, *Aspergillus nidulans*, *Penicillium digitatum*, *Magnaporthe oryzae*, *Mycosphaerella graminicola* (Andrade *et al.*, 2000; de Waard *et al.*, 2006; Del Sorbo *et al.*, 2000; do Nascimento *et al.*, 2002; Gulshan & Moye-Rowley, 2007; Gupta & Chattoo, 2008; Nakaune *et al.*, 2002; Stergiopoulos *et al.*, 2003 b; Zwiers & De Waard, 2000; Zwiers *et al.*, 2002). Membrane-spanning transporters also affect the sensitivity of fungal pathogens to azoles (Becher & Wirsel, 2012b; Cannon *et al.*, 2009).

A contribution of efflux mechanisms to azole resistance was indicated by mutants of *B. cinerea* (de Waard *et al.*, 2006; Hayashi *et al.*, 2003; Vermeulen *et al.*, 2001), *M. graminicola*

(Zwiers *et al.*, 2002), *P. digitatum* (Nakaune *et al.*, 2002) and *A. nidulans* (Del Sorbo *et al.*, 1997). In these plant pathogens, azole resistance was associated with enhanced expression levels of specific ABC transporter genes. Reports revealed that strains with decreased fungicide accumulation and MDR phenotypes also exist in the field (Leroux & Walker, 2011).

A.5.2. Virulence-associated efflux pumps in plant pathogenic fungi

In several plant pathogens a contribution of ABC transporters to virulence has been demonstrated, too. Studies revealed that some transporters actively secrete host-specific and nonhost-specific toxins during infection. Some ABC transporters also play an essential role in protection against plant defence compounds during pathogenesis (de Waard *et al.*, 2006; Del Sorbo *et al.*, 2000). Such a role in pathogenicity has been demonstrated for the transporters encoded by the genes *ABC1* (ABC-G subfamily) and *ABC4* (ABC-A subfamily) from *M. oryzae*. Deletion mutants of both genes are non-pathogenic on rice and barley, which indicates an important role of these transporters during infection. Since mutants of *ABC1* showed no differences in their sensitivity to antifungal compounds (tebuconazole, metconazole, fenarimol and cycloheximide) in comparison to the wild type strain, this indicates that this transporter is not involved in drug resistance (Urban *et al.*, 1999).

In contrast, *ABC4* (ABC-G) subfamily mutants showed increased sensitivity to certain antifungal compounds (miconazole and cycloheximide) and also to the phytoalexins resveratrol, implying a role in multidrug resistance and virulence (Gupta & Chattoo, 2008). In the wheat pathogen *M. graminicola* an ABC transporter encoded by *MgAtr4* functions as a virulence factor affecting the colonization of substomatal cavities in wheat leaves (Stergiopoulos *et al.*, 2003 b). Expression studies in this fungus showed also that fungicides and plant defence compounds such as eugenol can up-regulate the ABC transporter genes *Mgatr1* and *Mgatr2*, belonging to the ABC-G subfamily (Zwiers *et al.*, 2002). Disruption of *BcatrB* (ABC-G subfamily) from *B. cinerea* increased the sensitivity to fungicides as well to resveratrol. The mutants showed lower virulence on grapevine leaves, which suggests that the encoded protein contributes to both virulence and fungicide sensitivity (Del Sorbo *et al.*, 2000; Schoonbeek *et al.*, 2001).

BcatrB exports also camalexin and has been documented to be a virulence factor on *Arabidopsis thaliana* (Stefanato *et al.*, 2009). ABC transporters in other plant pathogenic fungi such as *Gibberella pulicaris* (Fleissner *et al.*, 2002), *Nectria haematococca* (Coleman *et*

al., 2011), *Aspergillus fumigatus* (Paul *et al.*, 2013) and *Beauveria bassiana* (Song *et al.*, 2013) also contribute to virulence.

A.6. Selection of candidate genes

A.6.1. Classification

In this work, two genes encoding ABC transporters of *Fusarium graminearum* were selected for functional characterization. The first candidate protein is encoded by the gene FGSG_17046 and will be referred to as FgABC2. The second transporter protein is encoded by the gene FGSG_04580 and will be referred to as FgABC3. Topology prediction using computer programs such as TMHMM version 2.0 predicted 12 transmembrane helices in the TMDs of FgABC2 indicating a $(TMD_6\text{-}NBD)_2$ topology with two large extracellular loops between the first two transmembrane helices. This placed FgABC2 in the ABC-A subfamily of ABC transporters. On the other hand, FgABC3 was predicted to belong to the PDR subfamily (ABC-G group I) with 12 predicted transmembrane helices within the protein in a $(NBD\text{-}TMD_6)_2$ topology (Kovalchuk and Driessen 2010; Becher, Weihmann et al. 2011).

A.6.2. Microarray analyses

In previous studies, the capability of *F. graminearum* to develop azole resistance and the molecular mechanisms leading to it were investigated. Cultivation of the wild type strain NRRL 13383 in the presence of a sublethal concentration of tebuconazole allowed to recover isolates with enhanced tolerance to that fungicide (Becher *et al.*, 2010). Two distinct mycelial morphotypes called P1 and P2 were recovered and characterized. These differ in the levels of vegetative fitness, the acquired tebuconazole resistance, cross resistance to amine fungicides, virulence and mycotoxin production.

This suggested that also in *F. graminearum* may exist more than one mechanism leading to azole resistance as observed in other plant and human pathogenic fungi (Becher & Wirsel, 2012). The transcriptomic responses of *F. graminearum* occurring *in vitro* after a treatment with tebuconazole were investigated using a microarray approach (Becher *et al.*, 2011). In that study strong responses were observed for some genes of the sterol biosynthesis pathway, noticeably *FgCyp51A* to *FgCyp51C* that encode the molecular target of azole action, cytochrome P450 sterol 14α-demethylase. Furthermore, this study revealed that several genes encoding ABC transporters were significantly upregulated by tebuconazole treatment.

A.6.3. Selection for deletion

In this work two genes FGSG_17046 *(FgABC2)* and FGSG_04580 (*FgABC3*) encoding full-size ABC transporters were deleted to determine their contribution to fungicide resistance and virulence, and their putative effects on mycotoxin production. Selected ABC transporter genes were functionally analysed in two genetic backgrounds, i.e. NRRL 13383 and PH-1, to assess whether the respective genomic context may influence the effect of gene deletion. Expression of *FgABC2 and FgABC3* was previously analysed in microarray experiments and shown to be up-regulated after tebuconazole treatment in comparison to untreated controls 1.6-, 2.3-fold, respectively, in strains PH-1 (15-ADON) and 2.3-, 9.5-fold, respectively in NRRL (NIV chemotype) (Becher *et al.*, 2011; Becher *et al*., unpublished data).

The transcript levels of *FgABC2* and *FgABC3* were also determined by qRT-PCR in both wild type strains after a treatment with tebuconazole. The transcript levels for both selected genes were increased 1.8-, 1.5-fold, respectively in PH-1 and 3.1-, 3.4-fold, respectively in NRRL 13383 (Abou Ammar *et al.*, 2013).

A.7. Aim of this work

The occurrence of multidrug resistance (MDR) in fungal pathogens is a growing global problem in medicine and agriculture. As indicated above, MDR is often associated with overproduction of multidrug-efflux proteins of the ATP- binding- cassette (ABC) superfamily. The goal of this present work was to investigate the potential roles of ABC-transporters in the plant pathogen *Fusarium graminearum* and their significance in protecting the fungus against antifungal compounds. Furthermore, the role of ABC transporters in virulence and mycotoxin production was of interest. Two selected ABC transporter-encoding genes belonging to two different ABC transporter subfamilies were selected according to their expression data in the presence of azole fungicides.

The candidate genes were chosen for functional analyses and characterization using a targeted gene deletion strategy. The results of this work were expected to shed light on mechanisms of fungicide resistance in this fungus, and on the contribution of ABC transporters to fungicide adaptation under *in vitro* and green house conditions. Furthermore, the aim was to study the impact of the deletion of these selected transporter protein encoding genes on the fungus´ pathogenicity on the host plant and on mycotoxin production. These data were expected to show more conclusively the physiological functions of ABC transporters in

Fusarium graminearum. Understanding the role of ABC transporters in fungicide resistance may help establishing new control strategies of this plant pathogen.

Materials and methods

B.1. Organisms

B.1.1. Fungi

B.1.1.1. Strains

The strains NRRL 31084 (= PH-1) and NRRL 13383 of *Fusarium graminearum* were used in this study as wild type references. PH-1 was isolated from wheat grain in Michigan in 1996 and its genome sequence is available (Cuomo *et al.*, 2007). PH-1 has the 15-ADON chemotype that connects to higher aggressiveness on wheat than the NIV chemotype (Foroud & Eudes, 2009). As outlined above, PH-1 had been used in a previous microarray study investigating the transcriptomic responses to triazole treatment in *F. graminearum* (Becher, 2011). NRRL 13383 was originally isolated from infected maize in Iran and has the NIV chemotype, which associates with a higher aggressiveness on maize (Carter *et al.*, 2002; Lee *et al.*, 2009). NIV is also somewhat more problematic than DON with respect to toxication of food and feed (Minervini *et al.*, 2004). Since the genome sequence of strain NRRL 13383 is not known, it is uncertain to which extent it differs from PH-1 and how that may affect microarray experiments, which were designed based on the PH-1 genome. NRRL 13383 as well as the procedures used for its growth, sporulation and storage were described previously (Becher *et al.*, 2011).

B.1.1.2. Cultivation and storage

Strains were revitalised from permanent cultures by inoculation of PDA plates (see G.1.1) that were then used to inoculate subsequently cultures for growth assays, conidiation and DNA isolation. For single spore isolation, strains were cultivated on SNA medium (see G.1.1) in petri dishes with diameters of 55 or 90 mm containing 100 µg/ml of the respective antibiotic (see G.1.2) in darkness at 23°C for PH-1 and at 28°C for NRRL 13383. For permanent storage, macroconidia were collected in 1 ml of 30% glycerol from MBB cultures. Stock solutions were frozen at -80°C in 2 ml safelock tubes. To revitalise the strains from such permanent stocks, small portions of the frozen solutions were quickly transferred with a spatula to PDA plates without allowing the frozen stock to thaw. For DNA isolation, five

blocks of a five day old PDA culture were inoculated in 50 ml of PD liquid medium in a 100 ml Erlenmeyer flask. Liquid cultures were incubated for 7 d at 25°C and 110 rpm in a Multitron thermo incubator (Infors AG, Bottmingen-Basel, Switzerland). To obtain macroconidia for virulence and fungicide assays, PDA petri dishes were inoculated using frozen material from the stock cultures as described above. After 5 d of growth, 50 ml of liquid Mung Bean Broth (MBB) (see G.1.1) were inoculated with five blocks of agar in a 250 ml Erlenmeyer flask. MBB liquid cultures were incubated for 7 d on a Polymax 1040 shaker (Heidolph Instruments GmbH und Co.KG, Schwabach) in darkness at 100 rpm and 23°C. Macroconidia were harvested by filtration through Miracloth nylon mesh (Merck, Darmstadt, Germany) and centrifugation (Hermle ZK 380, swing out rotor; Hermle Labortechnik GmbH, Wehingen) at 3000x g in a 50 ml tube for 10 min. Sedimented macroconidia were transferred to a 2 ml safelock tube, and washed twice with 0.02% Tween 20 and counted in a haemocytometer (Brand, Wertheim, Germany).

B.1.1.3 Analyses of growth rate and conidiation capacity

Vegetative growth rates were determined on PDA plates (Ø 90 mm) at 15°C, 23°C and 30°C. Mycelial plugs (Ø 5 mm) were taken from margins of colonies grown on PDA at 23°C for five days and used for inoculation. Two measurements of colony diameters were taken daily during seven days and averaged. Each variant was replicated four times. The capacity of fungal strains to produce macroconidia was determined in 50 ml Mung Bean Broth (MBB) (see G.1.1) in 250 ml Erlenmeyer flasks inoculated with five mycelial plugs per flask as above. Cultures were incubated at 23°C with 100 rpm for 7 days. Conidia were harvested by filtering through Miracloth (Merck, Darmstadt, Germany) and collected by centrifugation at 3000x g for 10 min. Sedimented macroconidia were transferred to a 2 ml safelock tube, and washed twice with 0.02% Tween 20 and conidial density was determined using a haemocytometer (Brand, Wertheim, Germany). Each strain was grown in four cultures and conidia were counted twice. Statistical significances were determined by T-test ($p<0.05$) as implemented in the Microsoft Excel 2010 software. Germination efficiency of macroconidia was determined on glass slides inoculated with 20 µl of a conidial suspension (10^4 ml^{-1}), covered with a cover glass and incubated on three layers of moistened paper towels inside a 120x120x17 mm plastic dish (Greiner Bio-One, Solingen, Germany) at 23°C for 24 h under illumination with near-UV light (L18W/73, Osram, Munich, Germany). Germinated and

ungerminated conidia were counted twice in four replicates per strain. Statistical analysis was carried out as described above (Abou-Ammar et al., 2013).

B.1.2. Plants

B.1.2.1. Cultivars used

Virulence assays used wheat cultivar Kadrilj (SW Seed Hadmersleben, Hadmersleben, Germany), maize cultivar Golden Jubilee (Territorial Seed Company, Cottage Grove, OR, USA) and barley cultivar Barke (Saatzucht Josef Breun, Herzogenaurach, Germany).

B.1.2.2. Infection assays

B.1.2.2.1. Wheat ear infection assay

"Cultivation of wheat in the greenhouse and environmentally controlled growth chambers and ear inoculation was described earlier (Becher et al., 2010). *F. graminearum* strains were point-inoculated into the ninth spikelet of wheat cultivar Kadrilj (SW Seed Hadmersleben, Hadmersleben, Germany) when it reached anthesis (6-8 weeks old plants)" (Abou-Ammar et al., 2013). Inoculum suspensions were prepared from MBB liquid cultures (see G.1.1) and the density of macroconidia was determined in a counting cell chamber (Thoma type) using dilutions of 1:30, 1:40 and 1:60. Values were normalized according to the dilution factors. Spore suspensions used as inoculum were adjusted to concentrations of $3x10^4$ macroconidia per ml. Wheat ears were inoculated with 10 µl droplets, which were carefully placed with a pipette into the 9th earlet, near to the gynoecium. "The inoculum consisted of 300 macroconidia suspended in 10 µl of 0.02% Tween 20. For each strain tested, at least ten wheat heads were inoculated and covered with plastic bags misted with water to maintain high humidity. The bags were removed after 2 days post inoculation (dpi) and the incubation continued at 25°C, 70% relative humidity until 14 dpi. The development of bleached spikelets in the heads was recorded daily" (Abou-Ammar et al., 2013). Statistical significances were determined by T-test ($p<0.05$) as implemented in the Microsoft Excel 2010 software.

B.1.2.2.2. Maize stem infection assay

“Maize plants cultivar Golden Jubilee (Territorial Seed Company, Cottage Grove, OR, USA) were cultivated for six weeks in a greenhouse at 24°C with 50% relative humidity and a 14 h photoperiod, which employed lamps (Plantstar 600 Watt E40, Osram, Munich, Germany). For each strain tested, at least five plants were inoculated by punching a hole into the stem at the first internode using a sterile toothpick, followed by injection of 1000 macroconidia in 10 µL of 0.02% Tween 20. The control plants were inoculated with 0.02% Tween 20. The hole was covered with Parafilm for 7 d to maintain high humidity and exclude other organisms. At 14 dpi, the stalks were split longitudinally and the symptoms were documented by photography. The extent of the necrotic area was quantified using ImageJ software version 1.46” (Abou-Ammar et al., 2013). Statistical significances were determined by T-test ($p<0.05$) as implemented in the Microsoft Excel 2010 software.

B.1.2.2.3. Barley ear infection assay

“Barley cultivar Barke (Saatzucht Josef Breun, Herzogenaurach, Germany) was cultivated for ten weeks in a greenhouse using the same conditions as described for maize. For each strain tested, sixteen mature ears were inoculated employing a glass flacon to spray 2000 macroconidia in 2 ml of 0.02% Tween 20 onto each ear. The inoculated ears were enclosed in a misted plastic bag for 2 d. After an additional incubation of 12 d, the number of bleached spikelets was recorded for each head. Statistical analysis was carried out as above” (Abou-Ammar et al., 2013)

B.2. Chemicals

All chemicals were obtained from Carl Roth GmbH (Karlsruhe, Germany) und Sigma-Aldrich Chemie GmbH (Steinheim, Germany) unless it is otherwise mentioned. Oligonucleotides were purchased from biomers.net (GmbH, Ulm, Germany). Enzymes with the appropriate buffers were purchased from Fermentas (GmbH, St. Leon-Rot, Germany), and New England Biolabs NEB (Frankfurt am Main, Germany). Media, buffers and solutions used in this study were listed in the appendix (see G.1).

B.3. Molecular methods

B.3.1. Procedures for nucleic acid isolation

B.3.1.1. Preparation of fungal genomic DNA

B.3.1.1.1. Mini preparation of fungal genomic DNA

For small-scales, about 50 mg of mycelia (3-4 days old liquid culture in 3 ml PDB) was dried on paper filter, crushed in 800 µl of DNA lysis buffer (see G.1.2) in a 2 ml safe lock tube with one steel ball in a Retsch mill (Qiagen, Hilden, Germany; 30 rotations/s for 30 seconds). A single extraction of the aqueous phase was performed with 800 µl of Phenol-Chloroform-Isoamylalcohol 25:24:1 (PCI; Rotiphenol). A centrifugation was performed in an Eppendorf centrifuge 5403 (Eppendorf, Hamburg, Germany) (14500 g for 10 min at 4°C). The supernatant was transferred to a fresh 2 ml safelock tube. The DNA was precipitated by adding one volume of isopropanol and centrifuged (6800 g at 4°C for 15 min). The resulting pellet was washed twice with 70% (v/v) ethanol and then dried under the sterile bench. The DNA pellet was re-suspended in 30-50 µl of 1x TE buffer (see G.1.2) or bidistilled water and stored at -20°C.

B.3.1.1.2. Maxi preparation of genomic DNA

The isolation of fungal genomic DNA (gDNA) followed published methods (Dobbeling *et al.*, 1997). For large-scales of fungal DNA preparation, 500 mg of mycelia, from about 5-7 days old liquid cultures in 50 ml PDB was dried on paper filter and ground in liquid nitrogen to a frozen, homogenous powder using a mortar and a pestle. The powder was transferred to a 2 ml safelock tube, 800 µl of extraction buffer I were added (see G.1.2). Afterward, this solution was mixed on a vortexer, incubated on ice for 10 min and mixed again. The tubes were centrifuged in an Eppendorf centrifuge 5403 (Eppendorf, Hamburg, Germany) (14500x g at 4°C for 15 min). The supernatant was transferred to a fresh 2 mL safelock tube and extracted with phenol:chloroform (1:1). The DNA was precipitated by addition of one volume of isopropanol and centrifuged at (6800x g at 4°C for 15 min). After removal of the supernatant, the pellet was shortly dried under the sterile bench and then resuspended in 700 µL of extraction buffer II (see G.1.2).

RNA and remaining proteins were removed by addition of 3.5 µl of (10 mg/ml) RNase A (Roche Diagnostics, Mannheim, Germany) and a 30 min treatment at 37°C that was followed by the addition of 10 µl of (14-22 mg/ml) proteinase K (Roche Diagnostics) and an incubation at 60°C for 30 min. The supernatant was extracted with 700 µl of phenol-chloroform (1:1) and precipitated by the same volume of isopropanol as before. The precipitated DNA was centrifuged (20 min, 14500x g, 4°C), washed with 70% EtOH and centrifuged as before. The supernatant was removed and the pellet was shortly dried under the sterile bench or by vacuum centrifugation (UNIVAPO 100 H, Uni Equip, Dresden, Germany). Finally, the pellet was suspended in H_2O_{bidest} or 1x TE buffer (see G.1.2) and stored at -20°C. Alternatively, the isolation of genomic DNA was performed using the PeqLab DNA extraction kit using the provided instructions (PEQLAB Biotechnology, Erlangen, Germany).

B.3.1.2. Determination of DNA concentration

Quantity and quality controls of DNA preparations were determined with a NanoDrop ND-1000 (Thermo Scientific, Braunschweig, Germany) and visually controlled by gel electrophoresis.

B.3.1.3. Polymerase chain reaction

B.3.1.3.1. Standard PCR

PCR reactions were performed in Biometra T-Personal or T-Professional thermocyclers (Biometra Analytika, Göttingen, Germany) or in a PTC-150 thermal cycler (MJ Research, Waltham, USA). The enzymes and buffers used were from Fermentas (native Taq polymerase) and from NEB GmbH, Frankfurt a. M. (Phusion DNA Polymerase). Standard PCR was applied to examine insertion events in fungal transformants using Taq polymerase in 20 µl reactions.

Reaction mixtures had the following composition:

2 µl PCR-Puffer 10x
1 µl dNTPs (10 mM)
1 µl forward/reverse primers (2 µM)
0.2 µl Taq-Polymerase (5 units/µl)

DNA (1-50 ng)

H_2O_{bidest} was added to a final volume of 20 µl.

The PCR program used to amplify DNA with Taq-Polymerase was the following:

Table B.1: PCR reactions to test for insertion events in fungal strains

N°	Step	Temperature	Time
1	initial denaturation	94°C	2 min
2	denaturation	94°C	30 sec
3	annealing	Tm* + 3-5°C	30 sec
4	elongation	72°C	1 min/kb
	repeat steps 2-4 for 35 times		
5	final elongation	72°C	5 min
6	hold	12°C	∞

* Melting temperatures of primers (Tm) were calculated with the help of an equation (Tm= 69.5±C+ (0.41x%GC)-650/bp) and by using the Clone Manager version 9 computer program. The primer sequences were analysed for specificity by using Primer Blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the *F. graminearum* genome database (http://www.FGDB).

B.3.1.3.2. Fusion PCR

For the generation of constructs that were used for transformation, all PCRs were performed using Phusion DNA Polymerase (NEB GmbH, Frankfurt a. M) in volumes of 20 µl or 50 µl PCR according to the manufacturer's instructions. For these reactions, the Tm of the chosen primers was calculated using the software from Finnzymes (http://www.thermoscientificbio.com/webtools/tmc/). Primers were designed so they had annealing temperatures of 60-63° C.

B.3.1.3.3. Double-Joint PCR

DNA constructs, which were used to delete target genes by transformation of fungal protoplasts, were generated by the DJ-PCR method (Yu et al., 2004). In a first reaction, the 5' and 3' flanks of the target gene and the resistance cassette were independently amplified. One of the primers used to amplify a given flank, the so-called inner primer, included an

5´/3´overhang complementary to the last 20 bases of one the universal primers used to amplify the selection marker cassette. Primers used are listed in the appendix (see G.3). Marker genes employed for selection encoded nourseothricin acetyltransferase (*nat1*) and neomycin phosphotransferase resistance (*npt*) for homologous replacement of FGSG_17046 and FGSG_04580 genes, respectively. Templates for amplification of these resistance cassettes were the plasmids pNR1 (Malonek *et al.*, 2004) and pII99 (Namiki *et al.*, 2001) (see Fig.1 A&B). DNA fragments comprising these marker genes including heterologous constitutive promoters were generated by PCR and then fused by DJ-PCR with the left and right flanks of the respective target gene via the homologous overhangs at the inner primers described above. Phusion High Fidelity DNA Polymerase (NEB) was chosen because of its 3'-5' proofreading activity and its high synthesis rate of 2-4 kb/min. Therefore, the standard PCR protocol was modified by reducing the elongation times according to the sizes of the amplicons and by shortening the denaturation step at a higher temperature (see Table B.2). Reaction mixtures (50 µl) had the following composition:

10 µl HF-Buffer 5x
1 µl dNTPs (10 mM)
2 µl forward/reverse primers (2 µM)
0.25 µl Phusion Polymerase (2 units/ µl)
DNA (20-50 ng)
H_2O_{bidest} was added to a final volume of 50 µl.

Table B.2: First step of DJ-PCR program to amplify the flanking regions of target genes and the resistance cassette:

N°	Step	Temperature	Time
1	initial denaturation	98°C	1:30 min
2	denaturation	98°C	30 sec
3	annealing	Tm	30 sec
4	elongation	72° C	30 sec/kb
	repeat steps 2-4 for 35 times		
5	final elongation	72°C	5 min
6	hold	12°C	∞

The three PCR products were purified as described in the Sure Clean protocol (Bioline, Luckenwalde, Germany). These DNA fragments were then combined and used as template in a second PCR. In this reaction, different weights of the three fragments were tried. The amounts were 2:6:2 ng, 5:15:5 ng and 10:30:10 ng for the left flank / the resistance marker

cassette / the right flank. Reaction conditions were applied that facilitated first the fusion of the three fragments and then the amplification of the fusion products (see Table B.3). The reaction mixture (50 µl) had the following composition:

10 µl HF Puffer 5x

1 µl dNTPs (10 mM)
0.25 µl Phusion Polymerase (2 Units/µl)
products of first PCRs, in a ratio of 1:3:1 for 5'-flank - resistance cassette - 3'-flank
H_2O_{bidest} (to a total volume of 50 µl/reaction)

Table B.3: DJ-PCR program to fuse 5' and 3' flanking regions of the target gene with the resistance cassette

N°	Step	Temperature	Time
1	initial denaturation	98°C	1:30 min
2	denaturation	98°C	30 sec
3	annealing	Tm	30 sec
4	elongation	72°C	1 min
	repeat steps 2-4 for 10 times		
5	denaturation	98°C	30 sec
6	elongation	72°C	1:30
	repeat steps 5-6 for 25 times		
7	final elongation	72°C	5 min
8	hold	12°C	∞

The reaction products were purified using the Gene JET PCR purification kit (Fisher Scientific, Schwerte, Germany) and used as templates for a nested PCR in a third step, which used a standard cycling protocol (see Table B.4). The reactions mixture of the nested PCR (50 µl) had the following composition:

10 µl HF Puffer 5x
1 µl dNTPs (10 mM)
2 µl forward/reverse nested-primers (2 µM)
0.25 µl Phusion Polymerase (2 Units/µl)
20-50 ng of purified fusion fragment
H_2O_{bidest} (to a total volume of 50 µl/reaction)

Table B.4: Nested PCR program to amplify the deletion cassettes

N°	Step	Temperature	Time
1	initial denaturation	98°C	1:30 min
2	denaturation	98°C	30 sec
3	annealing	Tm	30 sec
4	elongation	72°C	15-30 sec/kb
	repeat steps 2- 4 for 35 times		
5	final elongation	72°C	5 min
6	hold	12°C	∞

Deletion constructs were amplified and purified using the Sure Clean protocol, as described by the manufacturer (Qiagen, Hilden, Germany). A yield of 5-8 µg of the purified deletion construct was needed at this step to perform later fungal transformation (see paragraph B.3.3). Alternatively, the correct band was excised from the gel and ligated into plasmid pJET 1.2 blunt (Fermentas) and transformed into *Escherichia coli* (see paragraph B.3.3).

B.3.2. Gel electrophoretic separation of nucleic acids

Gels of 0.7% to 2% agarose (w/v; Seakem LE-Agarose; Biozym Scientific GmbH, Hess. Oldendorf) were used to separate DNA fragments according to their size. Agarose powder was boiled in 1x TAE buffer in a microwave (see G.1.2). The hot solution was mixed and cooled down to less than 60°C and then was poured into a gel tray.

DNA samples containing $1/6^{th}$ volumes of loading buffer (see G.1.2) were separated in a constant electrical field of 2 to 12 V/cm in a gel electrophoresis chamber filled with 1x TAE. Gels were stained with ethidium bromide (1 µg/ml) for 10 to 15 min, shortly rinsed with water, and were visualised with UV light (320 nm) and digitally recorded with an Alpha Imager (Biozym Scientific GmbH, Hess. Oldendorf). DNA fragments of defined lengths (GeneRuler DNA Ladder Mix, Fermentas) were used as size standards.

B.3.3. Cloning of DNA fragments and transformation of *Escherichia coli*

Purified DNA fragments were cloned into pJET 1.2 blunt (Fermentas). Cloning was performed according to the manufacturer's protocol and (Sambrook & Russel, 2001). Competent *E. coli* (strain XL1-blue) was prepared and transformed according to (Chung *et al.*, 1989).

B.3.3.1. Isolation of plasmid DNA from *Escherichia coli*

The isolation of plasmid DNA was performed according to (Birnboim, 1983). Three ml of LB medium (see G.1.1), amended with 100 µg/ ml of the corresponding antibiotic (ampicillin), were added to a 15 ml tube and inoculated with a colony of a recombinant *E. coli* strain, which grew overnight at 37°C and 250 rpm. This culture was centrifuged at 3000x g for 5 minutes at room temperature (CC 380, Hermle Labortechnik GmbH, and Wehingen) and the supernatant discarded. The Pellet was suspended in 100 µl of buffer P1 (see G.1.2) and the suspension transferred into a 1.5 ml safelock tube. After the addition of 1µl (10 mg/mL) RNase A and 100 µl buffer P2 (see G.1.2) and the tube inverted slightly several times. Then 100 µl buffer P3 (see G.1.2) have been added and the tube was inverted prior to centrifugation (Eppendorf GmbH, Hamburg, centrifuge 5417R) at 4°C and 14500x g for ten minutes. The supernatant was transferred in a new 1.5 ml reaction tube and 800 µl 96% ethanol were added. This was followed by incubation at-20°C for an hour. Later, a second centrifugation was performed for 10 minutes at 14500x g and 4°C. The supernatant was removed, discarded and the pellet was resuspended in 30-50 µl of TE buffer (see G.1.2).

B.3.4. Generation and validation of *F. graminearum* transformants

Protoplasts were used in this work for fungal transformation. For the preparation of protoplasts, $5x10^6$ macroconidia were incubated for 12 h in 100 ml of YEPD (see G.1.1) at 28°C and 175 rpm. The mycelium was recovered on a sterile paper filter and then incubated for 4 h at 30°C and 90 rpm in 20 ml of protoplasting mix (500 mg driselase, 1 mg chitinase, 100 mg lysing enzyme of *Trichoderma harzianum* in 1.2 M KCl) (see G.1.2). Protoplasts were harvested at R.T. by centrifugation at 1000x g and suspended in 1 ml STC buffer (see G.1.2) (1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5). A transformation reaction contained 10^6 protoplasts in 100 µl STC buffer, 50 µl 30% PEG 8000 and 8 µg DNA of the deletion construct in 50 µl water. After incubation for 20 min at RT and 50 rpm, 2 ml 30% PEG 8000 were added, gently mixed, and 5 min later 4 ml STC buffer were added. Aliquots of 600 µL were mixed with 15 ml molten regeneration medium (see G.1.1) and poured into a Petri dish. After incubation for 16-20 h at 25°C, 15 ml of molten regeneration medium containing the antibiotic appropriate for the marker gene used for selection, either nourseothricin or G418 at concentrations of 200 µg/ml, was poured onto the surface of the

agar. Colonies that started growing after 4 d were harvested to obtain single spore isolates as described (see paragraph B.3.5).

B.3.5. Generation of monokaryotic strains

Colonies grown on the transformation plates were transferred to a microtiter plate with 12 wells, each containing 2 ml of solid selection medium (PDA with 100 µg/ml of the respective antibiotic). After two to three days of growth at 25°C, mycelial pieces were transferred from the hyphal front to SNA (Ø 55 mm) to induce sporulation. After 10-14 days of growth at 25°C in darkness, macroconidia were collected by rinsing with 1 ml of sterile, dist. water. One to two hundred microliter of undiluted spore suspension was inoculated on PDA with 100 µg/ml of the respective antibiotic (Ø 55 mm). Single colonies that were well separated on the plate were transferred to a fresh SNA petri dish with 100 µg/ml of the respective antibiotic. For each transformation, twenty to twenty-five independend single-spored transformants were further selected for PCR to test for the deletion of the target gene (Gaensewig, 2010).

B.3.6. Southern hybridisation

To analyse the integration site and the numbers of the integrated constructs, genomic Southern blot analyses were performed on clones that were pre-selected by PCR analyses.

B.3.6.1. Generation of DIG labelled probes

Using standard PCR (see paragraph B.3.1.3.1) hybridisation probes were generated, except that for the labelling the regular dNTP mix was replaced by the DIG (digoxygenin) - labelling Kit (Roche Diagnostics) that contains (2 mM dATP; dCTP; dGTP; 0.19 mM dTTP and 0.1 mM DIG-11-dUTP, alkali labile). Agarose gel electrophoresis was performed to verify the labelling (see paragraph B.3.2). In agarose gels DIG-labelled fragments appeared larger compared to the non-labelled fragments used as reference, due to the incorporated digoxygenin. The probe was purified then using the Gene JET PCR purification kit.

B.3.6.2. Digestion of genomic DNA

Samples of DNA in (5-8 µg) were digested overnight with the appropriate restriction enzymes and buffers in a total volume of 30-50 µl and incubated at the appropriate temperature. The completeness of the reaction was examined by electrophoresis of a 5% aliquot on an agarose mini gel.

B.3.6.3. Capillary transfer of the digested gDNA

The completely digested gDNA was separated on 0.7% TAE agarose gel (2 V/cm; 16 h) (see paragraph B.3.2). DNA separation was visualised and documented as above (see paragraph B.3.2). The gel was destained with distilled water for about 15 min, then depurinated with 0.25 M HCl and denaturated with 0.4 M NaOH, both carried out for 30 min at RT. The DNA fragments were blotted using the downward capillary method onto a positively charged nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, Freiburg) (Brown, 1999). The membrane (gel-sized) was drained in 20x SSC (see G.1.2) (filter should not get dry during transfer), and placed on three gel-sized filter papers drained in 20x SSC (Whatman 3MM, Whatman GmbH. Dassel, Germany), and placed on about 5 cm stack of paper towels. The gel was placed carefully and tightly onto the membrane, which was only touched with forceps, and air bubbles were avoided. The gel was then covered with three gel-sized 20x SSC-drained Whatman filters. Two glass petri dishes (12 cm diameter) were placed beside the blot and filled with 20x SSC. Then a long rectangular Whatman filter paper was drained with 20x SSC and placed from the left to the right side of the blot to build a bridge, which touched the upper filter paper on the top and hung down on both sides to the supplying buffer reservoirs. This filter paper bridge should not touch the blot on either side in order to keep the paper towels dry and to keep the capillary transfer working. Finally, a glass plate (gel sized) and a weight of about 250 g were put on the top and the blotting continued for 4 to 6 h. After blotting the gel was re-stained with ethidium bromide as above to document the completeness of DNA transfer. Then the transferred DNA was cross-linked to the filter by an incubation of 1-2 hours at 80- 90°C in a hybridization oven (Biozym Scientific GmbH, Hess. Oldendorf, Germany).

B.3.6.4. Hybridization

After cross-linking, the membrane was placed in a glass tube and incubated with 20 ml of pre-hybridization buffer (see G.1.2) at 65°C for 2 h in the hybridization oven mentioned above. Thereafter, the DIG-UTP-labelled probe (300 ng) was denaturated at 100°C for 2 min in a heating block, chilled briefly on ice, added to the hybridization buffer in the tube and incubated overnight at 65°C. To analyse the transformants for the correct integration of a single copy of the deletion cassette at the targeted locus in the genome, two blots were performed. The first filter was hybridized with a probe that was specific for the wild type allele of the target gene to indicate which clones are putative deletion mutants and which clones still have the original locus and thus may carry ectopic integrations. The second filter was hybridised with a probe that detected the resistance marker gene to check whether putative deletion mutants may carry additional ectopic copies of the transformed DNA fragment.

B.3.6.5. Detection of the bound probe

The membrane was washed in the tube for 5 min with 2x, 0.5x and 0.25x SSC for 10, 15, 15 min, respectively. Thereafter, it was equilibrated for 1 min with buffer M (see G.1.2). Afterwards, the membrane was incubated for 2 h with the blocking solution and subsequently with the anti-DIG antibody-coupled alkaline phosphatase (diluted 1:10000 in the same blocking buffer; anti-DIG AB, Fab-fragment; Roche Diagnostics) with the blocking buffer for 1 h at RT. The unbound antibodies were washed off twice for 15 min with the washing buffer at RT. The membrane was washed with the detection buffer for 1 min and then removed from the glass tube and placed in a plastic bag (Carl Roth GmbH). CSPD solution (0.25 M CSPD ready-to-use; Roche Diagnostics) was diluted 1:20 in 1 ml of detection buffer and this solution was equally distributed onto the filter avoiding the generation of bubbles. Initial incubation was for 5 min in darkness. Then, the plastic bag was sealed and the membrane inside it was exposed to an X-ray film (Hyperfilm ESL; Amersham Pharmacia Biotech, Freiburg, Germany). Depending on the signal strength the exposure time ranged from 2 h to overnight (12-16 h). Finally, the film was developed with an Optimax TR device (MS Laborgeräte, Heidelberg, Germany). All buffers used are listed in the appendix (see G.1.2).

B.3.7. Complementation of deletion mutants with the wild type allele

To complement the deletion strains with the native wild type allele, co-transformation was applied, in which the resistance cassette in the deletion strains was replaced by homologous double cross over with the native ABC transporter gene at the native locus. In order to select such transformants, at the same time a second DNA fragment was transformed that carried another resistance cassette, which should integrate ectopically into the genome. *ΔFgABC2.NRRL.8* and *ΔFgABC3.NRRL.8* have been chosen for such complementation experiments. The nourseothricin resistance cassette in the strain *ΔFgABC2.NRRL.8* was replaced with the *FgABC2* wild type allele by co-transformation with the geneticin cassette as a resistance marker. The geneticin cassette was amplified from pII99 to yield a PCR product of 1251 bp. The amplification of the *FgABC2* allele from genomic DNA of the NRRL 13383 wild type strain yielded a fragment of 4836 bp. The PCR purification and transformation was done using protocols described above (see paragraphs B.3.2 and B.3.4) Nine µg of the *FgABC2* fragment and 6 µg of the geneticin resistance cassette were used to co-transform protoplasts.

Transformants that emerged from the regeneration medium with geneticin were transferred onto PDA with geneticin. These strains were then tested on PDA with nourseothricin to test for the loss of resistance against the old resistance marker. Transformants that were not able to grow on nourseothricin were considered as candidate strains of a successful complementation. Such strains were further analysed by three independent PCR tests. The first PCR tested for the presence or absence of the new resistance marker cassette. The second PCR tested for the presence or absence of the old resistance marker cassette. The third PCR analysed the targeted locus for amplicon size to prove the homologous re-integration of the wild type allele. The same strategy was applied for the complementation of *FgABC3*. The geneticin resistance cassette in *ΔFgABC3.NRRL.8* strain was replaced with the *FgABC3* wild type allele by co-transformation with the nourseothricin resistance marker cassette. The *FgABC3* allele had a size of 4580 bp and it was amplified from genomic DNA of NRRL 13383. The nourseothricin resistance cassette was amplified from pNR1 to yield a product of 1473 bp. Transformation of protoplast of strain *ΔFgABC3.NRRL.8* was performed as above.

B.3.8. Oligonucleotide sequences used in this study

All oligonucleotides used in these studies are listed in the Appendix (see G.3).

B.4. Determination of sensitivity to fungicides and plant metabolites

"The sensitivity of transformants to fungicides and plant compounds was tested on PDA plates (12 x 12 cm, Greiner Bio-One) amended with appropriate concentrations of a given substance. For each compound, we used three concentrations that were optimized in preliminary experiments. For azoles, we chose concentrations that roughly covered the ED_{50} and ED_{90} values determined earlier (Becher *et al.*, 2010). For the other substances, we first tested widely spaced concentrations and then narrowed down to concentrations that allowed for differentiation between the strains. The following fungicides were obtained as commercial formulations: azoxystrobin (Amistra, BASF), fenpropimorph (Corbel, BASF), metconazole (Caramba, BASF), prochloraz (Sportak, BASF) and tebuconazole (Folicur, Bayer). Pure active compounds, i.e. epoxyconazole, fenarimol, spiroxamine, boscalid and dithianon were obtained from Sigma-Aldrich (Schnelldorf, Germany), except for prothioconazole, which was kindly provided by Bayer. Tolnaftat (Sigma-Aldrich) was included as a control xenobiotic that has never been used in agriculture. Sensitivity of fungal strains against plant secondary metabolites 2-benzoxazolinone (= BOA), 3-(dimethylaminomethyl) indole (= gramine), 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one,4′,5,7-trihydroxy-flavanone (= naringenin) and 3,3′,4′,5,7-pentahydroxyflavone dihydrate (= quercetin) purchased from Sigma-Aldrich was tested in the same way. For all substances, stock solutions were prepared in DMSO; the final concentration of the solvent in culture media was at most 0.3%. Two µL of a suspension of macroconidia ($1x10^5$ ml^{-1}) were used as inoculum to assess the effect of the compounds on germination. Mycelial plugs on unamended PDA were used as inoculum to determine the effect of the compounds on vegetative growth. At least three plates were used for each compound and concentration. Incubation was carried out in the dark at 23°C for strains derived from NRRL 13383 and at 30°C for strains with PH-1 background. Colony area was determined on digital photographs using ImageJ software version 1.46 (http://rsbweb.nih.gov/ij/index.html). Statistical significances were determined by T-test (p<0.05) as implemented in the Microsoft Excel 2010 software" (Abou Ammar *et al.*, 2013).

B.5. Analysis of mycotoxin production *in vitro*

"*Fusarium* isolates were grown in rice media, culture material was extracted with acetonitrile/water and the extracts were defatted as described. Mycotoxins were separated by HPLC on an RP column (Polaris C18 ether, 10062 mm, 3 mm particle size; Agilent, Darmstadt, Germany) at 40°C at a flow rate of 0.2 ml/ min. The solvent system consisted of (A) water with 5% acetonitrile and (B) methanol, both containing 7 mM acetic acid. The elution gradient rose linearly from 10% to 98% of solvent B followed by washing and equilibration steps. The detection was performed by tandem mass spectrometry using triple quadruple 1200L (Varian, Darmstadt, Germany) after electrospray ionization in negative mode as described before.

For each isolate, at least five independent cultures were analysed. Statistical significances were determined by T-test ($p<0.05$) as implemented in the Microsoft Excel 2010 software" (Abou-Ammar et al., 2013).

C. Results

C.1. Selection of two ABC transporter genes for targeted deletion

Two candidate genes *FgABC2*, *FgABC3* encoding ABC transporters were selected from the *Fusarium graminearum* genome for targeted deletion mutagenesis. These candidate genes belong to two different ABC subfamilies and have been shown to be up-regulated in strains PH-1 (15-ADON chemotype) and NRRL 13383 (NIV chemotype) after tebuconazole treatment (see paragraph A.6). To examine how the genetic background may influence the effects of the mutation on fungicide sensitivity and virulence, deletions of these two genes were introduced in two wild strains representing the NIV and the 15-ADON chemotypes, i.e. NRRL 13383 and PH-1.

C.2. Deletion of *FgABC2* and *FgABC3*

FgABC2 (FGSG_17046) was replaced in *F. graminearum* wild type strains PH-1 and NRRL 13383 by transformation of protoplasts using a deletion construct that had been generated by the DJ-PCR method (Yu *et al.*, 2004) and that included a nourseothricin resistance cassette. The deletion construct was a fusion of 1217 bp of the 5´ region of *FgABC2*, 1748 bp of a nourseothricin acetyltransferase (*nat1*) resistance cassette (amplified from pNR1) and 1143 bp of the 3´ region of *FgABC2* (both the 5´ and 3´ region of the selected gene were amplified from genomic DNA of *F. graminearum* PH-1) (see paragraph B.3.1.3 and Fig. 1 A).

Correspondingly, *FgABC3* (FGSG_04580) was replaced in *F. graminearum* PH-1 and NRRL 13383. The deletion construct was a fusion of 896 bp of the 5´ region of *FgABC3*, 1251 bp of a geneticin resistance cassette (amplified from pII99) and 571 bp of the 3´ region of *FgABC3* (both the 5´ and 3´ region of the selected gene were amplified from genomic DNA of *F. graminearum* PH-1) (see paragraph B.3.1.3 and Fig. 2 A).

After transformation, single spore isolates were generated from the resulting transformants (see paragraph B.3.5), and genomic DNA was isolated from the resistant monokaryotic clones (see paragraph B.3.1.1.2). Standard PCR assays were performed targeting the wild type locus to obtain information about the success of the deletion in the transformants (see paragraph B.3.1.3.1 and Figs. 1 B, 2 B).

Clones, in which the band representing the wild type allele of the selected gene was not amplified by this PCR assay, were then selected for Southern blot analyses. Two nylon membranes carrying digested genomic DNA of such candidate clones were hybridized with a probe targeting the candidate ORF or the resistance marker, respectively. The first probe tested for the absence of the wild type allele to indicate which clones are putative deletion mutants and which clones still have the original locus and thus may carry ectopic integrations. The second probe tested whether putative deletion mutants may carry additional ectopic copies of the transformed DNA fragment (see paragraph B.3.6 and Figs. 1 C, 2 C). In both recipient strains several independent transformants were obtained for each candidate gene that harboured a single DNA integration at the target locus resulting from a double-cross-over event, representing a type III integration leading to the deletion of the selected transporter gene (Yu *et al.*, 2004).

For further experiments, two independent transformants were chosen for each deleted gene in each of the recipient strains (see Figs. 1 C, 2 C). The selected clones in the PH-1 background were *FgABC2.PH1.2*; *FgABC2.PH1.7*; *FgABC3.PH1.1*; *FgABC3.PH1.5*. In the NRRL 13383 background the selected clones were *FgABC2.NRRL.5*; *FgABC2.NRRL.8*; *FgABC3.NRRL.2*; *FgABC3.NRRL.8*.

C.3. Complementation of deletion mutants with wild type alleles of *FgABC2* and *FGABC3*

Complementation is a tool for the genetic characterization of deletion mutants. In this technique the wild type gene is re-introduced into a deletion mutant to confirm that the phenotypes observed in the deletion mutant are indeed the result of a single type III integration event and not an artefact occurred during transformation. Δ*FgABC2.NRRL.8* and Δ*FgABC3.NRRL.8* were chosen as recipient strains for complementation. A co-transformation method was applied, in which the resistance cassette in the deletion strains was replaced with the respective native ABC transporter gene (see paragraph B.3.7).

Accordingly, the nourseothricin resistance cassette in the Δ*FgABC2.NRRL.8* strain was replaced with the *FgABC2* wild type allele following protoplast transformation. At the same time a second DNA fragment was transformed carrying a geneticin resistance cassette, which should integrate ectopically somewhere in the genome.

In strain Δ*FgABC3.NRRL.8* the geneticin resistance cassette was replaced with the *FgABC3* wild type allele. Selection relied on another co-transformed DNA fragment carrying a hygromycin resistance cassette (see Fig. 3 A, Fig. 4 A).

Transformants that exhibited the expected phenotypes on tester media, i.e. a gain of resistance corresponding to the new marker and a loss of resistance matching to the old resistance marker were subjected to three different PCR tests. The first PCR detected the presence or absence of the new resistance marker cassette (see Fig. 3 B, Fig. 4 B). The second PCR tested for the presence or absence of the old resistance cassette that had been used to create the deletion (see Figs. 3 C, 4 C). The third PCR analysed the targeted loci for a change of amplicon size to prove the homologous re-integration of the native gene. The primers used for the last PCR bound outside of the transformed DNA (see Figs. 3 D, 4 D). In case of *FgABC2*, the deletion mutant yielded an amplicon size of 3841 bp, whereas strains with a successful complementation exhibited a band of 7258 bp as the WT did. In case of *FgABC3*, the deletion strain showed a band of 2792 bp, whereas the complemented strains and the WT had bands of 6123 bp.

To confirm the phenotypes observed for the deletion strains in virulence assays (see paragraph B.1.2.2) and fungicide sensitivity assays (see paragraph B.4), two independent complemented strains of Δ*FgABC3* were chosen for further experimentation. Since it eventually turned out during these assays that the deletion of *FgABC2* did not lead to any discernible phenotypes in comparison to the wild type strains, the complemented strains of *ΔFgABC2* were not used further on.

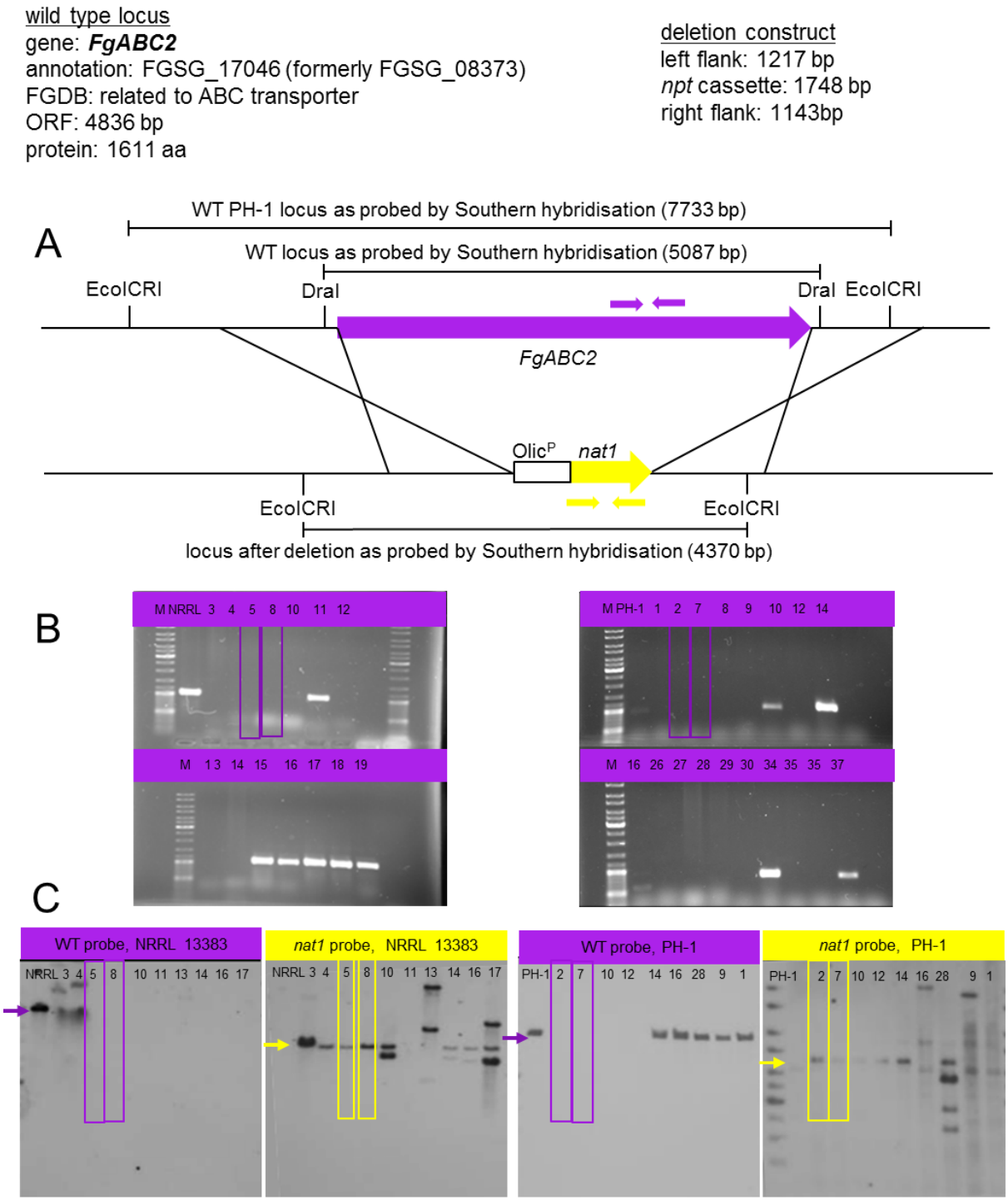

Fig. 1. **A)** Strategy to delete *FgABC2*. Lilla arrows give the positions of primers used to detect the wild type locus by PCR. Yellow arrows indicate the respective primers for the nourseothricin resistance marker (*nat1*). The same primers were used to generate the DIG-labeled probes used for Southern hybridisation**. B)** Results of PCR targeting the wild type locus. **C)** Results of Southern hybridisation detecting the wild type locus (lilla) and *nat1* (yellow). Arrows indicate the expected band. Boxed lanes indicate clones used for subsequent analyses. (taken from Abou Ammar *et al*., 2013).

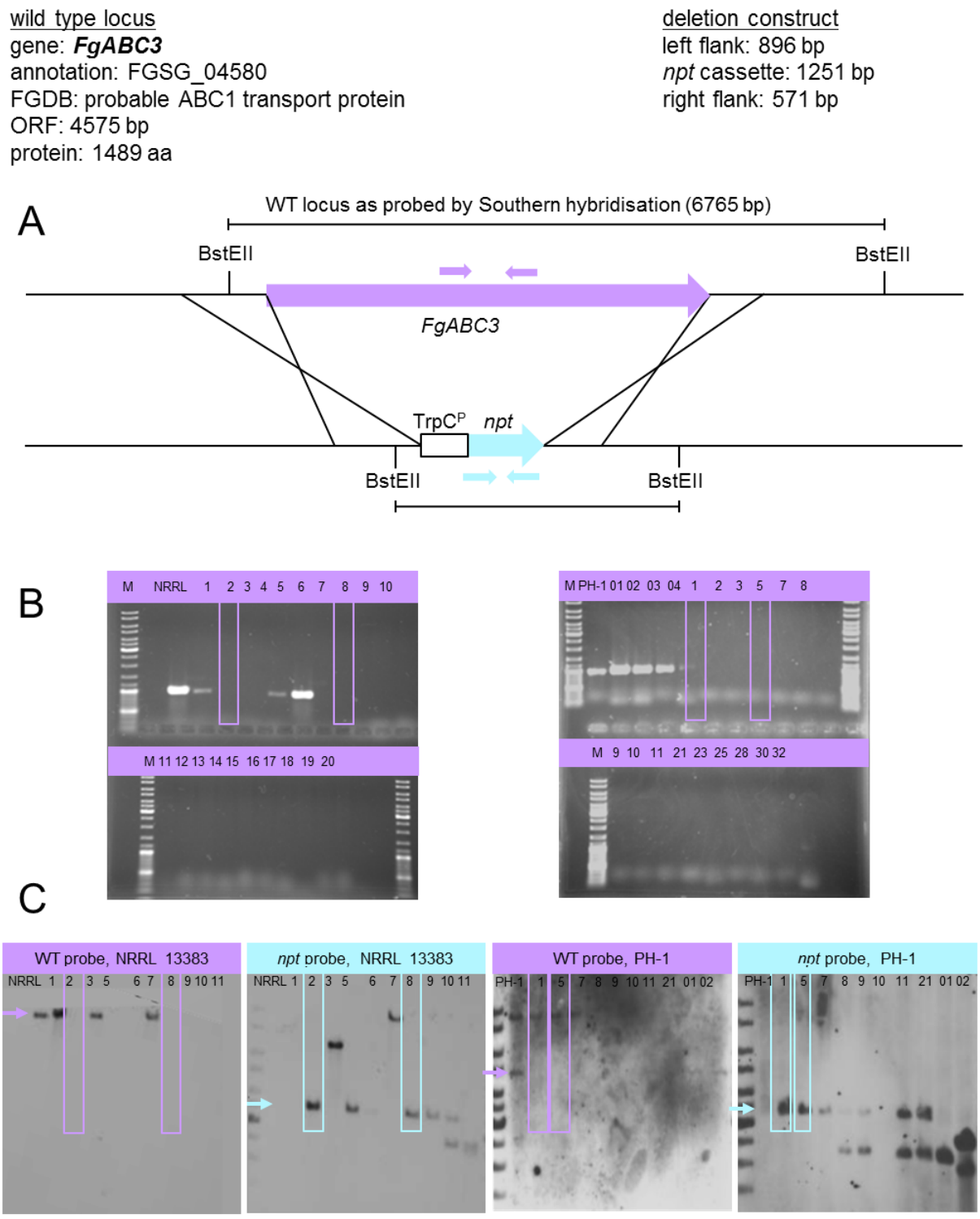

Fig. 2. **A**) Strategy to delete *FgABC3*. Light lilla arrows give the positions of primers used to detect the wild type locus by PCR. Light green arrows indicate the respective primers for the neomycin resistance marker (*npt*). The same primers were used to generate the DIG-labeled probes used for Southern hybridisation. **B)** Results of PCR targeting the wild type locus. **C)** Results of Southern hybridisation detecting the wild type locus (light lilla) and *npt* (light green). Arrows indicate the expected band. Boxed lanes indicate clones used for subsequent analyses. (taken from Abou Ammar *et al*., 2013).

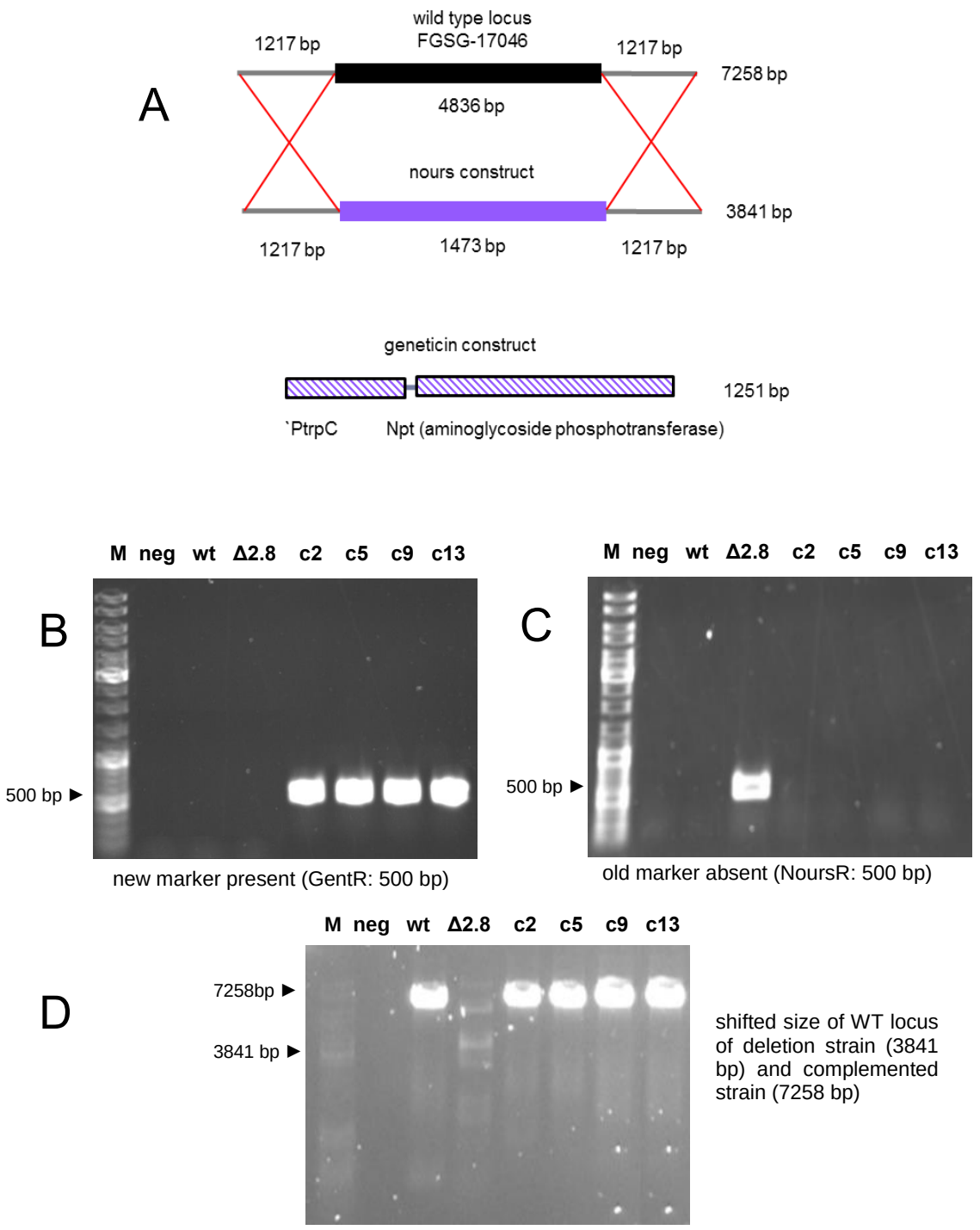

Fig. 3. **Complementation of *FgABC2*. A)** The nourseothricin resistance cassette in *ΔFgABC2.NRRL.8* was replaced with the *FgABC2* WT allele. The geneticin resistance cassette was co-transformed and integrated ectopically. **B)** The first PCR detected the presence or absence of the new resistance marker cassette. **C)** The second PCR tested for the presence or absence of the old cassette. **D)** The third PCR analysed the targeted loci for a different amplicon size to prove the homologous re-integration of the native gene in the genomic DNA. M for the Marker (Gene Ruler DNA Ladder Mix, Fermentas, St. Leon-Rot, Germany) was used as size standard.

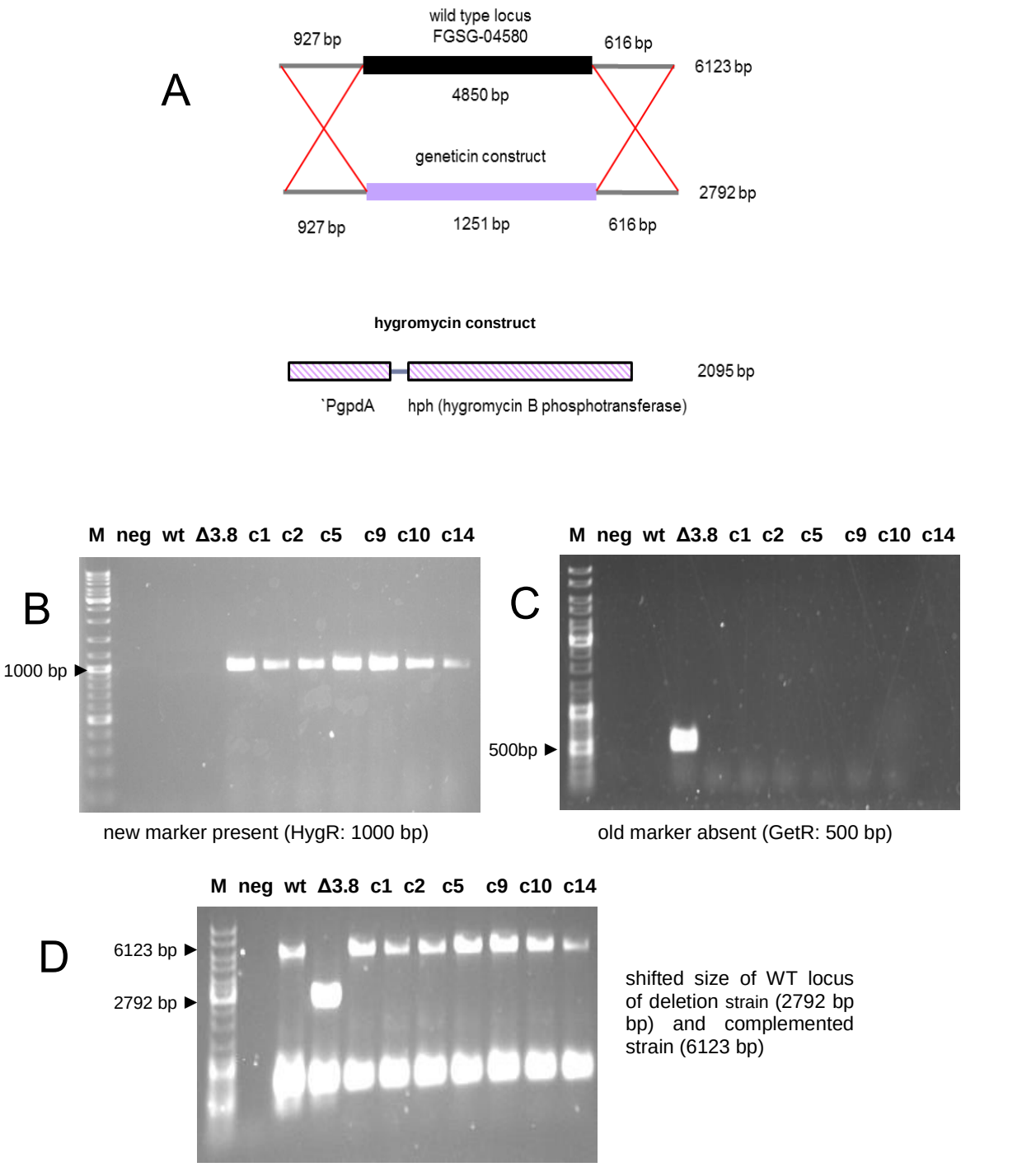

Fig. 4. **Complementation of *FgABC3*. A)** The geneticin resistance cassette in the *ΔFgABC3.NRRL.8* was replaced with *FgABC3* allele and introduced with the hygromicin cassette as a resistance marker. **B)** The first PCR detected the presence or absence of the new resistance marker cassette. **C)** The second PCR tested for the presence or absence of the old cassette. **D)** The third PCR analysed the targeted loci for a different amplicon sizes order to prove the homologous re-integration of the native gene in the genomic DNA**.** M for the Marker (GeneRuler DNA Ladder Mix, Fermentas, St. Leon-Rot, Germany) was used as size standard.

C.4. Characterization of Δ*FgABC2* and *ΔFgABC3* transformants and complemented strains

C.4.1. Vegetative fitness and growth assays

Growth assays were performed in order to assess whether the deletion of *FgABC2* and *FgABC3* would generally affect the resulting transformants with respect to vegetative growth and asexual reproduction (see paragraph B.1.1.3). For each deletion, growth rates on PDA plates were examined at three temperatures (15, 25 and 30°C) (see Fig. 5). The formation of conidia as well as their germination *in vitro* was quantified in two transformants for each gene deletion (see Fig. 6 A and B).

None of the deletions led to any significant change in any of the three assays that have been performed. This was true for the transformants in both backgrounds PH-1 and NRRL 13383. However, there existed a difference in growth rates between the two backgrounds. PH-1 and the mutants derived from it grew somewhat faster than the strains from the other background (see Fig. 5). Conidia formation differed between both backgrounds, as the NRRL 13383 wild type and the mutants in this background showed higher conidia production in MBB medium compared to strains in the PH-1 background grown at the same conditions (see Fig. 6 A).

The complemented strains of *FgABC2* and *FgABC3* did not show any significant changes with respect to vegetative growth and asexual reproduction in comparison to NRRL 13383 (see Fig. 7 A and B).

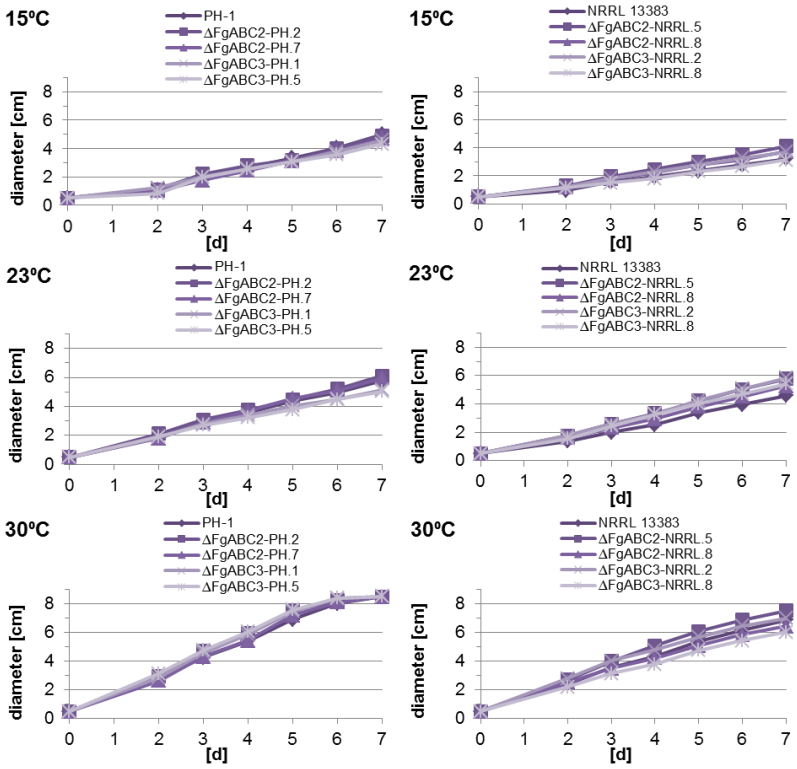

Fig. 5. Growth kinetics of deletion strains in NRRL 13383 and PH-1 backgrounds. Two deletion mutants for each gene are compared to the respective wild type strain at three temperatures on PDA medium (modified from (modified from Abou Ammar *et al*., 2013).

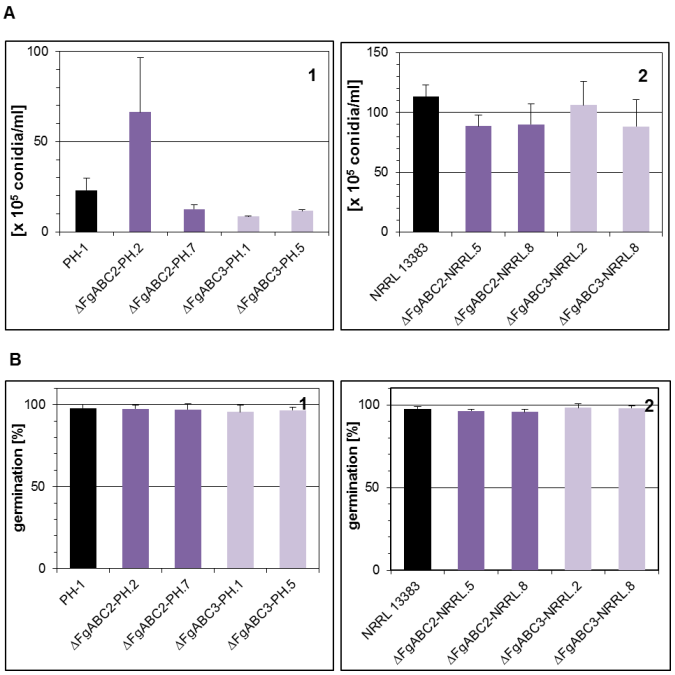

Fig. 6. A) Formation of macroconidia *in vitro.* For each deletion, two transformants of each genetic background are compared to the respective wild type strain. Data show the average conidial densities formed in MBB medium in four replicated cultures after incubation for 7 d at 23ºC. Error bars represent SE. None of the variations between the mutants and the wild type is significant. **B) Germination of macroconidia *in vitro.*** For each deletion, two transformants of each genetic background are compared to the respective wild type strain. Data shown give the average frequencies of germinated macroconidia on glass slides in four replicated cultures after incubation for 24 h at 23ºC. Error bars represent SD. **1)** PH-1 background, **2)** NRRL 13383 background (modified from (Abou Ammar *et al*., 2013)

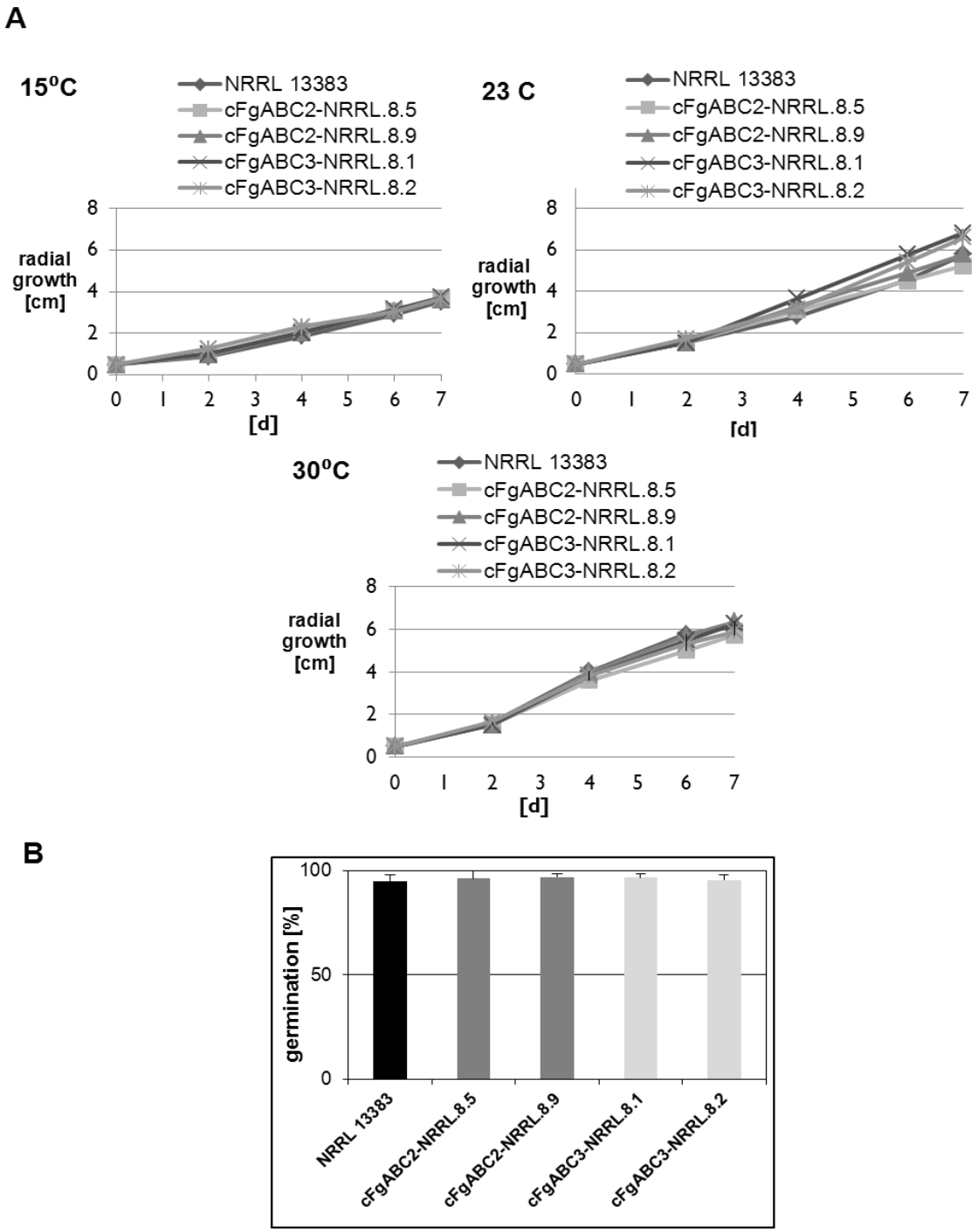

Fig. 7. Vegetative fitness of complemented strains in NRRL 13383 background. A) Growth kinetics *in vitro.* For each complementation, two transformants are compared to the respective wild type strain at three temperatures on PDA medium. **B)** Germination of macroconidia *in vitro.* For each complementation, two transformants are compared to the wild type strain NRRL 13383. Data shown give the average frequencies of germinated macroconidia on glass slides in four replicated cultures after incubation for 24 h at 23ºC.

C.4.2. Sensitivity assays of the deletion mutants

C.4.2.1. Fungicide sensitivity assays

In this work, the impact of the deletion of the ABC transporter genes *FgABC2* and *FgABC3* on fungicide sensitivity in *Fusarium graminearum* was characterized. Sensitivity assays have been applied (see paragraph B.4) to study the effects of 11 fungicides belonging to eight different chemical groups (anthraquinones, imidazoles, methoxy-acrylates, morpholines, pyridine-carboxamides, pyrimidines, spiroketal-amines and triazoles) on germination and vegetative growth of the transformants (Table C.1).

Table C.1. Sensitivity of deletion mutants to fungicides in WT backgrounds

			PH-1 background		NRRL 13383 background	
common name	**fungicide class**	**chemical grouping**	**conidia**	**mycelia**	**conidia**	**mycelia**
tebuconazole	SBI class I	triazole	WT	Δ*FgABC3*	Δ*FgABC3*	Δ*FgABC3*
prothioconazole	SBI class I	triazole	Δ*FgABC3*	Δ*FgABC3*	Δ*FgABC3*	Δ*FgABC3*
epoxyconazole	SBI class I	triazole	WT	WT	Δ*FgABC3*	Δ*FgABC3*
metconazole	SBI class I	triazole	WT	WT	WT	WT
fenarimol	SBI class I	pyrimidine	Δ*FgABC3*	Δ*FgABC3*	WT	Δ*FgABC3*
prochloraz	SBI class I	imidazole	WT	WT	WT	WT
fenpropimorph	SBI class II	morpholine	WT	WT	WT	WT
spiroxamine	SBI class II	spiroketal-amine	WT	WT	WT	WT
azoxystrobin	QoI	methoxy-acrylates	WT	WT	WT	WT
boscalid	SDHI	pyridine-carboxamide	WT	WT	WT	WT
dithianon	quinones	Anthraqui-none	WT	WT	WT	WT
tolnaftat	n.a.	n.a.	WT	WT	WT	WT

Designated mutations led to significantly ($p < 0.05$) increased sensitivity in comparison to the respective wild type strain. WT: no significantly altered sensitivity. Tolnaftat was included as a xenobiotic that has not been used in agriculture.

Sensitivity assays were performed by measuring the area covered by a given colony on PDA medium containing a specific concentration of each fungicide tested (see paragraph B.4). Such cultures were started by inoculating a defined number of conidia or a small mycelial plug onto the plates. Cultures from inoculations of conidia were typically more sensitive to fungicides than those inoculated with mycelial plugs, which was true for both backgrounds (see Figs. 8, 9).

In the background of NRRL 13383, Δ*FgABC3* mutants had significantly reduced tolerance for the triazoles tebuconazole, prothioconazole and epoxyconazole, but also to fenarimol, which is a pyrimidine fungicide but has the same target as the triazoles (both SBI class I) (see Table C.1 and Fig. 8). There existed no significant changes in sensitivity against fungicides belonging to other fungicide classes (SBI class II, QoI, SDHI, anthraquinone, as well as tolnaftat) (Table.C.1). In contrast to *FgABC3*, deletion of *FgABC2* in the same background (NRRL 13383) did not affect significantly the tolerance levels against any of the fungicides tested (see Table C.1 and Fig. 8).

The deletion of the same two ABC transporter genes in the PH-1 background affected the resulting mutants in a similar way as in NRRL 13383 (see Table C.1 and Fig. 9). However, strains of the PH-1 background were generally more sensitive to most fungicides when compared to the corresponding strains in the NRRL 13383 background. Although the differences between the wild type and the mutants in the PH-1 background were similar as seen for NRRL 13383, they were sometimes less severe, so that they were not statistically supported anymore (see Table C.1, Figs. 8, 9).

Sensitivities of two complemented strains of Δ*FgABC3* in the NRRL 13383 background to the azole fungicide tebuconazole were tested to demonstrate that the effects seen in the deletion mutants did result from the removal of the gene and not from artefacts that might have occurred during the transformation. Results obtained showed that the complementation strains indeed did recover from the reductions in fungicide sensitivity reaching levels that were similar to those of the wild type NRRL 13383 (see Fig. 10).

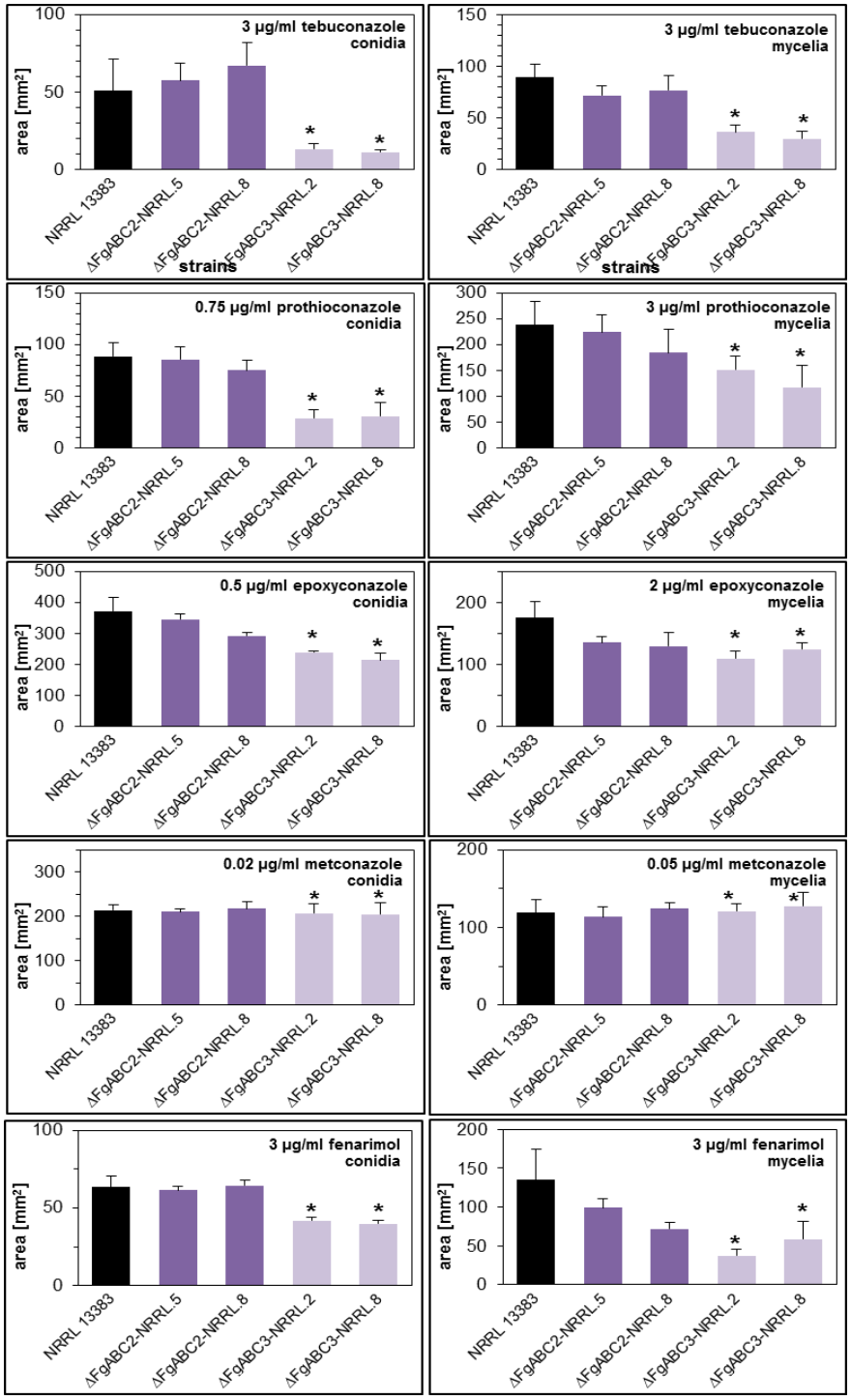

Fig. 8. Sensitivity to SBI class I fungicides. For each deletion, colonial areas of two transformants in the NRRL 13383 background were assessed on PDA amended with the indicated concentration of fungicides. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type. Strains tested: NRRL 13383, Δ*FgABC2-NRRL.5*, Δ*FgABC2-NRRL.8*, Δ*FgABC3-NRRL.2*, Δ*FgABC3-NRRL.8*. (modified from Abou Ammar *et al*., 2013).

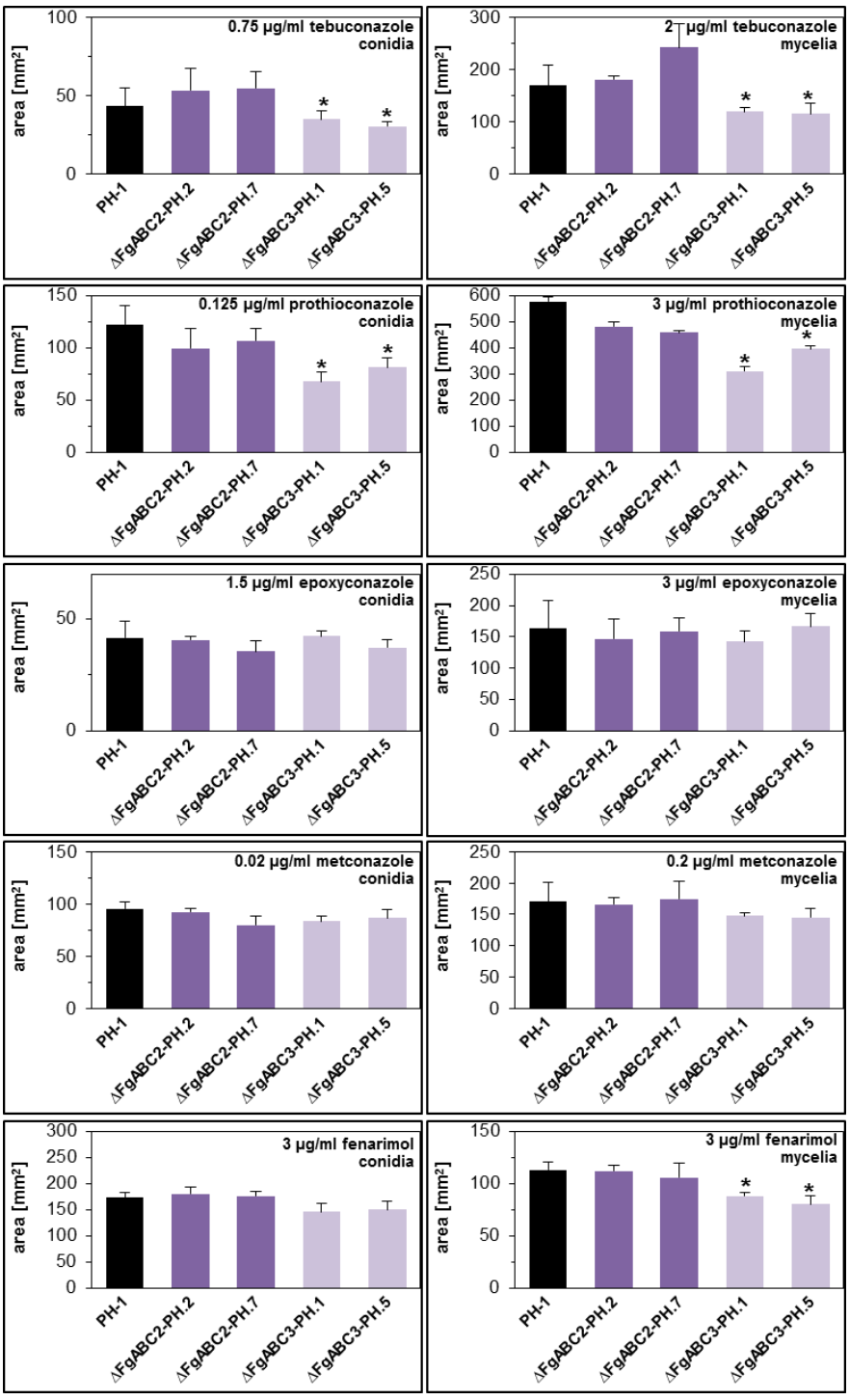

Fig. 9. Sensitivity to SBI class I fungicides. For each deletion, colonial areas of two transformants in the PH-1 background were assessed on PDA amended with the indicated concentration of fungicides. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type. Strains tested: PH-1, Δ*FgABC2-PH.2*, Δ*FgABC2-PH.7*, Δ*FgABC3-PH.1*, Δ*FgABC3-PH.5.* (modified from Abou Ammar *et al*., 2013).

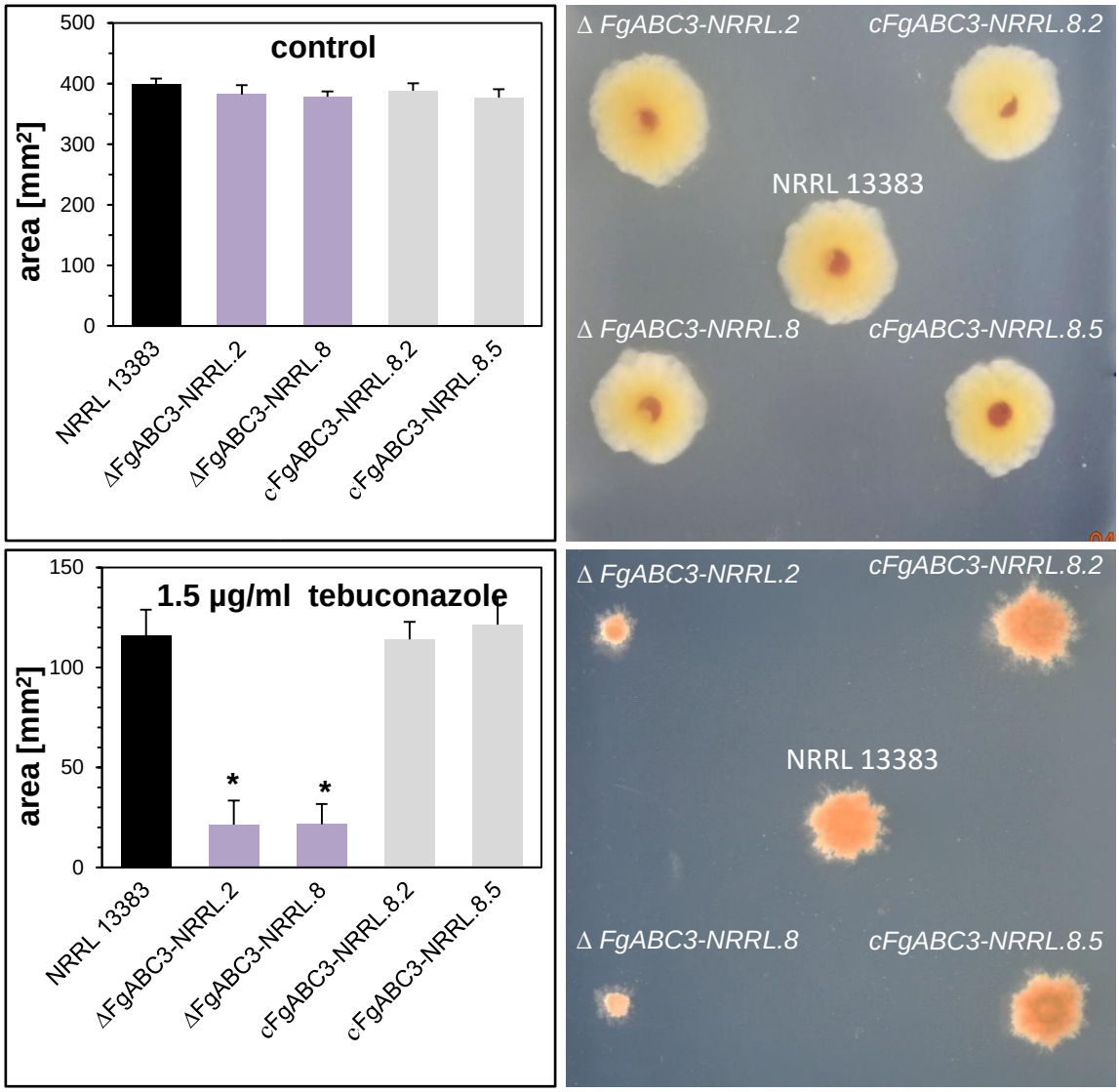

Fig. 10. Sensitivity to tebuconazole of complemented strains. Colonial areas were assessed on PDA amended with the indicated concentration of fungicide. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type NRRL 13383, strains tested: NRRL 13383, two deletion mutants (Δ*FgABC3-NRRL.2*, Δ*FgABC3-NRRL.8*), and two complemented strains (*cFgABC3-NRRL.8.2*, *cFgABC3-NRRL.8.5).*

C.4.2.2. Impact of SBI class I fungicides on hyphal morphology

Since the *FgABC3* deletion mutants were clearly affected in fungicide sensitivity (see Table C.1 and Figs. 8, 9) microscopic analyses were performed to visualize the impact of the SBI class I fungicides prothioconazole and fenarimol on their hyphal morphology (see Fig. 11 A and B). Concentrations used were chosen according to the results of the sensitivity assays that had been performed previously (see Figs. 8, 9).

Hyphal morphology of all mutants grown in liquid cultures without fungicides resembled that of the respective wild type strains. However, treatment with 3 ppm of the tested fungicides induced changes in hyphal morphology in the Δ*FgABC3* mutants, but not in the WT and in the Δ*FgABC2* mutants (data of Δ*FgABC2* are not shown here as there were no differences to the respective wild type strain). Hyphae of Δ*FgABC3* appeared thicker and had bubble-like structures or swellings that emerged throughout the mycelium, but most often apically. Treatment of *Fusarium culmorum* with tebuconazole had shown the same effects, as reported previously (Kang & Buchenauer, 2000).

C.4.2.3. Sensitivity to cereal plant secondary metabolites

To address if the candidate transporter proteins could have roles in protection of the fungus from host defence compounds, the sensitivity of the transformants to four commercially available secondary metabolites with antifungal properties, which are produced by cereals, was tested. Sensitivity assays were performed in the same way as for fungicides to examine putative effects resulting from the deletion of a candidate gene. Different concentrations were tested ranging from 1 µg to 500 µg/ml. However, neither BOA, gramine, naringenin nor quercetin affected at any concentration the growth of any mutant in any background differently from the respective wild type strain (data are not shown).

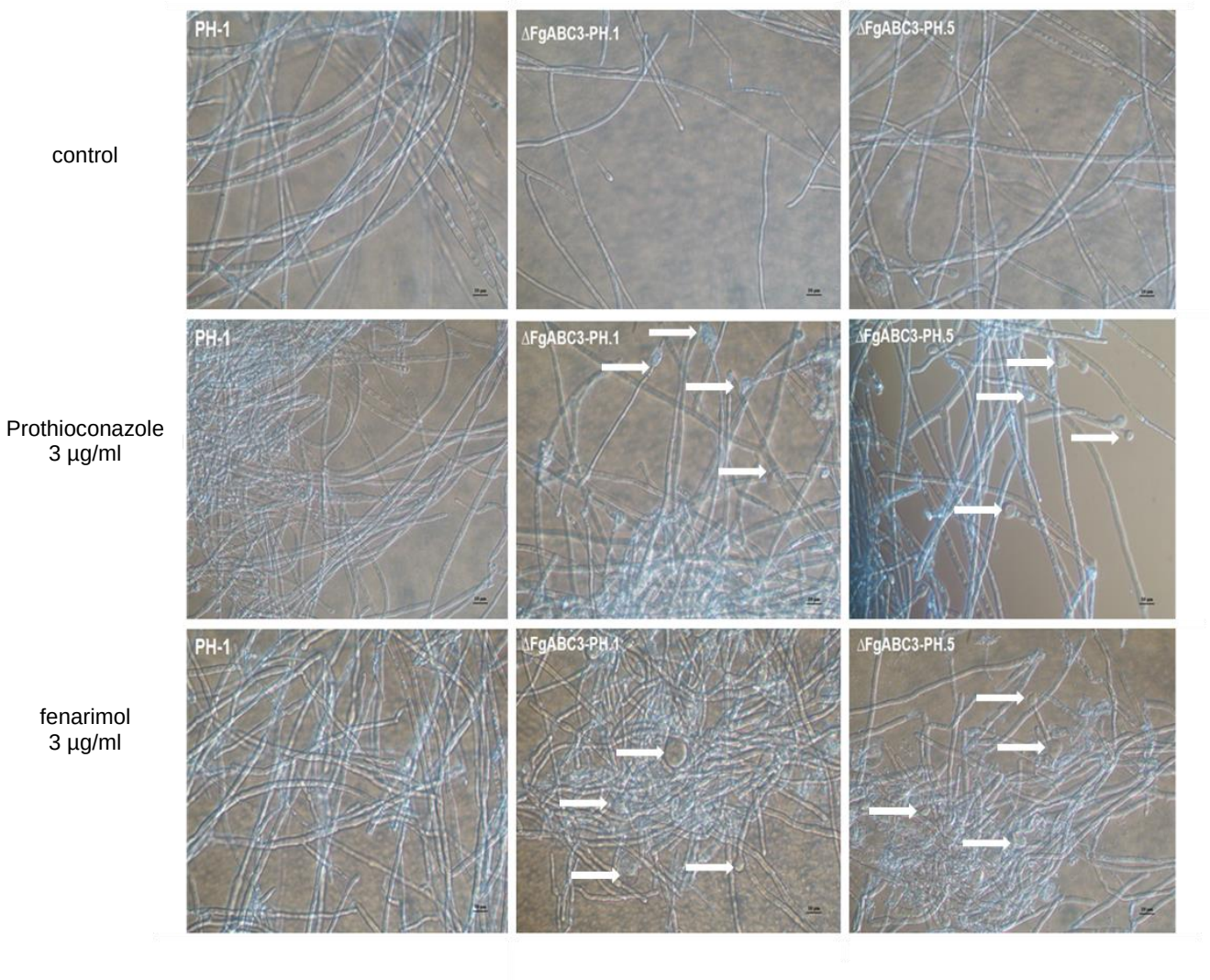

Fig. 11.A. Influence of SBI class I fungicides on hyphal morphology of Δ*FgABC3* mutants in the PH-1 background. For each strain, cultures containing 3 ppm of prothioconazole or fenarimol or no fungicide were grown for 4 d in liquid PDA. Only Δ*FgABC3* mutants are shown, since Δ*FgABC2* mutants did not differ from the wild type. Observation by bright field microscopy at 400x magnification. Arrows refer to the bubble-like structures.

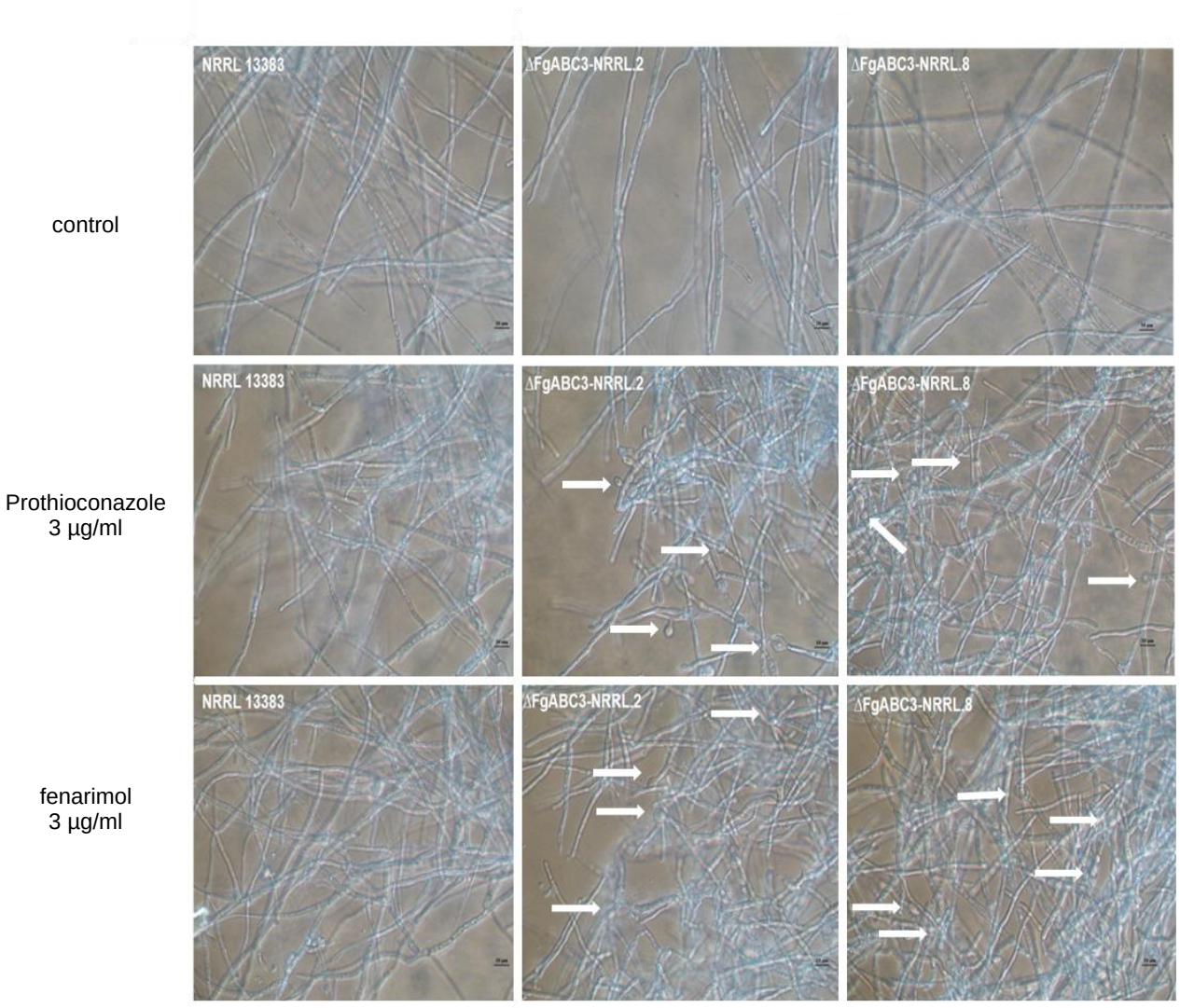

Fig. 11.B. Influence of SBI class I fungicides on hyphal morphology of Δ*FgABC3* mutants in the NRRL 13383 background. For each strain, cultures containing 3 ppm of prothioconazole or fenarimol or no fungicide were grown for 4 d in liquid PDA. Only Δ*FgABC3* mutants are shown, since Δ*FgABC2* mutants were like the wild type references. Observation by bright field microscopy at 400x magnification. Arrows refer to the bubble-like structures.

C.4.3.Virulence assays of the deletion mutants

Virulence assays on wheat using a point-inoculation method were performed (see paragraph B.1.2.2.1). For each deleted gene, conidia of two transformants of each background were pipetted into the central spikelets of wheat ears. Over the entire period monitored, the PH-1 strain was more aggressive on wheat than NRRL 13383, as the symptoms developed faster in the former strain leading to a higher percentage of bleached spikelets per head (see Fig. 12 A and B). Deletion of *FgABC3* caused a strong reduction of virulence in both backgrounds. Although the mutants were still able to cause local infections starting from the inoculated spikelet, they spread very slowly as compared to the corresponding wild type strains. At the end of the scoring period, in the PH-1 background the Δ*FgABC3* mutants had caused disease in only about one third of the spikelets, as compared to the wild type strain (see Figs. 12 A, 15). In the NRRL 13383 background, symptom development was even slower (see Figs. 12 B, 15).

In contrast, deletion mutants of *FgABC2* showed no reduction in virulence and symptom development resembled the corresponding wild type references (see Fig. 12 A and B).

As *F. graminearum* has a wide range of host plants it was assessed also whether the virulence factor discovered to be essential for infection of wheat might be also essential for the infection of other host species. For barley, a virulence assay was applied that included a spray inoculation of the entire head, whereas for maize an assay was used in which a conidial suspension was injected into wounded stems (see paragraphs B.1.2.2.2 and B.1.2.2.3). Interestingly, as observed in wheat, the deletion of the *FgABC3* gene similarly caused a considerable reduction in virulence in maize (see Figs. 13, 15) and barley (see Figs. 14, 15). When assessing the contribution of the genetic background, PH-1 was more aggressive on both plants in comparison to NRRL 13383 over the scoring period (14 dpi) as it was observed in wheat infection assays. Interestingly, the reduction in virulence was also confirmed for *FgABC3* mutants on maize and barley plants, which confirmed that this transporter plays a role in virulence irrespective of the host species. As seen in wheat, the deletion of *FgABC2* did not introduce any notable changes in virulence in the other two hosts irrespective of the genetic background of the fungus (see Fig. 13, 14).

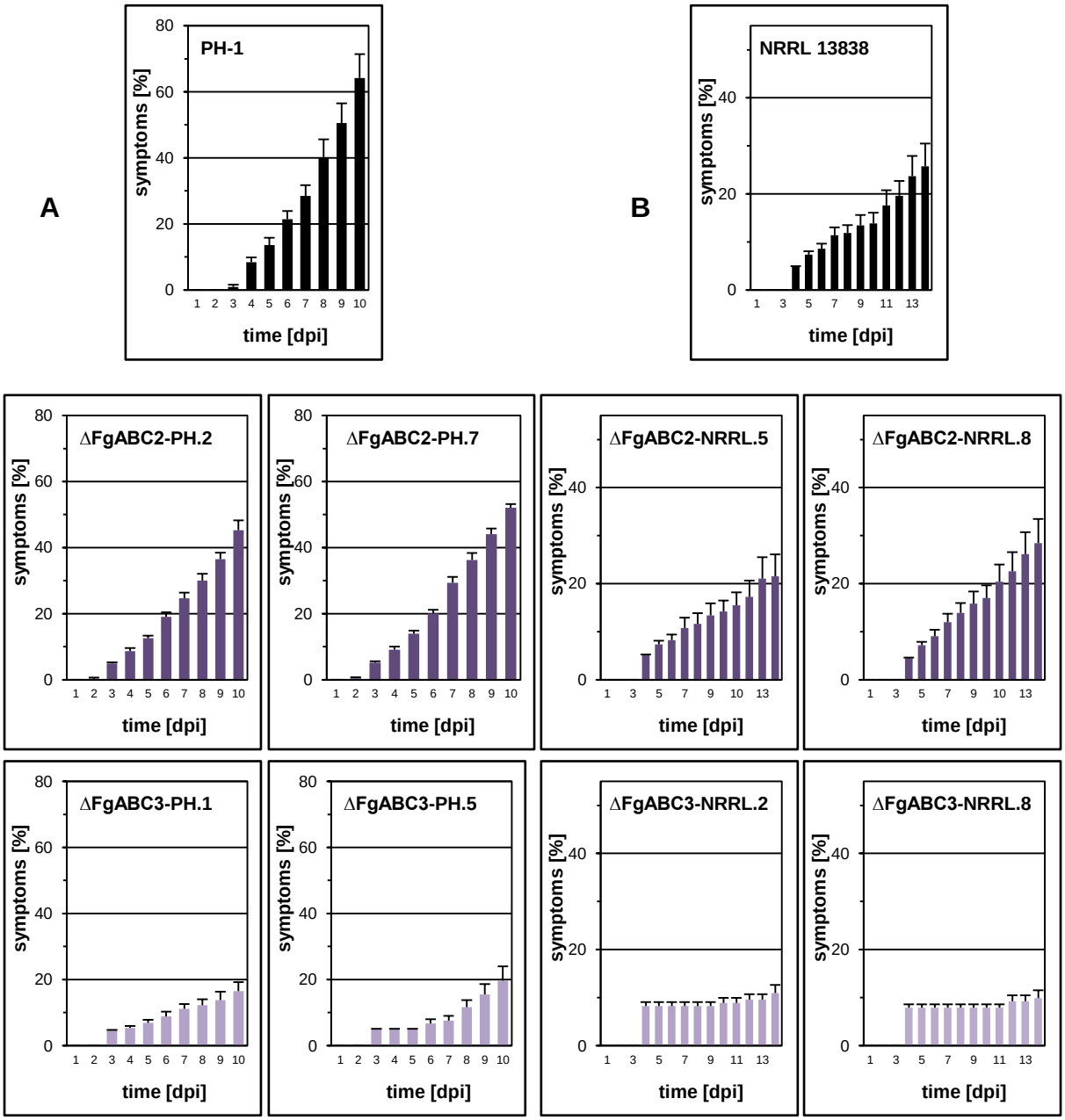

Fig. 12. Virulence on wheat heads. For each deletion, symptom development of two transformants of each genetic background is compared to the respective wild type strain for up to 14 dpi. Columns show the percentage of symptomatic spikelets in point-inoculated wheat heads. A) PH-1 background, B) NRRL 13383 background.

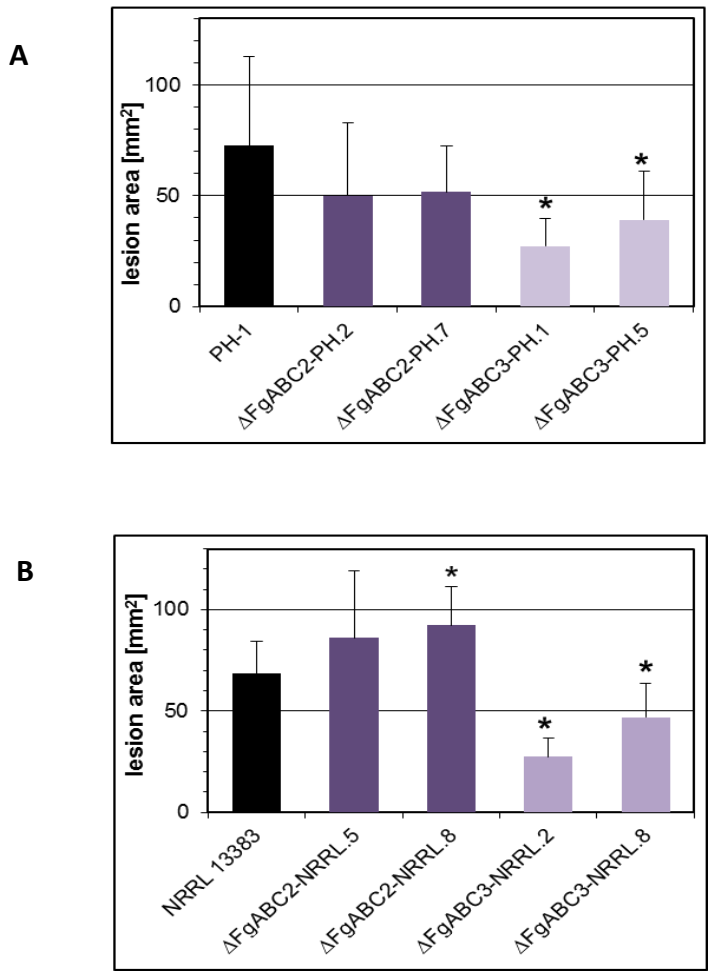

Fig. 13. Virulence on maize stems. For each deletion, two transformants of each genetic background are compared to the respective wild type strain. Columns show symptomatic lesion areas in maize stems that were harvested at 14 dpi and then split longitudinally. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type. **A)** PH-1 background, **B)** NRRL 13383 background.

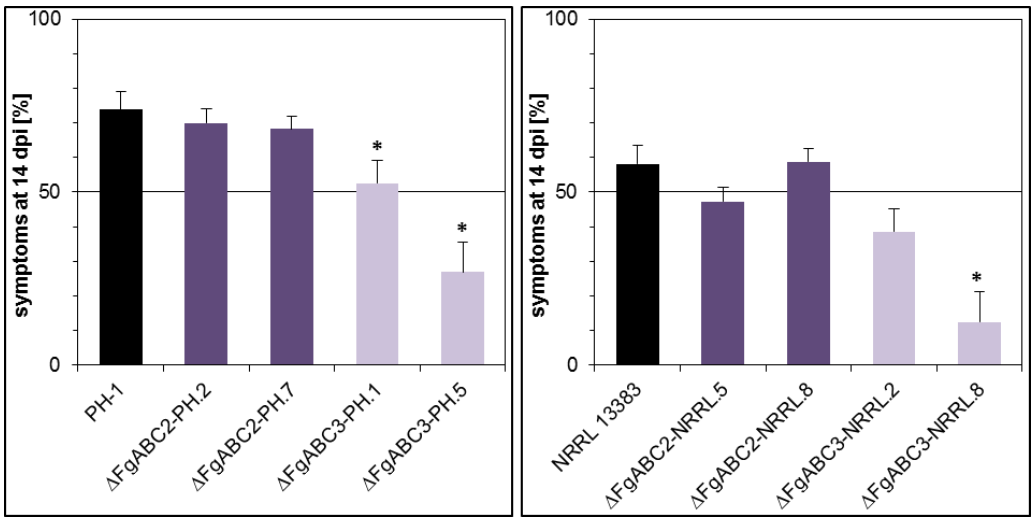

Fig. 14. Virulence on barley heads. For each deletion, two transformants of each genetic background are compared to the respective wild type strain. Columns show the percentage of symptomatic spikelets in spray-inoculated barley heads at 14 dpi. Error bars represent SE. **A)** PH-1 background, **B)** NRRL 13383 background.

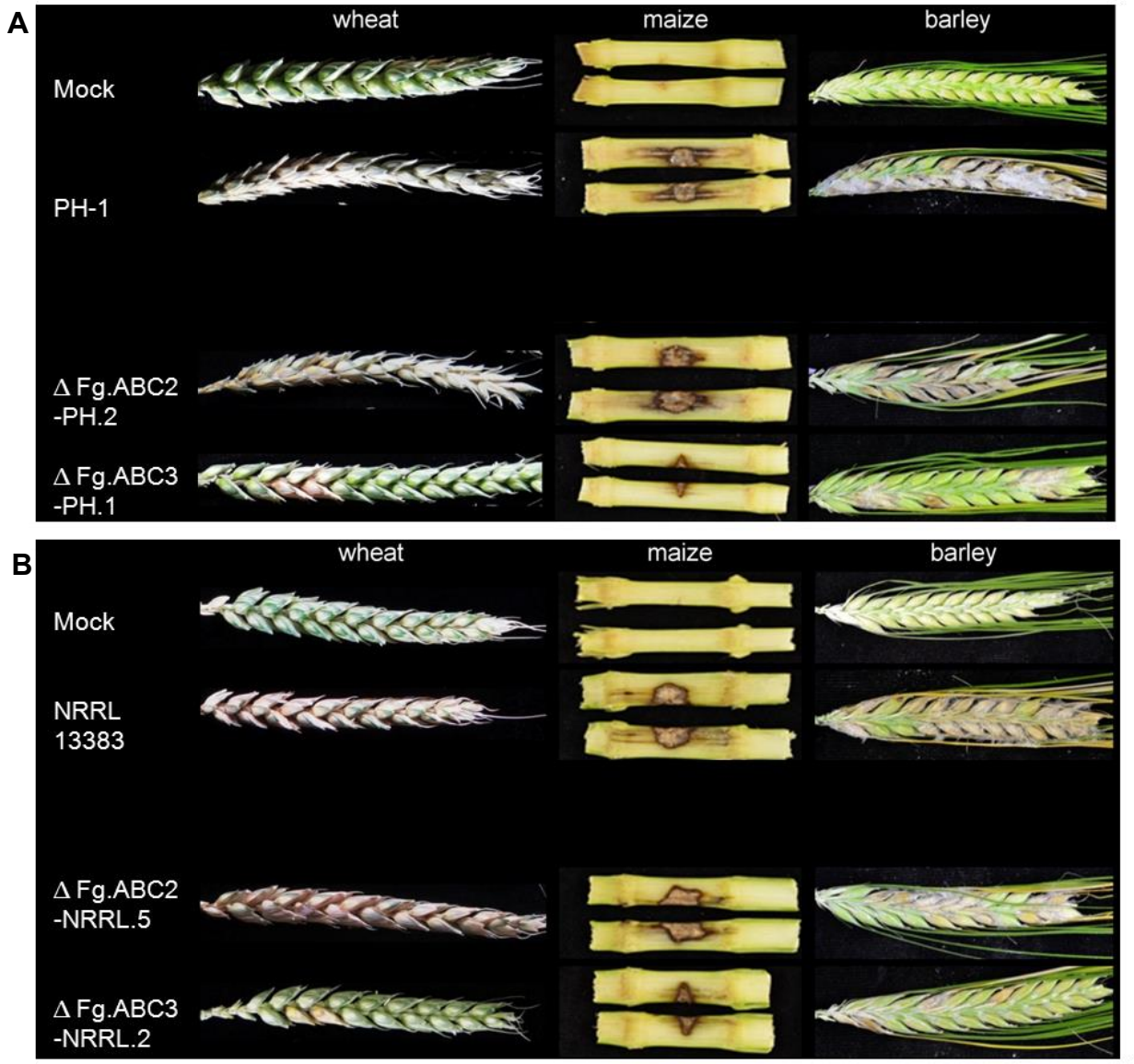

Fig. 15. Symptoms on infected cereals. Photos show symptoms occurring at the end of the monitoring, which was at 10 dpi for wheat, 14 dpi for maize and 14 dpi for barley. One representative example is provided for each genotype. Mocks were treated with 0.02% Tween 20. **A)** PH-1 background, **B)** NRRL 13383 background.

Complemented Δ*FgABC3* strains were also tested for virulence on the three host plants to show that the reduction in virulence of the *FgABC3* deletion mutants (see Figs. 12 to 15) resulted only from the deletion of this ABC transporter gene and was not caused by another mutation somewhere else in the genome that may had occurred during the transformation procedure. As outlined above (see paragraph C.3.), complemented strains were created in a deletion mutant of *FgABC3* in the NRRL 13383 background. Infection assays on wheat and maize yielded for both hosts virulence levels that were similar to those of the wild type reference NRRL 13383 (see Figs. 16, 17). This confirmed that the deletion of *FgABC3* did cause indeed the observed virulence phenotypes.

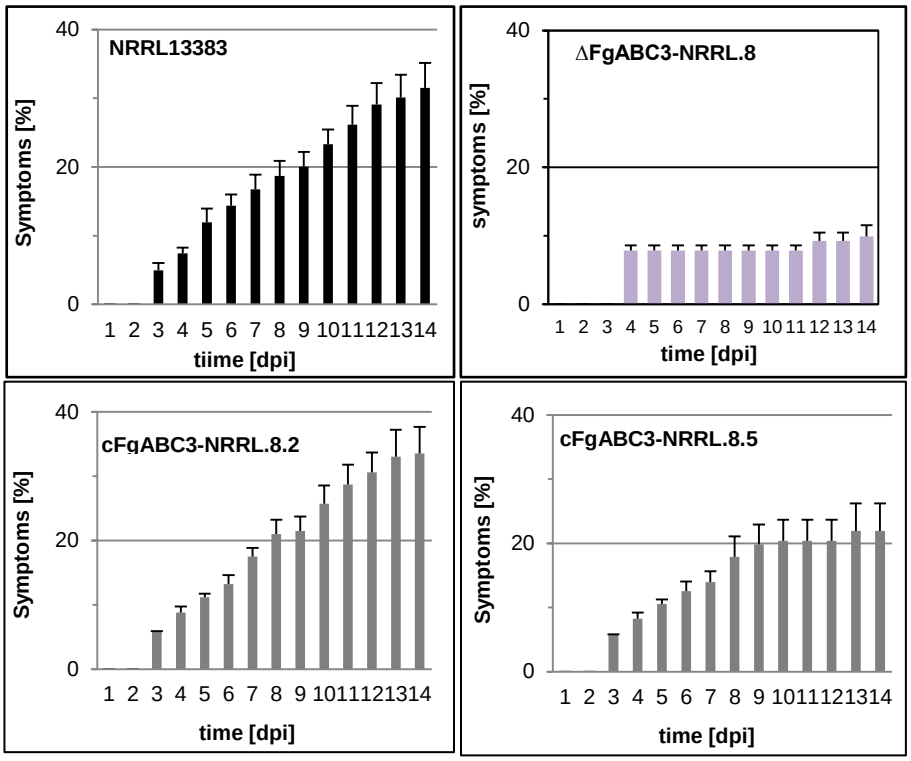

Fig. 16. Virulence on wheat heads. Strains: NRRL 13383, Δ*FgABC3* and the two complemented strains *cFgABC3.8.2, cFgABC3.8*.5**.** Symptom development of transformants is compared to the wild type strain for up to 14 dpi. Columns show the percentage of symptomatic spikelets in point-inoculated wheat heads.

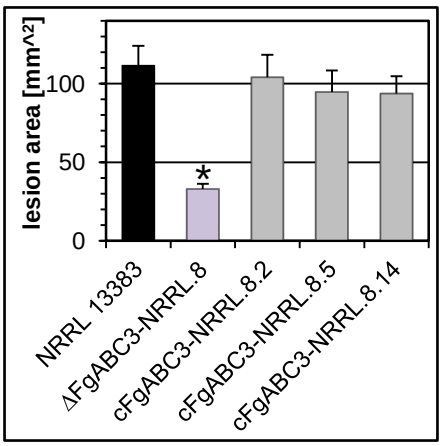

Fig. 17. Virulence on maize stems. Strains: NRRL 13383, Δ*FgABC3* and the three complemented strains *cFgABC3.8.2*, *cFgABC3.8.5 cFgABC3.8.14.* Columns show symptomatic lesion areas in maize stems that were harvested at 14 dpi and then split longitudinally. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type NRRL 13383 background.

C.4.4. Production of mycotoxins by the deletion mutants

Production of mycotoxins was determined in this work to examine whether the FgABC2 and FgABC3 transporters are involved in the secretion of fungal mycotoxins (see paragraph B.5). Thereby, the production of B-trichothecenes and zearalenone was assessed. In the PH-1 background (see Fig. 18), the levels of DON and 15-ADON produced *in vitro* were significantly increased in the two deletion mutants when compared to the wild type strain (Fig. 18 A and B), whereas the ZEA levels were similar in all strains (see Fig. 18 C). In the NRRL 13383 background (see Fig. 19), deletion of *FgABC3* led to lower NIV levels, whereas the *FgABC2* mutants resembled the wild type (see Fig. 19 D and E). Similar to the PH-1 background, none of the deletion mutants in NRRL 13383 produced ZEA at levels that differed significantly from the wild type (see Fig. 19 F). These experiments indicated that the strongly reduced virulence of the deletion mutants of *FgABC3* on the three host species was likely not caused by a reduced formation of trichothecenes (DON and NIV) that are documented as virulence factors. These results also revealed that there are slight differences in mycotoxin levels between the two deletions mutants. These differences are not significant except for the NIV production in NRRL 13383. However, considering the results for Δ*FgABC3* in the PH-1 background that showed a clear reduction in virulence while the levels of DON and 15-ADON were significantly increased as discussed above, such differences in trichothecene production would not explain the differences seen between the two deletion mutants in the virulence assays.

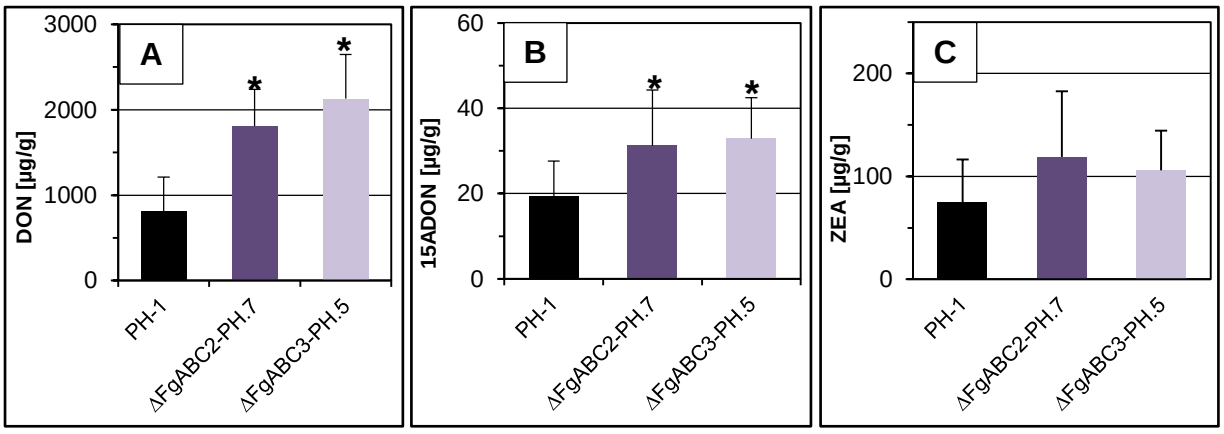

Fig. 18. Production of mycotoxins. For each deletion, one transformant is compared to the wild type strain PH-1. Columns show the amount of a given substance determined by HPLC-MS/MS. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type PH-1. **A)** DON; **B)** 15-ADON; **C)** ZEA.

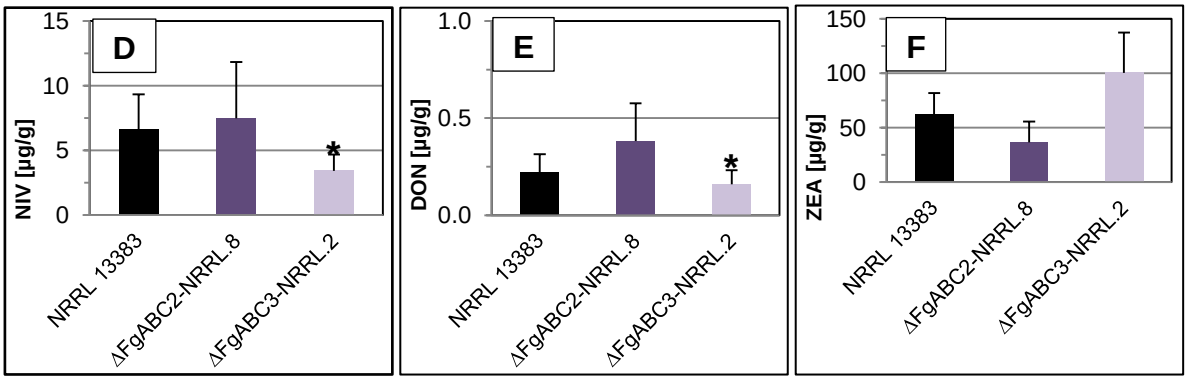

Fig. 19. Production of mycotoxins. For each deletion, one transformant is compared to the wild type strain NRRL 13383. Columns show the amount of a given substance determined by HPLC-MS/MS. Error bars represent SD. An asterisk indicates a significant difference between a mutant and the wild type NRRL 13383 background. **D)** NIV; **E)** 15-ADON; **F)** ZEA.

C.5. Functional analysis of *FgABC3* in an azole adapted strain of *Fusarium graminearum*

C.5.1. Deletion of *FgABC3* in P1-11

The results documented in this thesis have revealed that *FgABC3* has a role in tolerance to azole fungicides in two wild type backgrounds of *F. graminearum* since the corresponding deletion mutants exhibited increased sensitivity to this fungicide (see Table C.1and Figs. 8, 9, 10). Previously, *F. graminearum* strain NRRL 13383 had been exposed *in vitro* to tebuconazole for more than a month, which had allowed recovering of two types of isolates with increased tolerance to that fungicide (Becher *et al*., 2010). The same deletion construct that was used earlier to transform the wild type strains (see paragraph B.3.4 and Fig. 2) were used also to transform the azole resistant strain P1-11. This approach was applied to test if FgABC3 was involved in the long term adaptation process and if it is role in tolerance, which was confirmed during this work in the wild type strain, could be modified and which genetic changes could affect this role during fungicide adaptation (see Fig. 20 A). Obtained transformants were purified by single spore isolation and analysed by standard PCR (see Fig. 20 B) and Southern hybridisation (see Fig. 20 C) for the presence or absence of the *FgABC3* and the *npt* resistance marker genes to reveal the mode of DNA integration. Several independent transformants were found to carry the anticipated gene replacement of the target gene resulting from a single DNA integration event that led to a double cross-over (see Fig. 20 C). For further characterisation, two independent transformants were selected, i.e. Δ*FgABC3-P1-11.4*, Δ*FgABC3-P1-11.6*.

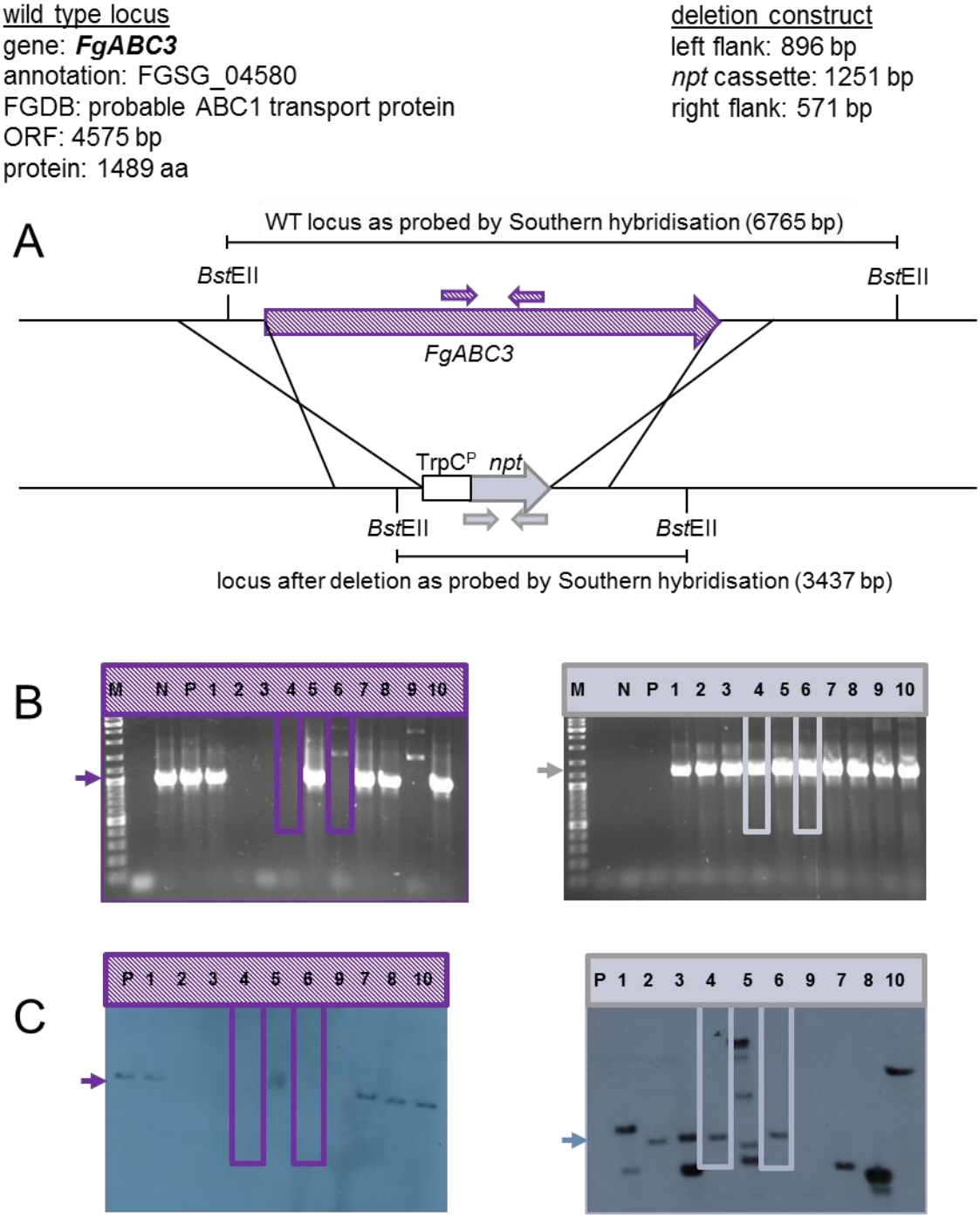

Fig. 20. Generation of *FgABC3* deletion mutants in P1-11. A) Strategy to delete *FgABC3*. Orange arrows give the positions of primers used to detect the wild type locus by PCR. Pink arrows indicate the respective primers for the neomycin resistance marker (*npt*). The same primers were used to generate the DIG-labeled probes used for Southern hybridisation. **B)** Results of PCR targeting the wild type locus (left) and *npt* (right). **C)** Results of Southern hybridisation detecting the wild type locus (left) and *npt* (right). Arrows indicate the expected bands. Boxed lanes indicate clones used for subsequent analyses. N is the abbreviation for NRRL 13383, P for P1-11, and M for the Marker (GeneRuler DNA Ladder Mix, Fermentas).

C.5.2. Vegetative fitness of *FgABC3* deletion mutants in P1-11

To characterise the vegetative fitness of the *FgABC3* deletion mutants in P1-11, growth rates and conidia formation *in vitro* was determined in the two selected transformants (see Fig. 21 A and B). As seen before in the two wild type backgrounds (see Figs. 5, 6), deletion of *FgABC3* did not significantly affect growth rate in the background of the azole-adapted strain (see Fig. 21 A). The capacity to form conidia in liquid culture did not differ significantly between NRRL 13383 and the adapted strain P1-11 due to the large standard deviations. However, a previous study did neither find differences for this trait between the two strains (Becher et al., 2010). The deletion strains were a bit ambiguous since one of the two mutants produced significantly more conidia than the progenitor strain P1-11, whereas the other formed levels of conidia that resembled those of P1-11. Two ectopic mutants used as controls, also resembled P1-11 (see Fig. 21 B).

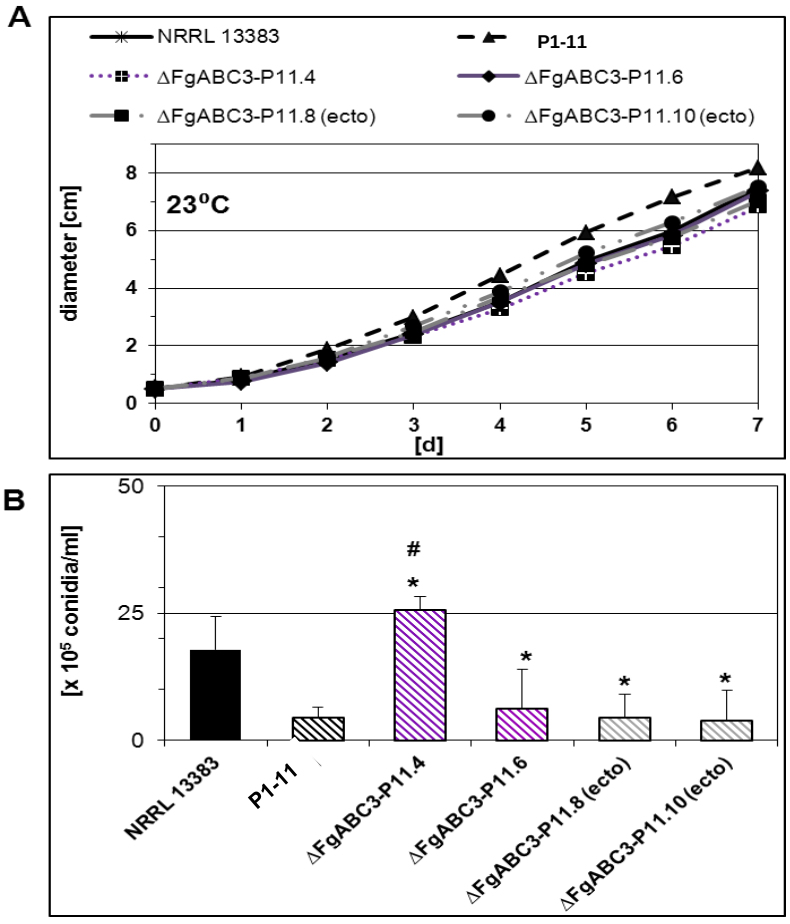

Fig. 21. Vegetative fitness of *FgABC3* deletion mutants in P1-11. A) Growth kinetics *in vitro*. Strains: Azole-adapted strain P1-11, two *FgABC3* deletion mutants in P1-11 and two ectopic strains were compared to the wild type strain NRRL 13383 on PDA medium at 23°C. Each data point represents the mean of four replicated cultures. **B)** Formation of macroconidia *in vitro.* Columns show the average conidial densities formed in MBB medium in four replicated cultures after incubation for 7 d at 23ºC. Error bars represent SE. (*) indicates significant differences to NRRL 13383, (#) significant differences to P1-11.

C.5.3. Fungicide sensitivities of the *FgABC3* deletion mutants in P1-11

To determine whether the deletion of *FgABC3* in P1-11 had the same effect on azole sensitivity as in NRRL 13383, the corresponding mutants were tested for their sensitivity to four fungicides belonging to the SBI class I (see Table C.2). Besides the two selected deletion mutants in the P1-11 background (see Fig. 20), two transformants were included in this assay carrying an ectopic integration of the transformed construct and thus still had the native *FgABC3* locus. As controls, the deletion mutants of *FgABC3* in the wild type background of NRRL 13383 were also included. As seen before in the wild type background (see Table C.1, Figs. 8, 9) the deletion of *FgABC3* in the background of P1-11 also led to significant reductions in tolerance to the triazoles tebuconazole, prothioconazole and epoxyconazole as well as to fenarimol, which is a pyrimidine fungicide but has the same target as the triazoles (SBI class I) (see Table C.2, Fig. 22).

The ectopic transformants did deviate neither notably from their progenitor strain P1-11 nor from the wild type reference NRRL 13383. On the other hand, P1-11 showed a significantly increased tolerance to the fungicides used when compared to NRRL 13383 thus confirming previous results (Becher *et al*, 2010). The *FgABC3* deletion mutants in the background of NRRL 13383, which were used as additional controls, showed again significant reductions in tolerance to these fungicides as shown above (see Fig. 8). Furthermore, the deletion mutants of *FgABC3* in the background of P1-11 showed significant reductions in fungicide tolerance compared to P1-11. These reductions seemed even a bit stronger than those observed between NRRL 13383 and the corresponding mutants obtained in that background (see Fig. 22). Nevertheless, the deletion mutants in P1-11 were somewhat more tolerant to azoles than those in NRRL 13383. Since the deletion of *FgABC3* in P1-11 led to similar outcomes as seen before in the corresponding wild type background it is proposed that this gene is involved in contributing to acquired azole resistance in the adapted strain.

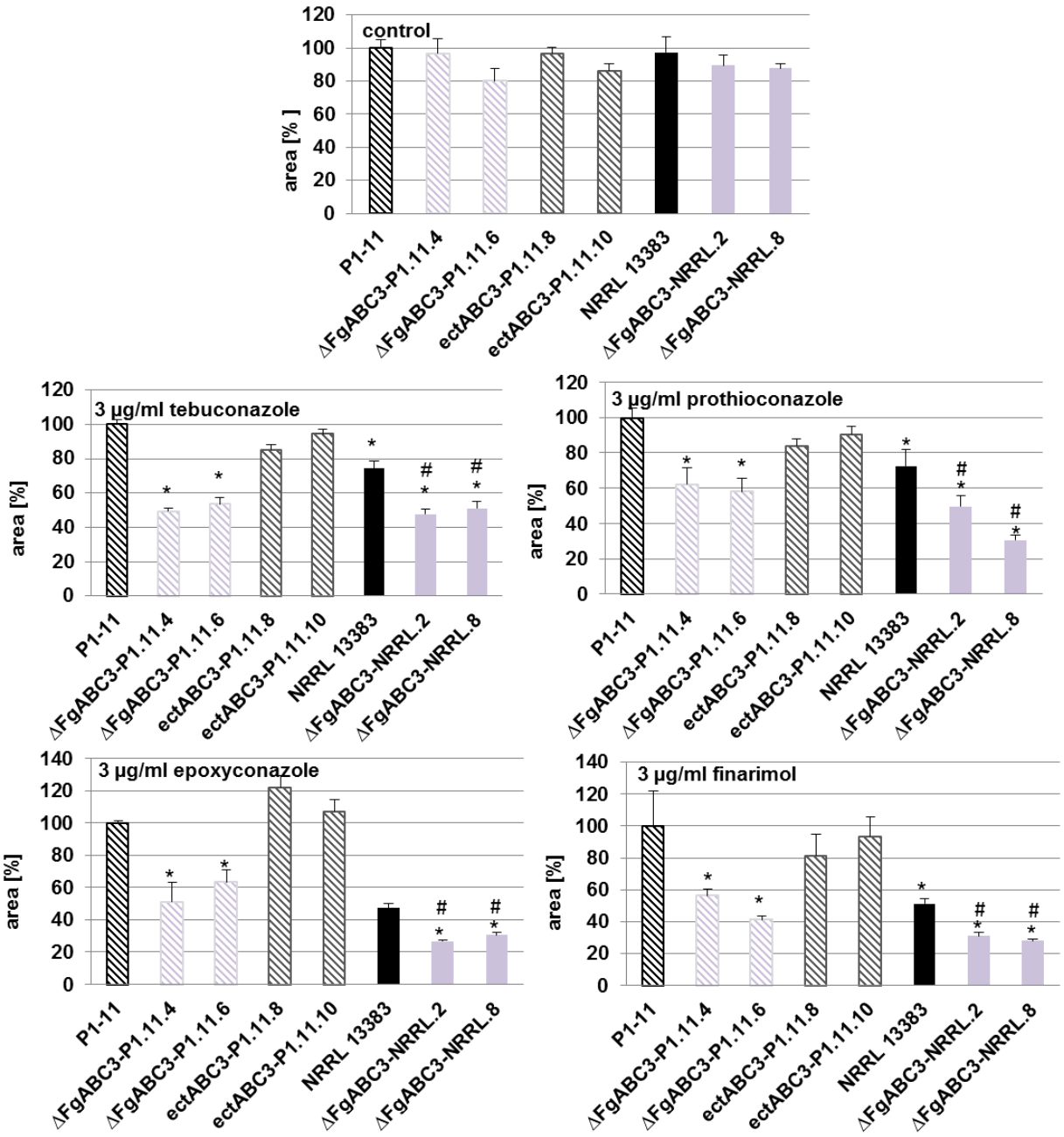

Fig. 22. Fungicide sensitivities of the *FgABC3* mutants. Strains: P1-11, NRRL 13383, two *FgABC3* deletion mutants in P1-11 (*ΔFgABC3.P1-11.4*, *ΔFgABC3.P1-11.6*), two ectopic mutants in P1-11 (*ectFgABC3.P1-11.8*, *ectFgABC3.P1-11.10*), two deletion mutants of *FgABC3* in NRRL 13383 (*ΔFgABC3.NRRL.2*, *ΔFgABC3.NRRL.8*). Error bars represent SD. (*): significant ($p < 0.05$) difference in comparison to strain P1-11. (#) significant ($p < 0.05$) difference in comparison to wild type strain NRRL 13383.

Table.C.2: Sensitivity to azole fungicides of *FgABC3* deletion mutants in P1-11.

			Δ*FgABC3* in P1-11 background
common name	**chemical grouping**	**fungicide class**	
tebuconazole	triazole	SBI class I	+
prothioconazole	triazole	SBI class I	+
epoxyconazole	triazole	SBI class I	+
fenarimol	pyrimidine	SBI class I	+

Assays used PDA plates amended with the indicated fungicides that were inoculated with mycelial plugs. (+) Deletion led to significantly ($p < 0.05$) increased sensitivity in comparison to the progenitor strain P1-11.

C.5.4. Virulence assays of the *FgABC3* deletion mutants in P1-11

As mentioned above (see paragraph C.4.3 and Figs. 12 to 15) the deletion of *FgABC3* in the wild background did not only impede the tolerance of the mutants to several fungicides of SBI class I but also their virulence. To assess whether virulence would be affected to the same extent when the same gene was deleted in the background of P1-11 such mutants were assayed on wheat and maize. For these experiments, two deletion mutants in the background of P1-11 were used, and as control one ectopic integration transformant obtained in the same transformation leading to these deletion mutants. Additional control strains were Δ*FgABC3* mutants in NRRL 13383 as well as P1-11 and NRRL 13383.

The comparison of P1-11 with the Δ*FgABC3* mutants in this background showed a strong reduction of virulence on wheat in the mutants (see Fig. 23). Thus, while in P1-11 about 30% of the spikletes were bleached, in the mutants only about 10% of the spikelets showed symptoms at 14 dpi. The mutants were still able to cause infections in the inoculated spikelet but further on they spread very slowly, while the ectopic transformant showed wild type levels of virulence and no significant differences to P1-11 (see Fig. 23). For comparison, in the wild type background, the reduction of symptoms between NRRL 13383 and the deletion mutants was 50%, and thus at a level comparable to the other background.

The virulence assay employing maize stems allowed for an easier application of fungicides than that using single earlets of wheat. Therefore, the experiment using maize was performed

in two variants, i.e. one without any fungicide (see Fig. 24 A) and one with the application of 50 µg/ml prothioconazole (see Fig. 24 B). In the former assay, compared to strain P1-11, deletion mutants of *FgABC3* in that background were significantly reduced in virulence. The same was seen when assessing the corresponding deletion mutants in the background of NRRL 13383. This wild type strain, P1-11 and the ectopic transformant exhibited similar levels of virulence on maize stems (see Fig. 24 A).

As Δ*FgABC3* mutants in the background of P1-11 and in NRRL 13383 have shown a clear reduction in tolerance against azole fungicides *in vitro* (see Fig. 22), it was interesting to assess whether this will also occur *in planta*. As explained above, this was tested by employing the maize stem assay. Comparison of the untreated variants of P1-11 and NRRL 13383 with their treated variants indicated the effectiveness of the fungicide treatment (Fig. 24 B). Although P1-11 exhibited slightly reduced symptom severity in the untreated variant compared to NRRL 13383, after treatment with prothioconazole it showed slightly increased severity. However, none of these differences was statistically significant. Corresponding differences were not observed when comparing the deletion mutants of *FgABC3* in both backgrounds since both exhibited similarly reduced symptom severities (see Fig. 24 B).

In conclusion, the comparison of the quantitative levels of fungicide resistance and virulence in two genetic backgrounds, i.e. NRRL 13383 versus P1-11, which carried a deletion of the same gene, suggested that there existed no antagostic or synergistic effects that may interact with FgABC3 in the azole adapted strains during the fungicide adaptation.

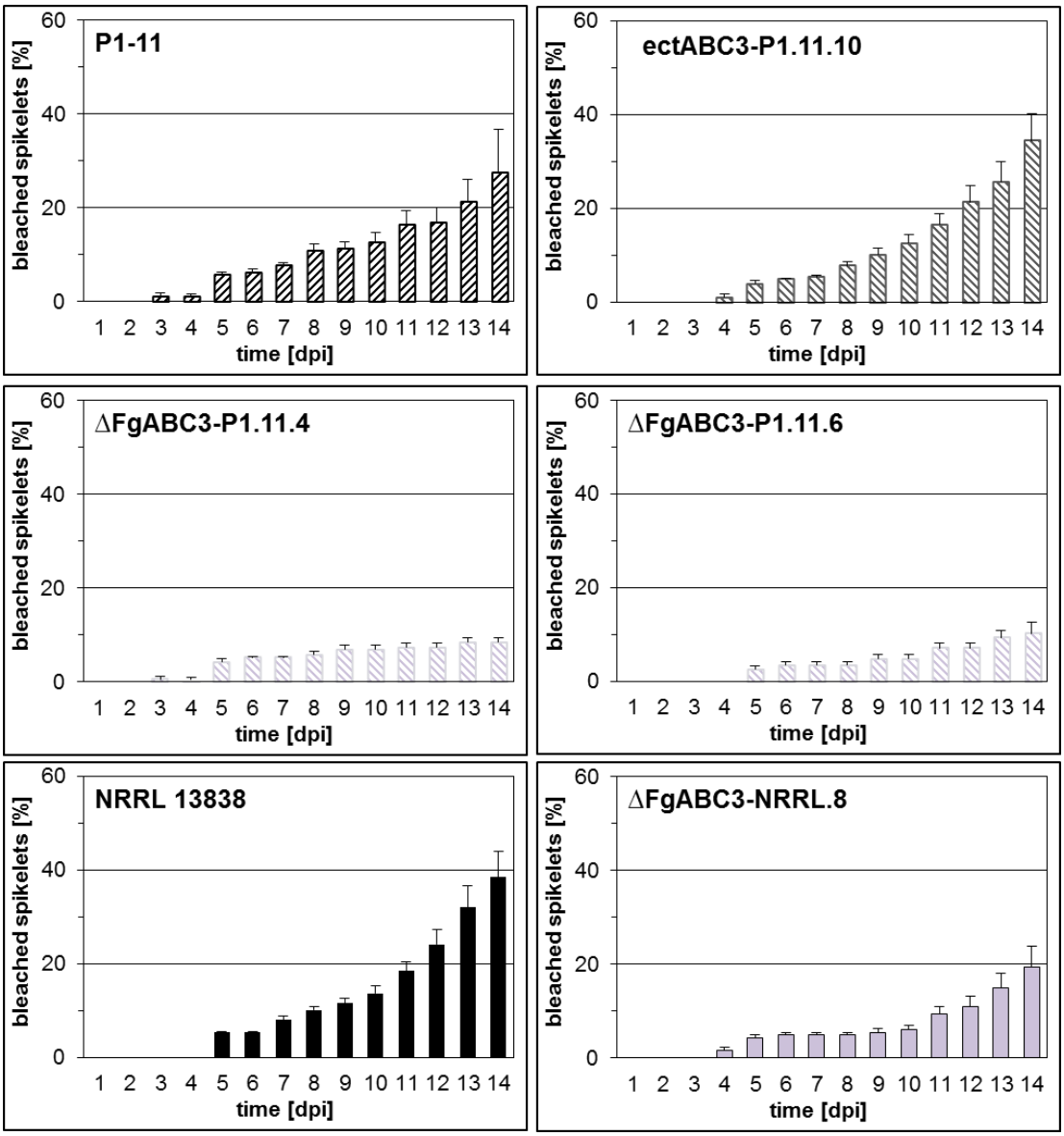

Fig. 23. Virulence of Δ*FgABC3* mutants in P1-11 on wheat heads. Symptom development of transformants was monitored until 14 dpi. Columns show the percentage of symptomatic spikelets in point-inoculated wheat heads. Error bars represent SE. Strains: P1-11, two deletion strains of *FgABC3* in P1-11 (*ΔFgABC3.P1-11.4*, *ΔFgABC3.P1-11.6*), ectopic transformant (*ectFgABC3.P1-11.10)*, wild type strain NRRL 13383, and ΔFgABC3.NRRL.8).

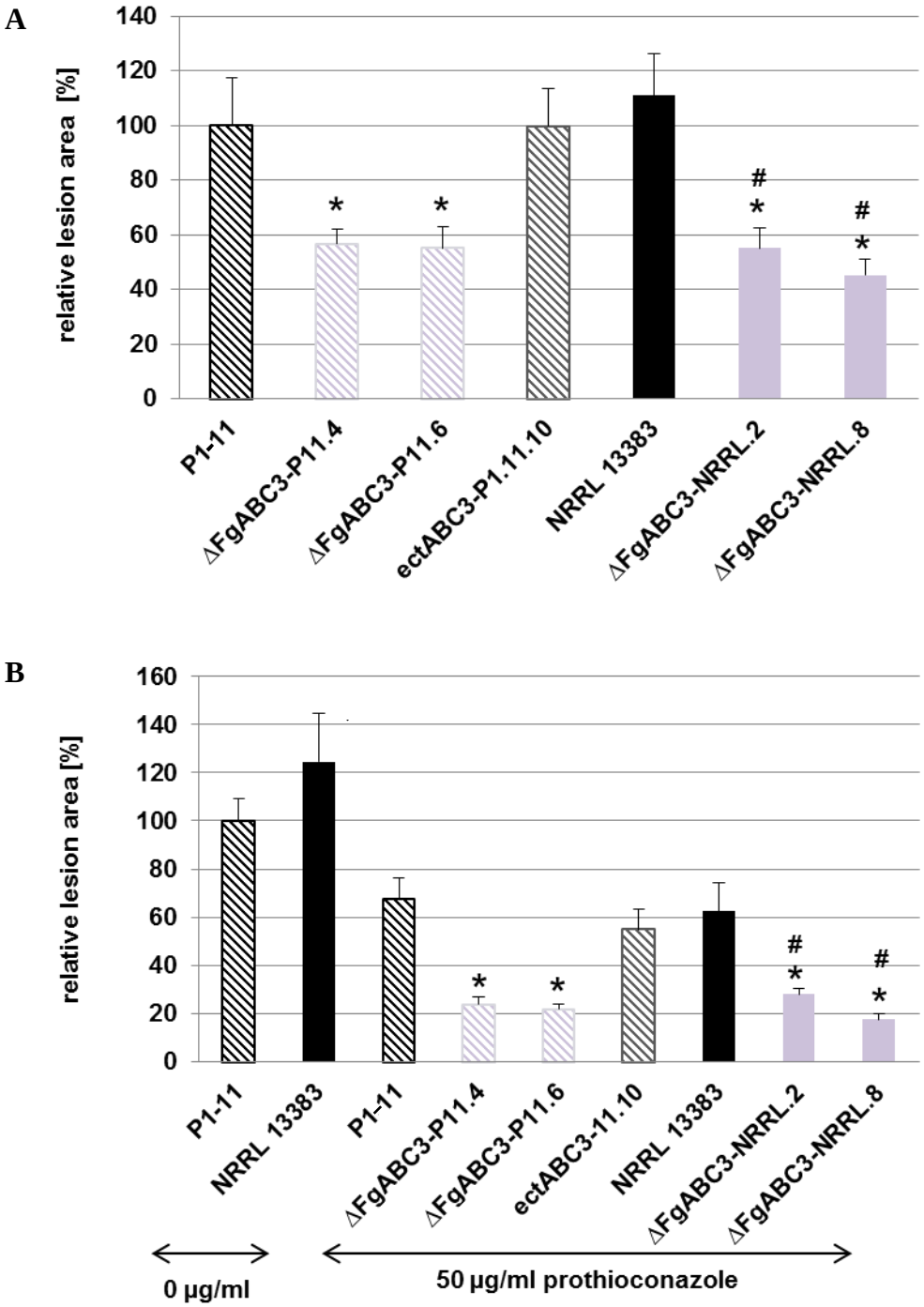

Fig. 24. Virulence of Δ*FgABC3* mutants in P1-11 on maize stems. A) Without fungicide**, B)** With 50 µg/ml prothioconazole. Strains: deletion mutants of *FgABC3* in P1-11 (*ΔFgABC3.P1-11.4*; *ΔFgABC3.P1-11.6*), ectopic transformant in P1-11 (*ectFgABC3.P1-11.10*), wild type strain NRRL 13383, deletion mutants of *FgABC3* in NRRL 13383 (*ΔFgABC3.NRRL.2*, *ΔFgABC3.NRRL.8*). Columns represent relative symptomatic areas normalised to untreated P1-11 in maize stems that were harvested at 14 dpi and then split longitudinally. Error bars represent SE.
(*) significant ($p < 0.05$) difference in comparison to the untreated strain P1-11.
(#) significant ($p < 0.05$) difference in comparison to the untreated strain NRRL 13383.

D. Discussion and perspectives

Membrane-bound transporter proteins are known to mediate azole resistance in fungal plant and human pathogens (Becher & Wirsel, 2012; Cannon *et al.*, 2009; de Waard *et al.*, 2006; Kovalchuk & Driessen, 2010; Paul & Moye-Rowley, 2014). Eukaryotic ABC transporters have been classified into subfamilies according to sequence homology and domain topology. The existing eukaryotic proteins have been grouped into major subfamilies, termed from ABC-A to ABC-I (Dassa, 2011; Dean *et al.*, 2001). Typically, fungal ABC transporters mediating drug resistance belong to the pleiotropic drug resistance proteins PDR (= ABC-G) and the multidrug resistance proteins MDR (= ABC-B) and to a lesser extent to the multidrug resistance-associated proteins MRP (= ABC-C) subfamilies, according to the yeast nomenclature classification system and the Human Genome Organisation scheme for classification of ABC transporters, respectively.

A previous microarray study found 15 ABC transporter genes transcriptionally upregulated in response to tebuconazole treatment in the plant pathogen *Fusarium graminearum* (Becher *et al.*, 2011). Taking advantage of those transcriptomic data and additional phylogenetic information, in this study two of these genes, *FgABC3* (FGSG_04580) and *FgABC2* (FGSG_17046) encoding full-size ABC transporters and belonging to different subfamilies, have been selected for functional analyses. These two genes were deleted to determine their contribution to fungicide tolerance, virulence on different host plants and mycotoxin production. My study shows that the deletion mutants in Δ*FgABC3* (FGSG_04580) acquired a higher sensitivity to several fungicides belonging to the SBI class I, but not to other fungicide classes that were also tested. Remarkably, the deletion of *FgABC3* caused a strong reduction of virulence on three economically important host plants, i.e. wheat, barley and maize. However, the deletion of the second selected gene, *FgABC2* (FGSG_17046), affected neither the sensitivity nor the aggressiveness of the mutants in comparison to two wild type reference strains representing the 15-ADON and NIV chemotypes.

Furthermore, the selected candidate gene *FgABC3* (FGSG_04580) was also deleted in the azole adapted strain P1-11, which was previously generated in our group (Becher *et al.*, 2010) to assess to which extent its resistance is dependent on this gene. The deletion of *FgABC3* (FGSG_04580) in P1-11 resulted in effects that were similar to those previously observed in

the wild type background. The mutants had lost acquired tolerance for azoles indicating that *FgABC3* is indeed mediating resistance in strain P1-11.

D.1. Features of the selected genes

D.1.1. Features of *FgABC2*

The open reading frame (ORF) of *FgABC2* is 4932 nt in length, contains one intron, and encodes a protein of 1625 amino acids (BROAD Institute; *F. graminearum* PH-1 (FG3): Supercontig 5: 2086499-2091430 +) corresponding to protein FGSG_17046. Software topology prediction programs predict 12 transmembrane helices and two large extracellular loops in the protein in a $(TMD_6\text{-}NBD)_2$ topology, placing FgABC2 within the ABC-A subfamily group I of ABC transporters (Kovalchuk & Driessen, 2010). The classification scheme of yeast does not assign a group to this transporter because it is missing in the Saccharomycotina. FgABC2 is the only protein in *F. graminearum* belonging to the ABC-A subfamily.

The ABC-A transporters of vertebrates are the best-characterized members of this subfamily playing a crucial role in lipid transport and metabolism. Thus, it has been suggested that transporters of this subfamily may mediate lipid transduction. The genomes of most Pezizomycotina including *F. graminearum* contain just a single gene for a full-length ABC-A transporter whereas some as *Magnaporthe oryzae*, *Botryotinia fuckeliana* and *Phaeosphaeria nodorum* contain two of such genes (Kovalchuk & Driessen, 2010).

D.1.2. Features of *FgABC3*

The *FgABC3* open reading frame (ORF) is 4575 nt in length, contains two introns, and encodes a protein of 1489 amino acids (BROAD Institute*; F. graminearum* PH-1 (FG3): Supercontig 2: 317465-322039 +) corresponding to protein FGSG_04580. Software for the prediction of topology predicted 12 transmembrane helices within the protein in a $(NBD\text{-}TMD_6)_2$ topology, placing FgABC3 within the pleiotropic drug resistance (PDR) subfamily of ABC transporters (Becher *et al.*, 2011; Kovalchuk & Driessen, 2010). Transporters within the ABC family have been reported to transport a wide range of compounds and to confer tolerance by the translocation of these compounds across the membrane, thereby

counteracting an increase in the intracellular concentration of toxic compounds (Higgins, 1992; Higgins, 2001).

Fungal PDR-type transporters were first studied in yeast and are well documented as proteins providing protection to cells through the removal of toxic compounds (Gulshan & Moye-Rowley, 2007; Kretschmer *et al.*, 2009; Sang *et al.*, 2015). PDR transporters, whose contribution to azole resistance have been analysed in detail, are for example CDR1 and CDR2 in *Candida albicans* and PDR5 in *Saccharomyces cerevisiae* (Golin & Ambudkar, 2015; Prasad & Goffeau, 2012; Prasad *et al.*, 2015). According to the yeast nomenclature classification system, *FgABC3* encodes a transporter protein that contains the conserved domains characteristic of the Pdr5-like family of ABC transporters that play an important role in azole resistance in *Saccharomyces cerevisiae* and *Candida* spp. (Kolaczkowski *et al.*, 1996; Leppert *et al.*, 1990; Zhang *et al.*, 2012b). According to an alternative classification scheme based on the Human Genome Organization scheme for classification of ABC transporters, FgABC3 belongs to the ABC-G group I subfamily, a class often associated with pleiotropic drug resistance (Gardiner *et al.*, 2013; Kovalchuk & Driessen, 2010). According to a recent analysis, 19 ABC-G transporter genes out of 62 potential ABC transporter genes have been predicted in the *F. graminearum* genome (Gardiner *et al.*, 2013; Kovalchuk & Driessen, 2010).

D.2. Effects of deletions on fungicide sensitivity

D.2.1. Deletion of *FgABC2* in wild type backgrounds

The two ABC transporter genes that were selected in this work for deletion mutagenesis were previously found upregulated after tebuconazole treatment in both wild type strains used in this work, NRRL 13383 and PH1-1, which have NIV and 15-ADON chemotypes, respectively (Abou Ammar *et al.*, 2013; Becher *et al.*, 2011). Deletions have been introduced in these two strains to examine to what extent the genetic background may affect the resulting phenotypes (Abou Ammar *et al.*, 2013). Deletion of *FgABC2* resulted neither in NRRL 13383 nor in PH-1 in significant changes in vitality or in a reduction of tolerance to any of the fungicide classes tested, when compared to the respective wild type strain (see paragraph C.4.2.1 and Figs. 8 and 9). These results suggest that *FgABC2* is not important to cope with azole stress at all or its deletion was functionally complemented by other transporters encoded by the genome. However, the latter explanation may be less likely for two reasons. On the one hand, FgABC2 is the only protein in *F. graminearum* belonging to the ABC-A subfamily.

Thus, there exists no highly similar protein that may have a similar function. On the other hand, although there exists no extensive literature on members of the ABC-A subfamily in fungi, studies revealed that this transporter protein subfamily is contracted generally in the Mycota in comparison to animals. It has been suggested that transporters of this subfamily may mediate lipid transport (Broccardo *et al.*, 1999; Kovalchuk & Driessen, 2010). In humans, it is suggested to be involved in the transfer of cholesterol and phospholipids from cells to high-density lipoprotein particles (Gupta & Chattoo, 2008; Young & Fielding, 1999). The ABC-A transporter protein subfamily is missing in the genomes of *S. cerevisiae* and some other fungi suggesting that these transporters are not essential for fungal viability and may only be required under specific physiological conditions (Kovalchuk & Driessen, 2010). Among plant pathogenic fungi, transporters residing in the ABC-A subfamily have been functionally characterised only in *M. oryzae*. MoABC4 (MGG_00937), which is in that fungus the protein most similar to FgABC2, had been reported to be important for appressorium formation and thus virulence using mutants inoculated onto barley leaves (Gupta & Chattoo, 2008; Kovalchuk & Driessen, 2010). These phenotypes, but also a higher sensitivity to some xenobiotics, were observed in a mutant recovered in a genetic forward screen employing *Agrobacterium tumefaciens*-mediated transformation. These phenotypes were not confirmed, however, by gene deletion or RNAi experiments afterwards. In this study, several phenotypes were assessed in at least two independent deletion mutants generated in each of two wild type strains, NRRL 13383 and PH-1. Although several fungicide classes were tested in addition to some plant secondary metabolites (see paragraphs C.4.2.1 and C.4.2.3) no significant differences between the Δ*FgABC2* mutants and the wild type strains were found in any assay. Therefore, it seems that the enhanced transcript abundance of this gene during azole treatment may have been a rather indirect effect.

D.2.2. Deletion of *FgABC3*

D.2.2.1. Deletion of *FgABC3* in wild type backgrounds

Deletion of *FgABC3* in both wild type strains, i.e. PH-1 and NRRL 13383, did not reveal significant changes in the viability of the mutants in comparison to the respective progenitor strain. This was true for different media and temperatures (see Fig. 5).

FgABC3 is not essential for the vegetative part of the life cycle of *F. graminearum*. Since mating assays were not conducted, it is unknown whether this protein may contribute to the

sexual part of the life cycle. This study revealed that the deletion of *FgABC3* caused an enhanced sensitivity to tebuconazole and other triazoles (see Figs. 8, 9, 11A and 11B) but not to different fungicide classes that were tested also in this study, i.e. anthraquinones, imidazoles, methoxy-acrylates, morpholines, pyridine-carboxamides, pyrimidines, spiroketal-amines. This reduction in tolerance to triazoles of Δ*FgABC3* mutants occurred in both wild type backgrounds after transformation of the same deletion construct (see paragraphs C.4.2.1, C.4.2.2 and Table C.1). Transformants with a reconstituted native ORF of *FgABC3* were generated by co-transformation of a deletion mutant of this gene in the NRRL 13383 background. These transformants exhibited tolerance levels that were again close to those of the wild type (see paragraph C.4.2.1 and Fig. 10). This clearly confirms that the increased sensitivity of the original Δ*FgABC3* mutants was indeed caused by the deletion of that gene and not by artefacts that could have occurred during the transformation process.

Considering that the genome of *F. graminearum* putatively comprises 62 genes encoding ABC transporters from which are 19 predicted as PDR transporters (Becher *et al.*, 2011; Gardiner *et al.*, 2013), it is notable that a single deletion already yielded such a strong phenotype in fungicide tolerance. It has been documented that beside the selected genes tested in this study, each ABC subfamily harbours additional genes, some of which also responded to tebuconazole with a more than twofold change in transcript levels (Becher *et al.*, 2011). Thus, distinct transcriptional responses only occurring for *FgABC3* are probably not the reason why none of the similar paralogs was able to sufficiently complement the deletion of this transporter protein. However, a specific post-transcriptional regulation of *FgABC3* may be considered to explain the insufficient complementation of its deletion mutant by similar paralogs. Alternatively, distinct substrate specificity of this transporter could be an explanation. It is assumed that details of the 3D structure determine the substrate specificity of ABC transporters. Full-size PDR transporters are comprised of two homologous halves each having an arrangement of domains as [NBD-TMD_6], which means that each half contains an N-terminal hydrophilic nucleotide binding domain (NBD) and a C-terminal transmembrane domain (TMD) comprising six transmembrane spans (TMSs) (Cannon *et al.*, 2009; Lamping *et al.*, 2010) (see paragraph A.5).

The NBDs bind and hydrolyse ATP that powers drug efflux, while the TMSs form a channel in the membrane and provide some substrate specificity to the protein. The substrates accepted by ABC transporters appear to be chemically unrelated (Prasad & Goffeau, 2012; Rawal *et al.*, 2013; Tanabe *et al.*, 2011; Tegos & Mylonakis, 2012). ATP binding and/or hydrolysis induces conformational changes that are transmitted from the NBDs to the TMDs,

resulting in drug translocation, drug efflux or resetting of the reaction cycle (Gupta *et al.*, 2011Gupta *et al.*, 2011; Rawal *et al.*, 2013).

Although most fungal PDR efflux pumps have relatively wide substrate specificities some transporters may have narrow specificities. Often these overlap among family members of ABC transporters and thus provide a broad spectrum protection against xenobiotics including the widely used triazole fungicides (Tegos & Mylonakis, 2012). In the model organism *Saccharomyces cerevisiae* it was documented that the substrate specificities of Pdr5p, Snq2p and Yor1p, all belonging to the PDR subfamily, are largely, but not totally, overlapping. From the nine ABC transporters belonging to this subfamily in yeast, Pdr5p contributes most to azole fungicide tolerance (Rogers et al., 2001). In the human pathogen *Candida albicans*, CaCdr1p and CaCdr2p are clinically important multidrug resistance transporters that are closely related but functionally distinguishable. These two transporters are orthologous to ScPdr5p and show induced expression under azole pressure. However, CaCd2p contributes less to triazole tolerance than CaCdr1p, which is considered more important in *C. albicans* (Cannon *et al.*, 2009; Holmes *et al.*, 2008; Tanabe *et al.*, 2011; Tsao *et al.*, 2009).

Since the Δ*FgABC3* mutants were still able to grow slowly at about 3 ppm of tebuconazole, there probably exists at least one transporter, which may belong to the ABC or the MFS families and which is involved in the efflux of azole fungicides in *Fusarium*. This could be expected considering that the genome of *F. graminearum* comprises 62 genes putatively encoding ABC transporter. Some of them were suggested to be involved in mechanisms alleviating the impact of azole fungicides (Gardiner *et al.*, 2013). Another possibility to explain the remaining level of tolerance to azoles in the deletion mutants of this gene would be intracellular mechanisms allowing the cell to cope with drug-induced stress. Such cellular stress responses are required for survival, both in the wild type and the mutants. This supposition is supported by studies showing a link between azole stress, cellular signal transduction and membrane stress responses (Cowen & Steinbach, 2008). These studies showed that transcription factors, which regulate fungal ABC transporters involved in drug resistance in different fungi, also regulate additional genes that are involved in minimizing the impact of the drug on the cell and enabling the cell to cope with stress. For example, in addition to CaCdr1 and CaCdr2 the transcription factor Tac1 of the human pathogen *C. albicans* also regulates a putative glutathione peroxidase, a sphingosine kinase, and a phospholipid flippase, suggesting roles in oxidative stress response and lipid metabolism (Cowen & Steinbach, 2008; Liu *et al.*, 2007).

In contrast to human pathogens, fewer functional genetic analyses were performed for PDR transporters mediating azole resistance in plant pathogenic fungi. Nevertheless, some studies recently revealed a contribution of members of the PDR subfamily (ABC-G) to protection of fungal plant pathogens against azole stress. For instance, in *Penicillium digitatum*, a pathogen of citrus, disruption mutants of *PMR1* (ABC-G group I) displayed increased sensitivity to azoles (Nakaune *et al.*, 2002). In *Botrytis cinerea*, a plant pathogen with a wide host range, gene deletion and overexpression experiments showed that *BcatrD* (ABC-G group I) mediates azole resistance (Hayashi *et al.*, 2003). The ABC transporters *MgAtr1* (ABC-G group III), *MgAtr2* (ABC-G group I) and *MgAtr4* (ABC-G group I) of *Mycosphaerella graminicola*, a pathogen infecting wheat, were heterologously expressed in a *S. cerevisiae* mutant in which six ABC transporter genes had been deleted. In each of these cases increased tolerance to azoles was observed (Zwiers *et al.*, 2002). However, the deletion or the disruption of the genes *MgAtr1* to *MgAtr5* in *M. graminicola*, did not cause changes in azole sensitivity (Zwiers *et al.*, 2003). In *Neurospora crassa*, CDR4, which is closely related to FgABC3, is the major contributor to azole resistance among four Pdr5p-like ABC transporter proteins existing in this fungus (Zhang *et al.*, 2012b). The Pezizomycotina have at least two phylogenetically distinguishable groups of Pdr5p homologues. One contains PMR1-like Pdr5p homologues in which FgABC3 is predicted to belong, while the other contains both AtrF-like and AtrB-like members. It has been suggested that PMR1-like but not the other group plays a key role in azole resistance in filamentous fungi (Zhang *et al.*, 2012b). (Gardiner *et al.*, 2013) also documented the involvement of FgABC3 in resistance to toxic compounds in *F. graminearum*. In that work, the compound benalaxyl had a strong inhibitory effect on a deletion mutant of *FgABC3*; however azoles were not tested in this work.

D.2.2.2. Deletion of *FgABC3* in the azole-adapted strain P1-11

In a previous microarray study azole treatment led in P1-11 to an 21-fold increase in the abundance of *FgABC3* transcripts in comparison to the corresponding wild type NRRL 13383, which is the progenitor of this adapted strain (Becher et al., unpublished results). Deletion of *FgABC3* in P1-11 did not affect the viability of the mutants but resulted in significantly reduced tolerance to azoles similar to the results obtained for the deletion of *FgABC3* in NRRL 13838 (see paragraph C.5.3 and Fig. 22). The fungicide sensitivity phenotype obtained after deletion of *FgABC3* in the adapted strain P1-11 and the results of the microarray experiment mentioned above strongly suggest an involvement of this transporter in the acquired tolerance of *Fusarium graminearum* after an *in vitro* long term azole fungicide

treatment. These results also show for the first time in a plant pathogen, the involvement of an ABC transporter in azole resistance in a well characterized *in vitro* adapted strain.

The significantly enhanced transcriptional induction in the adapted strain P1-11 observed for *FgABC3*, a transporter that was shown in this thesis to contribute to increased azole tolerance, raises the question how resistance might have emerged in this strain. Previous studies have shown that ABC transporters can contribute to reduced sensitivity to azoles in field isolates of *M. graminicola, Penicillium digitatum* and *Botrytis cinerea*. Such isolates were cross-resistant to unrelated chemicals and showed a transcriptional up-regulation in several ABC transporter genes (de Waard *et al.*, 2006; Leroux & Walker, 2011; Nakaune *et al.*, 2002; Zwiers *et al.*, 2002). The enhanced transcript levels of such genes may result from mutations at different genomic loci controlling their expression. Studies revealed that genetic variations in the proximal promoter regions of ABC transporter genes might contribute to the observed variation in drug responses (Hesselson *et al.*, 2009; Zhao *et al.*, 2004).

For example, four different (PDR) ABC transporters (PMR1, PMR3, PMR4 and PMR5) have been studied for their involvement in DMI-resistance in *Penicillium digitatum*, but only PMR1 and PMR5 appear to be involved in fungicide resistance. Several mutations were found in the promoter and the coding region of PMR5 in resistant strains (Sánchez-Torres & Tuset, 2011).

Thus, on the one hand the promotor region of the ABC transporter gene itself may receive a mutation that leads to an increased transcription rate. Alternatively, a mutation may occur in another gene whose encoded protein affects the transcript abundances of the ABC transporter gene. One example would be a gene encoding a transcription factor, which binds to the promotor of the ABC transporter-encoding gene. For instance, in *S. cerevisiae* PDR1 is known to regulate the ABC transporter gene *PDR5* (Carvajal *et al.*, 1997; Gao *et al.*, 2004; Gulshan & Moye-Rowley, 2007; Hellauer *et al.*, 2002; Paul & Moye-Rowley, 2014). Certain mutations in *PDR1* were found to increase the transcript levels of *PDR5*, which in turn increases the tolerance to fungicides (Carvajal *et al.*, 1997; Gao *et al.*, 2004; Gupta *et al.*, 2014; Rogers *et al.*, 2001). Such a mechanism has been suggested also for some field strains of *B. cinerea* exhibiting increased tolerance against fungicides (Kretschmer *et al.*, 2009).

These hypotheses need to be further investigated in *F. graminearum* taking advantage of results from transcriptome analyses and knowledge established previously in model organisms as *S. cerevisiae*, which could serve as a starting point for future analyses (Paul & Moye-Rowley, 2014).

D.3. Effects of gene deletion on virulence

D.3.1. Deletion of *FgABC2* in wild type backgrounds

Deletion mutants of *FgABC2* did not exhibit notable differences in virulence compared to the respective wild type strains, which was true for assays using wheat, maize and barley. *FgABC2* is the only protein in *F. graminearum* belonging to the ABC-A subfamily (see paragraph D.1.1).

The corresponding homolog in *M. oryzae* is *MoABC4* (MGG_00937), which is the most similar gene in plant pathogenic fungi that was functionally characterized so far (Kovalchuk & Driessen, 2010). This gene is important for pathogenesis and appressorium formation, as shown in infection assays employing barley leaves (Gupta & Chattoo, 2008). The role of *MoABC4* was discovered in an *Agrobacterium tumefaciens*-mediated mutagenesis approach. However, gene deletion required to confirm the role of *MoABC4* in virulence was not performed. Since there were no follow-up publications on MoABC4 it remains unsettled whether ABC-A subfamily proteins may be only important for virulence in certain pathosystems.

A function in lipid transport and metabolism seems more probable than drug transport for fungal ABC-A proteins, as based on functional studies on mammalian homologs. Thus, further studies are required to identify the ABC-A protein substrates in *F. graminearum*. As members of ABC-A transporter protein subfamily are absent in the yeast genome (Dean & Annilo, 2005; Kovalchuk & Driessen, 2010), heterologous expression of FgABC2 in yeast could give more information about its substrate specificity and confirm its putative involvement in lipid translocation. In *F. graminearum* lipid biosynthesis occurs during early stages of wheat colonization leading to sexual development and lipid oxidation occurs during developing of perithecia, as suggested by microarray experiments analyses (Guenther *et al.*, 2009; Hallen *et al.*, 2007). Thus, it may be interesting to test the Δ*FgABC2* strains for their ability to form perithecia *in vitro*. Furthermore, they could be tested for their ability to accumulate lipids in overwintering hyphae that provide reserves for survival and perithecium development of *F. graminearum.*

D.3.2. Deletion of *FgABC3*

D.3.2.1. Deletion of *FgABC3* in wild type backgrounds

In this work it was shown that the deletion of *FgABC3*, encoding an ABC-G type transporter, reduced the virulence on three host plants, wheat heads, barley heads and maize stems, suggesting an important role of FgABC3 and of the molecules transported by it, during pathogenesis (see paragraph C.4.3. and Figs. 12, 13, 14, 15). This virulence phenotype of the Δ*FgABC3* strains was further confirmed by a genetic complementation approach, in which the native target gene *FgABC3* was re-transformed into one of the mutants deficient in *FgABC3* (see paragraph B.3.7 and Fig. 4). Complementation strains showed wild type levels of virulence on the tested hosts, i.e. wheat and maize. This confirmed that the reduction in virulence of the deletion mutants resulted from the deletion of *FgABC3* and not from other unknown alterations in the fungal genome, which could have been introduced as an artefact during transformation.

The biological roles of several transporters in the PDR subfamily have been studied earlier, showing an involvement of some of these transporters in pathogenesis (de Waard *et al.*, 2006; Del Sorbo *et al.*, 2000; Urban *et al.*, 1999). "Later research detected a subclade within the ABC-G subfamily, called group I, which is distinctive to *Fusarium* spp. Functional characterisation of members of this subclade, i.e. of NhABC1 in *Nectria haematococca* (anamorph: *F. solani*) (Coleman *et al.*, 2011) and GpABC1 in *Gibberella pulicaris* (anamorph: *F. sambucinum*) (Fleissner *et al.*, 2002) demonstrated that the proteins are essential for full virulence" (Abou Ammar *et al.*, 2013). Searches by the program BLASTp, using FgABC3 as a query against the nonredundant database of the National Center for Biotechnology Information (NCBI) (Altschul *et al.*, 2005), indicated that FgABC3 is closely related to GpABC1 of *F. sambucinum* (90% amino acid sequence identity), which has a role in virulence on potato and resistance to the phytoalexin rishitin (Fleissner *et al.*, 2002; Gardiner *et al.*, 2013). Beyond, FgABC3 has about 80% amino acid sequence identity with NhABC1 from *F. solani* that is required for virulence on pea and resistance to the phytoalexin pisatin (Coleman *et al.*, 2011). Finally, FgABC3 has 63% amino acid sequence identity to the ABC1 transporter of *Magnaporthe oryzae*. Transcript abundance of *MoABC1* is inducible by a treatment of cultures with miconazole and metconazole, as well as the rice phytoalexin sakuranetin and the protein synthesis inhibitor hygromycin. The deletion of *MoABC1* yielded mutants severely reduced in virulence, which indicates that this transporter protein is required for pathogenicity of *M. oryzae* on rice (Urban *et al.*, 1999). Thus, from several plant

pathogenic fungi proteins have been described that are highly similar to FgABC3, contribute to virulence and have a presumed or experimentally tested role in protecting the fungus from host defence compounds.

A recent study (Gardiner *et al.*, 2013) assessed the transcript levels of all ABC-G members during infection of different tissues in wheat and barley using published Affymetrix microarray data (Guldener *et al.*, 2006; Lysoe *et al.*, 2011; Stephens *et al.*, 2008; Zhang *et al.*, 2012b). In general, the transcript levels of *FgABC3* (=FGSG_04580) were among the highest found for the 19 ABC-G genes of *F. graminearum*. During infection, transcript levels of that gene increased over time in all tissues analysed, reaching a maximum at 4 to 6 dpi in FHB and coinciding with the highly destructive colonization of the host. The results described above for the virulence of the deletion mutants of *FgABC3* (FGSG_04580) on wheat were confirmed by a recent report (Gardiner *et al.*, 2013). However, this report did not confirm the importance of *FgABC3* for virulence on barley, probably because these researchers did not use spray inoculation of heads as performed here, which simulates natural infection. Instead they inoculated stem bases of 14 d old seedlings, which may explain the difference. Since this thesis has shown that FgABC3 is needed to allow for full virulence in three different hosts, it is evident that this protein transports an unidentified important compound produced either by the host to mediate defence or by the fungus to support pathogenesis. The former explanation is favoured here, since the homologs of this gene in *Fusarium* spp. were also needed for full virulence and since it was reported that they might export phytoalexins produced by the host.

D.3.2.2. Deletion of *FgABC3* in the azole adapted strain P1-11

In this work, the aim was to consider the correlation of virulence and fungicide resistance. Recent studies showed a positive correlation between virulence and fungicide tolerance in the plant pathogen *M. graminicola.* Virulence and tolerance to a triazole fungicide in a large collection of isolates sampled across several host genotypes and geographic locations have been assayed (Cowger *et al.*, 2009; Yang *et al.*, 2013). Such studies are of practical importance to understand the emergence of infectious diseases and to devise sustainable fungal disease management. However, they are still rare in plant pathogens (Yang *et al.*, 2013).

In this work, the deletion of *FgABC3* in two different wild type backgrounds did not only impede the tolerance of the mutants to several fungicides of SBI class I but also their

virulence. To assess whether virulence would be affected at the same extent when the same gene was deleted in the background of the azole-adapted strain P1-11 such mutants were also assayed on wheat and maize. In addition, it was interesting to determine whether there exists an association between virulence and resistance to fungicides, as well to test how the adaptation procedure that had led to the recovery of P1-11 (Becher *et al.*, 2010) might have affected the role of FgABC3 in virulence (see paragraph C.5.4 and Figs. 23, 24**)**. The data show a clear reduction of fungal invasion into maize stems after the application of prothioconazole. Also, they confirm the sensitivity of the Δ*FgABC3* strains to azoles, which was determined *in vitro* in both wild type backgrounds. However, under the experimental conditions applied, the treatment of maize stems with prothioconazole did not significantly differentiate between the progenitor wild type and the resistant strain P1-11 (see Fig. 24). These data are in agreement with a previous study (Becher *et al.*, 2010), in which the adapted strain P1-11 showed in comparison to its progenitor NRRL 13383 increased tolerance to tebuconazole only *in vitro* but not on infected wheat heads. Similarly, in a recent study wheat heads were inoculated with a *F. culmorum* strain with an acquired tolerance to tebuconazole, which resulted in symptom severity and DON content that were similar to the progenitor strain (Serfling & Ordon, 2014). For both studies it cannot be ruled out that experimental difficulties in finding optimal conditions and concentrations for the treatment may have prevented detecting effects of the fungicide on virulence *in planta*.

As in the wild type NRRL 13383, the deletion of *FgABC3* in P1-11 caused a strong reduction of virulence on wheat and maize. In both backgrounds, the deletion diminished virulence at comparable levels. Therefore, FgABC3 contributes in both backgrounds to virulence likely in the same way. Mutations that arose during the selection of the adapted strain P1-11 led to increased tolerance to azoles. These unknown mutations do however not lead to antagonistic or synergistic effects on virulence. Mutants carrying an ectopic integration of the same construct used to generate the deletion mutants of *FgABC3* in the background of P1-11 were also recovered in the respective transformations and included in the virulence assays. They exhibited levels of virulence in both, wheat heads and maize stems that were similar to their progenitor strain P1-11 (see paragraph C.5.4 and Figs. 23, 24). Thus, these data confirmed, that solely the deletion of *FgABC3* had caused the reduction of virulence observed in mutants of the adapted strain.

D.4. Effects of gene deletion on mycotoxin production in wild type backgrounds

In this work, deletion mutants of *FgABC3* were generated in two genetic backgrounds representing different chemotypes. The resulting transformants were impeded in infections of wheat, barley and maize irrespective of their trichothecene chemotypes. To assess whether *FgABC3* may be involved in the transport of trichothecenes or zearalenone its deletion mutants were assayed for the production of these compounds *in vitro.*

Mutants generated in the PH-1 background, exhibited for all trichothecenes, especially DON, significantly increased levels when compared to the wild type strain. There existed only minor differences in the zearalenone levels between the mutants and the wild type. In the NRRL 13383 background, deletion of *FgABC3* led to lower NIV levels whereas only minor effects were observed for the ZEA levels. Assuming that the comparison between wild types and mutants will be similar for the situation *in planta*, these results obtained from *in vitro* cultures suggest that the reduction in virulence of the *FgABC3* deletion mutants is not the consequence of drastically reduced mycotoxin levels. DON is a documented virulence factor of *F. graminearum* and it is essential for spreading from the initially infected to the adjacent spikelets in wheat but not in barley heads (Cowger & Arellano, 2013; Hallen-Adams *et al.*, 2011; Jansen *et al.*, 2005; Yekkour *et al.*, 2015).

The deletion of *FgABC3* in PH-1 did not lead to diminished but to increased DON levels in the mutant. Yet, these mutants were less virulent on wheat, indicating that this transporter is not involved in DON excretion. However, this was almost expected since a specific transporter for DON has been described (Alexander *et al.*, 1999; Kimura *et al.*, 2007). At the moment, it is unknown why some trichothecenes reached higher concentrations in the deletion mutants. In contrast, NIV has been shown to contribute to virulence in maize (Desjardins *et al.*, 2008; Maier *et al.*, 2006). NIV levels in the NIV producing NRRL 13838 background were lower in the Δ*FgABC3* mutants in comparison to the wild type strain (see paragraph C.4.4 and Fig. 19). Assuming that the toxin levels produced *in vitro* correspond to those *in planta*, the reduced NIV levels of the Δ*FgABC3* mutants cannot explain why virulence was reduced in all three host plants tested. As discussed above, it is more likely that an unknown metabolite produced by all three hosts is the natural substrate for this transporter.

The results for the *in vitro* production of ZEA showed no significant effects of the *FgABC3* deletion in both wild type strains tested. However, *FgABC3* was previously identified in a microarray analysis as a down-regulated gene (*FgZRA1*) in a deletion mutant of *FgZEB2* (FGSG_02398) (Lee *et al.*, 2011). *FgZEB2* encodes the transcription factor regulating the

gene cluster for zearalenone biosynthesis (Kim *et al.*, 2005). Lee *et al*. (2011) showed that the deletion of *FgZRA1* (= *FgABC3* = FGSG_04580) resulted in reduction of ZEA in liquid SG medium, leading to the assumption that the encoded ABC transporter *FgZEB2/FgABC3* is responsible for exporting ZEA (Lee *et al.*, 2011). Additional phenotypes such as fungicide sensitivity and virulence were not analysed in this study. The differences between the two studies could be explained by the variation resulting from different genetic backgrounds, culture conditions and data acquisition (Abou Ammar *et al.*, 2013). Furthermore, additional data published previously suggested that ZEA is not involved in pathogenesis on wheat and barley because deletion mutants for the genes involved in ZEA biosynthesis were causing the same levels of FHB on these hosts as wild type strains (Gaffoor & Trail, 2006; Kim *et al.*, 2005). This supports the results of this work that showed a clear virulence phenotype in *FgABC3* deletion mutants in both wild types without significant changes in ZEA production. If the true function of FgABC3 would be to export ZEA, a virulence defect is not to be expected because, as discussed above, ZEA is not known to contribute to virulence. Thus, considering the literature and the results of the mycotoxin measurements performed in this study, it is proposed that FgABC3 may rather export host-derived defence molecules than to export fungal secondary metabolites. This rationale is supported by the decreased levels of disease symptoms irrespective whether the *FgABC3* deletion was introduced in the DON or the NIV chemotypes (Abou Ammar *et al.*, 2013).

Deletion of *FgABC2* showed in the background of PH-1 increased levels of DON and its acetylated variants, while there were no significant differences to the wild type NRRL 13383 in NIV production. Furthermore, levels of ZEA did not differ between the mutants and the corresponding wild type strains (see paragraph C.4.4). As the deletion of *FgABC2* had no effect on virulence on the tested host plants (see paragraph C.4.3, Figs. 12, 13, 14, 15), observed variations in trichothecene production do not seem to significantly affect the overall phenotypes tested.

D.5. Effects of gene deletion on fungal sensitivity to cereal plant secondary metabolites in wild type backgrounds

The sensitivity of Δ*FgABC3* to four cereal plant secondary metabolites was tested as it was mentioned above (see paragraphs B.4 and C.4.2.3). These assays were applied to examine if the deletion might have affected the sensitivity of the transformants to four commercially available secondary metabolites produced by cereals that have antifungal properties. However, up to concentrations of 500 ppm, none of the plant metabolites tested, 2-benzoxazolinone (BOA), gramine, naringenin and quercetin significantly affected vegetative growth rates of the Δ*FgABC3* in any background. These results are in agreement with a previous study, in which *FgABC3* deletion mutants (= FGSG_04580) were tested for sensitivity to grass defence compounds by a spore droplet germination assay. There existed no significant differences for sensitivity to BOA, gramine and tryptamine between a *FgABC3* deletion mutant and the corresponding wild type strain (Gardiner *et al.*, 2013). Data presented here indicate that FgABC3 appears to protect the fungus from yet unidentified cereal defence compounds during pathogenesis. Further research is needed to reveal the structure of this compound(s) and its/their biological functions. Recently identified phytoalexins from the *Poaceae* family could be used for future sensitivity assays with Δ*FgABC3* (Park *et al.*, 2013; Poloni & Schirawski, 2014).

Some studies have attempted to determine the actual concentration and the nature of phytoalexins directly in plant tissues in response to invading microorganisms using spectroscopic methods (Balmer *et al.*, 2013; Becker *et al.*, 2014; Ghosh *et al.*, 2002). Employing such techniques, analysis of secondary metabolites in infected host tissue during the later stages of pathogenesis, in which *FgABC3* is highly expressed, would be helpful in determining highly induced defence compounds. Comparison of the profiles from tissues of different host plants infected by the mutants and the wild type may give a hint to the substrate of FgABC3 and its importance during fungal invasion.

The Δ*FgABC2* mutants were also tested for a possible inhibition by the four cereal metabolites mentioned (see paragraph B.4). However, none of the compounds tested significantly affected the vegetative growth of the mutants. Thus, as discussed before, further analyses are needed to determine the substrate specificity of this transporter protein (see paragraphs D.2.1 and D.3.1).

D.6. Varying effects of gene deletions in different genetic backgrounds

In order to assess whether the genomic context may influence the effect of gene deletion, the two selected ABC transporter genes were functionally analysed in two genetic backgrounds, i.e. NRRL 13383 and PH-1, representing the NIV and 15-ADON chemotypes, respectively. Deletion mutants of *FgABC2* did not show significant differences to the respective wild type strain in their sensitivity to fungicides and in virulence, which was true for both both backgrounds. However, deletion of *FgABC3* caused in NRRL 13383 significantly reduced tolerances to certain class I sterol biosynthesis inhibitors. Although this effect did also occur in the background of PH-1, it was less prominent (see paragraph C.4.2).

This result could be explained by the variation of ABC transporter genes between the two selected strains that may differ in their numbers, sequences and regulation, which could cause putative compensatory effects. As the genome sequence of NRRL 13383 has not been published yet, it is unknown whether this strain has exactly the same set of ABC transporters as the reference strain PH-1. This hypothesis could be further investigated once the genome sequence of NRRL 13383 is available. Importantly, this work shows that alterations in fungicide sensitivities resulting from gene deletions may differ in their extents in different genomic contexts. A recent publication on a human pathogenic fungus, *Aspergillus fumigatus*, reported a similar finding for the ABC transporter genes *AbcA* and *AbcB* (ABC-G subfamily), which share high sequence similarity with *Pdr5* of *S. cerevisiae* (Paul et al., 2013). Deletion mutants of these genes were created in three different strains of *A. fumigatus*. Absence of *AbcB* caused an increased azole susceptibility in all backgrounds, while effects of a deletion of *AbcA* had variable phenotype in the three different backgrounds. Both works thus highlight the contribution of the overall genomic background to the results obtained in fungicide assays.

E. Summary

Fusarium graminearum is a plant pathogen infecting several important cereals, resulting in substantial yield losses and mycotoxin contamination of the grain. Triazole fungicides are used to control diseases caused by this fungus on a world-wide scale. Previously, research was initiated to study the capability of *F. graminearum* to develop azole resistance and the molecular mechanisms leading to it. The transcriptomic response of *F. graminearum* challenged *in vitro* with tebuconazole was investigated using a microarray, which indicated that 15 ABC transporter genes were up-regulated.

Based on these previous studies, two ABC transporter genes were selected for functional characterization during this work. The first transporter protein is encoded by the gene (*FGSG_17046*) and referred to as FgABC2. The second transporter is encoded by the gene (*FGSG_04580*) and referred to as FgABC3.

Deletion of these candidate genes was performed in two genetic backgrounds of *F. graminearum* i.e. NRRL 13383 and PH-1 representing the NIV (nivalenol) and 15-ADON (15-acetyl-deoxynivalenol) trichothecene chemotypes, respectively. Deletion of *FgABC3* belonging to group I of ABC-G subfamily of ABC transporters, considerably increased the sensitivity to the class I sterol biosynthesis inhibitors triazoles and fenarimol. However, cross sensitivity to other fungicide classes was not observed in the deletion mutants of this ABC transporter protein in both backgrounds tested.

FgABC3 was also deleted in the azole resistant strain P1-11 in order to test if the encoded protein mediated long-term fungicide adaptation in *F. graminearum.* This showed that the deletion of *FgABC3* in P1-11 did not affect the viability of the mutants but it resulted in significantly reduced tolerance to azoles similar to the results obtained for the deletion of *FgABC3* in NRRL 13838. These data thus suggest an involvement of this transporter in the acquired tolerance of *F. graminearum* in P1-11.

Assessing the contribution of the two ABC transporters to virulence of *F. graminearum* revealed that, irrespective of their chemotypes, deletion mutants of *FgABC3* were impeded in virulence on wheat, barley and maize. Phylogenetic context suggested that *FgABC3* may encode a transporter protecting the fungus from still unknown host-derived antifungal molecules produced during infection. These results show that ABC transporters may play important and diverse roles in both fungicide resistance and pathogenesis of *F. graminearum.*

In contrast to *FgABC3*, deletion of *FgABC2* revealed no significant deviation from the wild type strains representing the two backgrounds analysed with respect to both fungicide resistance and virulence. Thus, it seems that the enhanced transcript abundance of this gene during azole treatment may be a rather indirect effect to fungicide stress. Furthermore, FgABC2 is the only protein in *F. graminearum* belonging to the ABC-A subfamily, and this work suggests that this transporter protein might be required only under specific physiological conditions.

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G. Appendix

All solutions, unless mentioned, were autoclaved for 20 minutes at 121°C and 2 bar pressure. Sensitive solutions were filter sterilized using 0.2 µm pore CA membrane filters (Heinemann Labortechnik, Duderstadt, Germany).

G.1. Culture media, buffers and solutions

G.1.1. Culture media

M.1. Luria-bertani-medium (LB Medium)

10 g bacto trypton, 5 g yeast extract and 5 g NaCl were dissolved in 1 L H_2O_{bidest}. For solid medium, 15 g of agar-agar were added, too. Antibiotics and fungicides were added after the media had cooled down to circa 40°C at concentrations required for a given experiment.

M.2. Mung bean broth (MBB)

20 g of bio mung beans were boiled in 1 L of distilled water for 15 min, filtered through Buchner funnels with paper filters (Roth).

M.3. Potato dextrose agar & - broth (PDA, PDB)

For PDB, 24 g potato dextrose (BD, Heidelberg, Germany) were dissolved in 1 L of distilled water. For solid medium, 15 g of agar-agar (Roth) were added, as well. Antibiotics, fungicides or plant defence compounds were added to the media after cooling to 45-40°C according to the final concentration needed.

M.4. Regeneration medium (RM)

Solution A contained 0.5 g of yeast extract, 0.5 g of casein hydrolysate and 5 g of agar-agar dissolved in 0.5 L of distilled water. For solution B, 275 g of sucrose were dissolved in 0.5 L of distilled water. Solutions A and B were combined after autoclaving them separately.

M.5. Synthetischer nährstoffarmer Agar (SNA)

This minimal medium contains 1 g of KH_2PO_4, 1 g of KNO_3, 0.5 g $MgSO_4$, 0.5 g of KCl, 0.2 g glucose, 0.2 g sucrose, and 15 g agar agar per 1 L distilled water (Leslie & Summerell, 2006). Ingredients were dissolved and autoclaved as above. SNA used for selection was cooled down to circa 40°C and the respective antibiotic was added to reach a final concentration of 100 µg/ml.

M.6. Yeast extracts peptone dextrose (YEPD)

3 g of yeast extract and 10 g of trypto-peptone were dissolved in 950 ml distilled water and autoclaved. 50 ml of 40% glucose was autoclaved separately and added then to complete the volume to 1 L.

G.1.2. Buffers and solutions

G.1.2.1. Buffers and reagents

B.1. Blocking solution 10x (Southern blot)

10 g of blocking powder (Roche Diagnostic, Mannheim, Germany) were dissolved in 100 ml of maleic acid buffer in a microwave oven, autoclaved and stored at -20°C. 1x blocking reagent was obtained by diluting the stock solution 1:10 with maleic acid buffer.

B.2. Detection buffer (Southern blot)

100 mM NaCl, 100 mM Tris-HCl, pH 9.5

B.2. DNA extraction buffer I (Genomic DNA isolation)

Autoclaved solution of 7 M urea, 2% (w/v) SDS in 5 mM EDTA, pH 8

B.3. DNA extraction buffer II (Genomic DNA isolation)

Autoclaved solution of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8

B.4. Denaturation solution (Southern blot)

1.5 M NaCl, 0.5 M NaOH

B.5. Hybridization buffer (Southern blot)

5x SSC, 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) SDS, 1% (w/v) blocking reagent (Roche Diagnostics), dissolved in 1 L autoclaved maleic acid

B.6. Loading buffer 6 X (Gel electrophoresis)

Solution of 30% (v/v) glycerin, 0.25% (w/v) bromophenol blue in 60% 10x TAE buffer

B.7. Lysis buffer (DNA extraction mini prep)

1% SDS, 2% Triton X 100, 100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8

B.8. M buffer (Southern blot)

Solution of 0.15 M NaCl, 0.3% (v/v) Tween 20 in 0.1 M Maleic acid, pH 7.5

B.9. Maleic acid buffer (Southern blot)

Solution of 100 mM maleic acid and 150 mM NaCl, pH 7.5

B.10. PEG 8000 solution (Fungal transformation)

30% (w/v) polyethylenglykol 8000 in 10 mM Tris-HCl, 0.5 mM $CaCl_2$, pH 8.0

B.11. Protoplasting mix (Fungal transformation)

500 mg of driselase, 1 mg of chitinase and 100 mg of lysing enzyme of *Trichoderma harzianum* (all from Sigma-Aldrich Chemie GmbH, München) were dissolved in 20 ml of 1.2 M KCl and stirred gently for 30 min. The solution was filtrated through a 0.45 μM Millex-HA filter (Millipore GmbH, Schwalbach) with the help of a 20 ml syringe.

B.12. P1 buffer (Plasmid maxi preparation)

50 mM Tris-HCl, 10 mM EDTA; pH 8

B.13. P2 buffer (Plasmid maxi preparation)

0.2 M NaOH, 1,0% SDS

B.14. P3 buffer (Plasmid maxi preparation)

2.55 M Potassium acetate, pH 4.8

B.15. STC buffer (Fungal transformation)

Autoclaved solution of 1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5 sterile filtered through a 0.45 µM Millex-HA filter, Millipore GmbH, Schwalbach)

B.16. Stripping solution (Southern blot)

0.05% (w/v) SDS, 3 M NaCl, 0.3 M sodium citrate, pH 7.5

B.17. TAE 50x (Gel electrophoresis)

2 M Tris, 0.05 M EDTA, 1 M acetic acid, pH 8.5

B.18. TE buffer (nucleic acids extraction)

0.1 mM EDTA in 10 mM Tris-HCl, pH 8

B.19. Transfer buffer 20x SSC solution (Southern blot)

3 M NaCl in 0.3 M sodium citrate

B.20. Wash buffer 2 x (Southern blot)

2 x SSC, 0.1% (w/v) SDS

B.21. Wash buffer 0.5 x (Southern blot)

0.5 x SSC, 0.1% (w/v) SDS

B.22. Wash buffer 0.25 x (Southern blot)

0.25 x SSC, 0.1% (w/v) SDS

G.1.2.2. Stock solutions

S.1. Ampicillin stock solution

100 mg/ml Ampicillin in bidistilled H_2O, sterile filtrated, stored at -20°C

S.2. Geneticin stock solution

100 mg/ml G418 Disulphate (For Medium, Ltd, England) in bidistilled H_2O, sterile filtrated stored at -20°C.

S.3. Hygromycin stock solution

100 mg/ml Hygromycin B (InvivoGen, Toulouse, France) in bidistilled H_2O, sterile filtrated stored at -20°C.

S.4. Nourseothricin stock solution

100 mg/ml Nourseothricin (Werner Bio Agents, Jena, Germany) in bidistilled H_2O, sterile filtrated, stored at -20°C.

G.2. Vectors

Table G2.1. List of vectors used

Vector name	Description	Reference
pJET2.1/blunt	(Fermentas, St. Leon-Rot) „High copy“ plasmid for cloning PCR-fragments with blunt ends and amplification in *E. coli*. Selection marker: ampicillin-Resistance	Fermentas, St. Leon-Rot
pNR1	Plasmid with insert for expressing nourseothricin-resistance	Malonek *et al*. (2004)
pII99	Plasmid with insert for expressing geneticin-resistance	Namiki *et al*. (2001)
pAN7-1	Plasmid with insert for expressing hygromycin-resistance	Punt *et al*. (1987)

G.3. Oligonucleotides used

Name of primer	sequence 5´ to 3´	target
Fg2.Fw1	ACGGAACTTTGCACGACTTCTC	*FgABC2*
Fg2.Nours.Rv	GTGCAACTGACAGTCGTACAGATGATTCAATGCAGAGTGGCAGAG	*FgABC2*
Fg2.Nours.Fw	GTCTGGAGTCTCACTAGCTTGTGTATCCCACTATTGTCCTAATCC	*FgABC2*
Fg2.Rv1	GACGACGAACTGATCTTGAC	*FgABC2*
Fg2.Fw2	ATATGGACCGGGCGACGTTG	*FgABC2*
Fg2.Rv2	GAGAGCCAATGCCGCGAAAC	*FgABC2*
Fg3.Fw1	CACTTCCATCATGCCTCCATC	*FgABC3*
Fg3.G418.Rv	GTGCAACTGACAGTCGTACATATGTTTGCGCTGCAAGAC	*FgABC3*
Fg3.G418.Fw	GGTCTGGAGTCTCACTAGCTTAAACTTGAGAGCTCGCGCTCTTTC	*FgABC3*
Fg3.Rv1	TATGCTGTGGGCTTGCAATTGG	*FgABC3*
Fg3.Fw2	CCGGCGATGAACTCCCATTAC	*FgABC3*
Fg3.Rv2	TGCTTGTTGGCGCTGACAC	*FgABC3*
uni-nours.F1	TGTACGACTGTCAGTTGCACATTCGGGCCGGATTG	*pNR1*
uni-nours.R1	AAGCTAGTGAGACTCCAGACACCGATGAAACGATTCTCAAC	*pNR1*
uni-G418.F1	TGTACGACTGTCAGTTGCACAGCGCGTTGTTGGATTAAG	*pII99*
uni-G418.R1	AAGCTAGTGAGACTCCAGACCTCAGAAGAACTCGTCAAGAAG	*pII99*
nours.Fw2	CTCTTGACGACACGGCTTAC	*nat1*
nours.Rv2	GGCAGGGCATGCTCATGTAG	*nat1*
Fg2.probe.Fw	ACAGTATTGCAAGTGGCCGGAAG	*FgABC2*
Fg2.probe.Rv	ACACTTCACCACGCCGGATAC	*FgABC2*
G418.Fw2	CTTGGGTGGAGAGGCTATTC	*npt*
G418.Rv2	CACCATGATATTCGGCAAGCAG	*npt*
Fg3.probe.Fw	GTTGCATTCATCGCCTCGTCAC	*FgABC3*
Fg3.probe.Rv	AACCCTGGATGTTGGCGACTTC	*FgABC3*

*** see (Abou Ammar et al., 2013)**

Curriculum vitae

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Scientific publications

- **Abou Ammar, G**., Tryono, R., Doll, K., Karlovsky, P., Deising, H. B. & Wirsel, S.G. (2013). Identification of ABC transporter genes of *Fusarium graminearum* with roles in azole tolerance and/or virulence. *PloS One* 8, e79042.

- **Abou Ammar, G.,** Tryono, R., Becher, R., Deising, H. B., Wirsel, S.G. (2014). Contribution of ABC transporters to azole resistance and virulence in *Fusarium graminearum.* IN: Modern fungicides and antifungal compounds, eds: Dehne, H.W., Deising, B., Fraaije, U., Gisi, D.;Hermann, D., Mehl, A., Oerke, E. C., Russell, P. E., Stammler, G., Kuck, K. H., Lyr, H. Vol. VII, pp 117-122. DPG, Braunschweig.

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Declaration (Erklärung)

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

Halle (Saale),

_____

Ghada Abou Ammar

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Datum **Unterschrift des Antragstellers**